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Chromosomal variation of the leaf-mining *Liodrosophila castanea* (Diptera, Drosophilidae), with the results of crossing experiments

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Chromosomal variation was found in three insular populations of *Liodrosophila castanea* (Diptera, Drosophilidae). Both Yakushima and Okinawa strains possessed 2n=12 chromosomes, 5 pairs of acrocentric autosomes and sex chromosomes, in the metaphase plates of larval brain cells. Of them a pair of middle-sized autosomes were heteropyknotic from the prophase to early metaphase during the cell division. Tsushima strain had 2n=10 chromosomes, 4 pairs of acrocentric autosomes and sex chromosomes. All nuclear plates of Tsushima strain contained small supernumerary chromosomes ranging from 2 to 9 in number and lacked any heterochromatic autosome. Morphologically, any discriminative character was not found between different chromosomal strains, excepting the nearly bare cercus in Yakushima and Okinawa strains (pubescent in Tsushima's). Male flies mated well with different strain females in crossing experiments without any mating preference, and produced fertile F_1 males and females in both directions of crosses. The present results demonstrate that these insular strains with different karyotypes should be regarded as chromosomal races of *Liodrosophila castanea*.

1. Introduction

Liodrosophila castanea was described by Okada and Chung (1960) from Chon-Nam district, Huk-san Island, Korea, and since then its collection records had been restricted to southern parts of the Korean Peninsula including adjacent islands (Lee, 1966; Okada, 1988). We had a chance to collect drosophilid flies in three islands, Tsushima, Yakushima and Okinawa, of southern Japan, and obtained questionable specimens by net sweeping on herbaceous plants. Prof. M. J. Toda (Hokkaido University) kindly identified the specimens as *L. castanea*, and noticed slight morphological variations in the specimens from Yakushima and Okinawa.

We carried out karyotype analyses in a total of 11 iso-female strains from these local populations and found chromosomal variation. Then, we conducted crossing experiments for examining the reproductive isolation and compared the morphology, including the genitalia, between the different chromosomal strains.

2. Material and methods

2.1 Geographic strains

Eleven strains of *L. castanea* were employed: four were collected at Sumo, Tsushima Island (henceforth designated as TS), Nagasaki Prefecture, in September, 1996, four at Kurio, Yakushima Island (YK), Kagoshima Prefecture, in October, 1996, and three at Experimental Forests of Ryukyu University, Yona, Okinawa Prefecture (OK), in March, 1997 and in September, 1998. All living strains used for karyotype observation and crossing experiments were established from a single inseminated female caught in the field.

2.2 Rearing method

Liodrosophila castanea is a leaf-mining species and cannot be maintained in standard *Drosophila* medium. A new culturing method was adopted in the present study. Leaves of *Spinacia oleracea* L. were mashed by a blender machine and then frozen in refrigerator to kill larvae of other insects. They were placed on

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filter paper, added with a small piece of apple for inhibiting mold, and then kept in a glass vial (30 mm in diameter, 100 mm in height). For establishing an iso-female strain, a wild-caught female was slightly etherized and identified, and kept in a glass vial with food described above at 18°C under continuous light. Mashed leaves were added on the shortage of food for larvae. Hatched larvae entered into a layer of mashed leaves and filter paper with sap, developed well in the layer and pupated on its surface.

2.3 Chromosome preparation

Neuroblasts of 3rd instar larvae were used to prepare mitotic chromosomes as follows: treated with a mixture solution of 0.1mg/ml of colchicines and 1% of sodium citrate for 45–60 min, fixed with a solution (carbinol: acetic acid=3: 1) for 30 min, stained with 4% Giemsa solution for 60 min, and then air-dried (Imai et al., 1977). About 30 nuclear plates were examined for each strain. Photos of metaphase chromosomes were taken by an analog camera (Olympus PM-6) and then saved as digital figures using a film-scanner (Nikon APS IX240).

2.4 Morphological observation

We compared some metric characters and the genital structures between the strains with different chromosome constitutions in the present study. Male and female terminalia were detached from the body, and treated with 10% KOH solution at 80°C for several minutes, and observed in a droplet of glycerin under a compound microscope. Twenty-three quantitative characters (Table 1) were measured with an ocular micrometer: body length (BL), thorax length (TL) including scutellum, wing length (WL), wing width (WW), ratio of frons/head width (FW/HW), numbers of dorsal (db of Ar) and ventral branches (vb of Ar) of arista, gena/maximum width of eye (G/E), length ratio of 2nd oral/vibrissa (Or2/Or1), length ratio of proclinate/posterior reclinate seta (Orb3/Orb1), length ratio of upper/lower humeral setae (U/L of Hu), length ratio of anterior/posterior dorsocentral setae (DcA/DcP), length distance/cross distance of dorsocentals (Ld/Cd of Dc), length ratio of posterior/anterior scutellar setae (SctP/SctA), distance from SctA to Sctp/distance between SctPs (SctA-SctP/SctPs), sterno-index, and seven wing indices (costal, 4V, 4C, 5x, Ac, M, C3-fringe). The first four characters, BL, TL, WL and WW, were measured separately for each sex.

Some quantitative characters such as the body size are quite variable according to rearing conditions. Five of mature females were allowed to oviposit in a glass vial with food, and the vials were renewed every three or four days, to avoid the overpopulation of larvae. When young flies emerged, they were transferred to a new vial at the same intervals. Twenty individuals (10 males and 10 females) were selected randomly and examined.

2.5 Crossing experiments

When adult flies emerged, they were collected every 24 hrs, sorted for sexes, and maintained in vials. All crossing experiments were carried out using 8- to 12-day-old flies, since *L. castanea* is a relatively rapid breeder and most adults reach to the mature stage for reproduction within 8 days after eclosion. Five males and five alien or same-strain females were put together into a vial (30 mm in diameter, 100 mm in height). After 48 hrs, the females were taken out, dissected in Ringer's solution, and examined for sperm in the spermathecae and the seminal receptacle. About 50–100 females were checked in each crossing. The index of pre-zygotic isolation for each cross-pair was estimated by the formula of Coyne and Orr (1989, 1997): 1– (% of heterogamic matings (i.e., A♀×B♂)) / (% of homogamic matings (A♀×A♂)). This index gives the value of 1 when isolation is perfect and the value of 0 for free crossings.

For mate-choice experiments, the choice-by-male method was adopted. The tip of the right or left wing was marked with red ink for recognizing the strains. Five males and ten (five own and five alien-strain) females were placed together, and the females were dissected in Ringer's solution for sperm after 48 hrs. The degree of mating preference was evaluated by Stalker's (1942) isolation index (I.I.): I.I.=[% of Homogamic (+) − % of Heterogamic (+)]/[% of Homogamic (+) + % of Heterogamic (+)], where Homogamic (+) and Heterogamic (+) mean females inseminated by same-strain and alien-strain males, respectively. This formula gives the value of 1 for completely assortative mating preference and 0 for lack of mating preference.

When hybrid adults were obtained, F_1 fertility was checked by backcrossing between the hybrids and the parental strains.

3. Results and discussion

Figs 1A and B show the male metaphase configu-

rations of the Yakusima (YK) and Tsushima (TS) strains of *L. castanea*, respectively. This is the first report on the karyotype of the genus *Liodrosophila* (Clayton and Guest, 1986; Clayton, 1998). The metaphase configurations of YK comprised 12 chromosomes in the diploid number with 5 pairs of acrocentric autosomes, relatively large acrocenric X, and submeta- or meta-centric Y. Two autosomes indicated with the arrows in Fig. 1A were denser in condensation than the other autosomes, probably due to heteropyknosis, from the prophase to early metaphase during the cell division, which implies rich heterochromatic elements of these autosomes. The same chromosomal constitution of 2n=12 with a pair of heteropyknotic autosomes was found in three Okinawa (OK) strains of *L. castanea*.

On the other hand, brain cell preparations of TS strains showed 2n=10 chromosomes, and all were acrocentric. Metaphase plates of TS lacked two heterochromatic autosomes found in those of YK and OK and had small supernumerary chromosomes (indicated with the arrows in Fig. 1B). These dot-like chromosomes were densely stained, and its number per nuclear plate was quite variable, ranging from 2 to 9, so far as we examined about 240 cells.

Figs 1C and D show the metaphase plates of hybrids in the crosses between YK females and TS males and in its reciprocal crosses, respectively. All nuclear plates of F_1 larval brain cells showed 2n=11 chromosomes and a few of dot-like supernumerary ones (indicated with the arrows in Figs 1C, D), suggesting that in the nuclear plate of F_1 offspring six chromosomes might have derived from YK but the other five and a few dot-like chromosomes from TS.

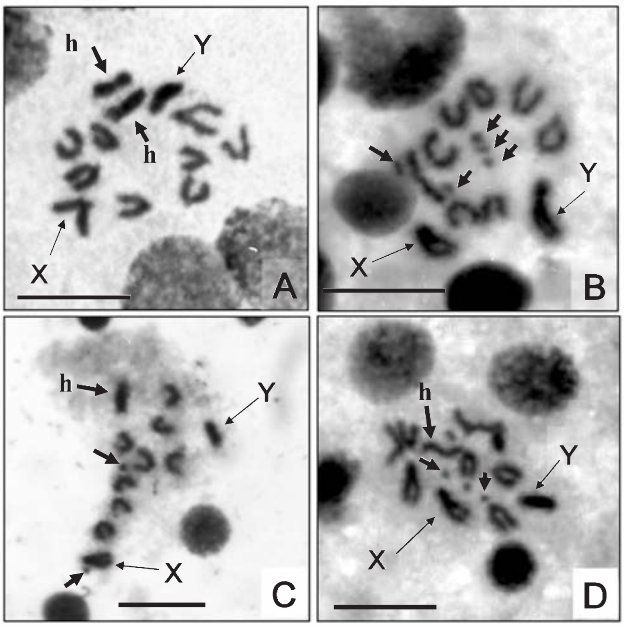
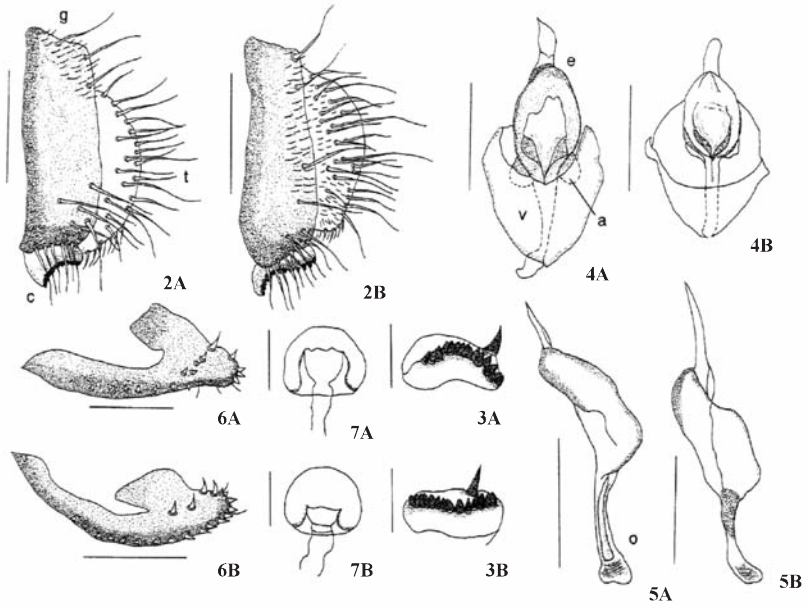


Figure 1 : Male metaphase chromosomes of *Liodrosophila castanea*. A, Yakushima strain; B, Tsushima strain; C, F_1 in the cross between Yakushima♀×Tsushima♂; D, F_1 in the cross between Tsushima♀×Yakushima♂. Bars=10μm.

Figs 2–7 show the male and female genitalia of YK (A) and TS (B) strains, respectively. The genital characters of YK were as follows: epandrium dark brown, pubescent on dorsal portion, with ca. 3 setae on upper, ca. 5 setae on lower and ca. 6 setae along ventral margin (Fig. 2A); surstylus with ca. 15 stout setae and a large seta near the apex on inner surface (Fig. 3A); cercus almost bare, hemispherical, with ca. 18 long setae and several short setulae on ventral margin (Fig. 2A); aedeagus grayish brown, apically pointed, club-shaped at lateral view (Figs 4–5A); aedeagal apodeme grayish brown (Fig. 5A); hypanrium narrowing distally (Fig. 4A); paramere brown, minute; oviscapt black on ventral margin, roundish at caudal tip, with ca. 17 orange marginal and ca. 5 lateral ovisensilla (Fig. 6A); spermatheca brown, with introversion half of outer capsule height (Fig. 7A). Of them, the most remarkable difference was seen in the pubescence on the cercus between YK and TS: the cercus was bare in YK, but widely pubescent in TS (Fig. 2B). In addition, the epandrium was pubescent only on the upper portion in YK, but on medial to upper portion in TS *castanea* (Figs 2A, B), and the spermathecal introversion duct was slightly higher in YK than in TS (Figs 7A, B). However, these characters in the epandrium and spermatheca varied among individuals within the same local strains, with the extreme case of YK strains resembling TS strains. Any discriminative difference was not detected in the surstylus (Fig. 3B), the aedeagus and its adjacent organs (Figs 4–5B), or the female terminalia (Fig. 6B) between YK and TS strains.

Table 1 gives the results of comparisons for the 23 quantitative characters between YK and TS strains. Twelve out of 23 characters (Nos. 2–4, 9, 10, 14, 15, 18–22) showed statistically significant differences between the strains, but with overlapped ranges between them in all characters.

Table 2 gives the percentage ratios of successful mating between YK and TS strains in no-choice method. The two strains mated well with each other in both directions, but the ratios of successful mating were slightly lower in the crosses between the differ-



Figures 2–7：Male and female genitalia of Yakushima (A) and Tsushima (B) strains of *Liodrosophila castanea*. 2, Epandrium and cercus; 3, surstylus; 4, aedeagus and adjacent organs in ventral view; 5, aedeagus in lateral view; 6, oviscapt; 7, spermatheca. Abbreviations: a, paramere; c, surstylus; e, aedeagus; g, epandrium; o, aedeagal apodeme; t, cercus, v, hypandrium. Bars: 0.1 mm in Figs. 1, 5; 0.05 mm in other figures.

Table 1：Variations of quantitative characters in two chromosomal strains of *Liodrosophila castanea*

Quantitative character*		Yakushima			Tsushima			Difference between strains (t-test)
		Mean±S.D.	Range	(n)	Mean±S.D.	Range	(n)	
1. BL(mm)	♂	1.96±0.14	1.80–2.20	(10)	1.86±0.23	1.60–2.36	(10)	ns
	♀	2.12±0.16	1.81–2.40	(10)	2.12±0.27	1.76–2.68	(10)	ns
2. TL(mm)	♂	0.77±0.05	0.72–0.88	(10)	0.70±0.04	0.66–0.80	(10)	$P<0.01$
	♀	0.81±0.05	0.72–0.88	(10)	0.76±0.05	0.68–0.84	(10)	$0.01<P<0.05$
3. WL(mm)	♂	2.02±0.10	1.84–2.12	(10)	1.79±0.08	1.68–1.92	(10)	$P<0.01$
	♀	2.08±0.07	1.96–2.20	(10)	1.94±0.08	1.84–2.12	(10)	$P<0.01$
4. WW(mm)	♂	0.77±0.04	0.72–0.84	(10)	0.66±0.02	0.62–0.72	(10)	$P<0.01$
	♀	0.80±0.03	0.76–0.84	(10)	0.73±0.06	0.68–0.88	(10)	$P<0.01$
Head								
5. FW/HW		0.50±0.02	0.47–0.53	(20)	0.49±0.02	0.45–0.56	(20)	ns
6. db of Ar		4.75±0.44	4.0–6.0	(20)	4.45±0.60	4.0–6.0	(20)	ns
7. vb of Ar		2.00±0.00	2	(20)	2.00±0.00	2	(20)	ns
8. G/E		0.21±0.05	0.13–0.30	(20)	0.20±0.03	0.15–0.27	(20)	ns**
9. Or2/Or1		0.78±0.11	0.60–1.00	(20)	0.90±0.11	0.67–1.00	(20)	$P<0.01$
10. Orb3/Orb1		0.62±0.05	0.50–0.69	(20)	0.68±0.05	0.60–0.75	(20)	$P<0.01$
Thorax								
11. U/L of Hu		0.96±0.10	0.67–1.00	(20)	1.01±0.08	0.86–1.33	(20)	ns
12. DcA/DcP		0.71±0.07	0.62–0.85	(20)	0.65±0.08	0.44–0.79	(20)	ns
13. Ld/Cd of Dc		0.62±0.05	0.54–0.74	(20)	0.55±0.08	0.48–0.77	(20)	ns**
14. SctP/SctA		0.65±0.11	0.33–0.79	(20)	0.56±0.06	0.45–0.65	(20)	$P<0.01$**
15. SctA-SctP/SctPs		0.72±0.08	0.57–0.86	(20)	0.64±0.05	0.53–0.71	(20)	$P<0.01$
16. Sterno-index		0.30±0.08	0.18–0.50	(20)	0.26±0.05	0.20–0.36	(20)	ns**
Wing indices								
17. C		1.61±0.11	1.46–1.87	(20)	1.63±0.12	1.45–1.90	(20)	ns
18. 4V		1.83±0.08	1.68–1.94	(20)	1.91±0.10	1.65–2.06	(20)	$P<0.01$
19. 4C		1.30±0.05	1.21–1.41	(20)	1.36±0.09	1.21–1.57	(20)	$0.01<P<0.05$**
20. 5x		1.65±0.14	1.42–1.90	(20)	1.61±0.18	1.38–2.00	(20)	$P<0.01$
21. Ac		3.48±0.26	3.07–4.17	(20)	3.64±0.32	3.08–4.40	(20)	$P<0.01$
22. M		0.52±0.04	0.45–0.59	(20)	0.49±0.05	0.42–0.58	(20)	$0.01<P<0.05$
23. C3-fringe		0.60±0.06	0.50–0.71	(20)	0.63±0.06	0.55–0.80	(20)	ns

* Abbreviations are explained in the text.
** Aspin-Welch method was adopted in the case of unequal variance (F-test, $\alpha=0.05$).

ent strains than in the crosses within the same strain. Although asymmetric mating preference has been frequently reported in closely related species or subspecies of *Drosophila* (Stalker, 1942; Watanabe and Kawanishi, 1979; Coyne and Orr, 1997; Wang et al., 2007), the indices of pre-zygotic isolation were nearly identical between the reciprocal crosses, being 0.338 between YK females and TS males and 0.275 between TS females and YK males.

Table 3 shows the results of crosses by the choice-by-male method. In both crosses the isolation index was nearly 0, indicating the absence of mating preference of males for females with different karyotypes.

We maintained the culturing vials containing both YK and TS flies for 2 weeks, and hybrid offspring appeared in both crossing directions. In the backcross, F_1 males mated to its parental females and produced fertile adults. Further, any syndrome of hybrid deficiency, such as viability, longevity or fecundity, was not observed in the adult flies obtained in the backcrosses or of F_2. This means a lack of post-mating isolation between the two chromosomal strains.

It is reasonable to assume that extra-chromosomes involved in the nuclear plates of TS strains might have derived from fragmentations of heterochromatic autosomes in those of YK and OK strains, and thus the latter would be more primitive in the karyotype evolution. At the present knowledge, these two types of *L. castanea* are allopatric in the geographic range, but any reproductive isolation was not observed in the crosses between these strains. These results imply that two geographic populations with the different karyotypes can be regarded as chromosomal races, rather than subspecies, although a slight morphological difference was found in the male genitalia (Mayr, 1974; Stamos, 2003).

Supernumerary chromosomes have been known in many taxonomic groups of invertebrates: for example, White (1972) demonstrated the chromosomal evolution and speciation by extra chromosomes in the grasshopper *Moraba viatica*. However, adaptive significance of extra-chromosomes in the natural population of *L. castanea* is quite open to question. This species has been presently known from Korea, China and southern districts of Japan (Okada 1988; Zhang et al., 1996; Bächli, 2007), and further studies on the karyotype and biology of this species are indispensable to clarify its genetic differentiation in relation to adaptation to local environments in many populations, especially in Kyushu locating between Tsushima and Yakushima.

Table 2 : Percentages of inseminated females in the crosses between two geographic strains of *Liodrosophila castanea*, Yakushima (YK) and Tsushima (TS), by no choice method. The numbers in parentheses and square brackets give the number of females examined and the pre-zygotic isolation index by Coyne and Orr (1989), respectively.

♂/♀	YK	TS
YK	82.4(68)	59.7 (67)[0.275]
TS	49.3(71)[0.338]	74.5(145)

Table 3 : Numbers and percentages (in parentheses) of females inseminated (+) and uninseminated (−) in crosses between two geographic strains of *Liodrosophila castanea*, Yakushima (YK) and Tsushima (TS), by choice-by-male method, and the calculated sexual isolation indices (I.I.) with the results of statistical tests.

Crosses		Homogamic		Heterogamic		I.I.
Female	Male	+	−	+	−	
YK+TS	YK	38(82.6)	7(17.4)	27(56.3)	21(43.7)	0.189^{ns*}
YK+TS	TS	34(72.3)	13(27.7)	28(59.6)	19(40.4)	0.096^{ns}

* ns: statistically non-significant.

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