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Taxonomic Problems in the *Drosophila melanica* Species Group (Diptera: Drosophilidae) from Southern China, with Special Reference to Karyotypes and Reproductive Isolation

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Karyotypes and reproductive isolation were studied in two allopatric populations of *Drosophila tsigana*, one from Guizhou Province in southern China and the other from Hokkaido in northern Japan, and in one population of a closely related species, *D. longiserrata*, from Guizhou. In metaphase plates of larval brain cells, both geographic strains of *Drosophila tsigana* showed 2n=10 chromosomes, with 2 pairs of metacentric (V-shape), 2 pairs of acrocentric (R-shape), and 1 pair of dot-liked (D-shape) chromosomes. *Drosophila longiserrata* showed the same number, 10 chromosomes, comprising 2V, 1J (sub-metacentric chromosome), 1R, and 1D. X chromosomes of both species were acrocentric, the presumed ancestral form. Premating isolation was complete between *D. tsigana* and *D. longiserrata*, and successful mating was also limited in crosses between the two geographic populations of *D. tsigana*, especially in crosses between Japanese (JP) females and Guizhou (GZ) males. F_1 hybrids were obtained only from crosses between GZ females and JP males, and fertilities of both F_1 females and males were quite incomplete. The results of morphological observations, karyotypic analyses, and crossing experiments clearly showed that the GZ and JP populations of "*D. tsigana*" were highly divergent from each other and that each population should be recognized as a biologically valid species. The present morphological observations and chromosomal analyses, together with the original descriptions, strongly suggest that "Guizhou *D. tsigana*" might be conspecific with *D. bisetata* Toda, 1988 from Myanmar, and that *D. longiserrata* might be conspecific with *D. afer* Tan, Hsu, and Sheng, 1949 from Meitan, Guizhou.

Key words: *Drosophila melanica* group, *D. tsigana*, isolating mechanism, karyotype, southern China

INTRODUCTION

Most members of the *Drosophila melanica* species group in the *virilis-repleta* radiation inhabit temperate deciduous forests in the northern Hemisphere (Throckmorton, 1975; Levitan, 1982). Five species of the *melanica* group are hitherto known from the Old World: *D. afer* Tan, Hsu, and Sheng, 1949; *D. bisetata* Toda, 1988; *D. longiserrata* Toda, 1988; *D. moriwakii* Okada and Kurokawa, 1957 (of questionable systematic position: Levitan, 1982; Beppu, 1988; Wang *et al*., 2006); and *D. tsigana* Burla and Gloor, 1952. *Drosophila tsigana* has a distributional pattern in Eurasia that is disjunct between Europe (including Austria, France, Hungary, Portugal, and European parts of Russia; Bächli, personal communication) and Northeast Asia, including Jilin and Liaoning Provinces of China (Zhang *et al*., 1996), with geographic variation in external morphology; its eastern Asiatic population was at one time identified as a separate species, *Drosophila pengi* Okada and Kurokawa, 1957 (Watabe *et al*., 1990).

The *melanica* group has been less explored in southern parts of China. Tan *et al.* (1949) reported only *D. afer* from Meitan, Guizhou Province, but the phylogenetic position of this species has been quite uncertain due to a lack of information since the original description.

We recently collected two species of the *melanica* group from Anshun, Guizhou and succeeded in establishing isofemale strains from them for the study of cytology and reproductive isolation. Prof. M. J. Toda carefully examined the male genitalia of these two species and tentatively identified them as *D. tsigana* and *D. longiserrata*. The latter identification represents a new record for China.

Although the phylogenetic relationships of the *melanica*

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species group were studied extensively and intensively by Stalker (1966, 1972), most of the species studied were from the New World, and very few species from the Old World were investigated. In the course of a phylogenic study of the *Drosophila virilis* section using DNA sequences, we found *D. tsigana*, with two geographic populations from Guizhou and Japan, to be paraphyletic in the molecular tree: Guizhou *D. tsigana* is more closely related to a sympatric species, *D. longiserrata*, than to Japanese *D. tsigana*, with high probability (Wang *et al*., 2006). Furthermore, Guizhou *D. tsigana*, like European *D. tsigana*, was found to be darker in color and smaller in body size than Japanese *D. tsigana* (Watabe *et al*., 1990), although we could not find any diagnostic characters in genitalia structure to discriminate between them.

In order to study isolating mechanisms, we performed crossing experiments between the two allopatric populations of *D. tsigana* by no-choice and mate-choice methods. We also checked crossability between *D. tsigana* and *D. longiserrata*. Finally, in the context of our results, we examined karyotypes of the two *D. tsigana* populations and *D. longiserrata*. Based on all the results obtained, we discuss the geographic distribution and taxonomic status of *D. tsigana* and *D. longiserrata*, in relation to two members of the *melanica* group reported from Myanmar (Toda, 1988).

MATERIALS AND METHODS

Three geographic strains of *D. tsigana* were used, two (designated GZ-1 and -2) collected from Anshun (ca. 700 m in altitude), Guizhou, China in August 2002, and the third (JP) collected from Sapporo (ca. 300 m), Hokkaido, Japan in August 2004. One strain of *D. longiserrata* from the same collection site as *D. tsigana* GZ-1 and -2 was also employed. Each living strain for crossing experiments and karyotype observations was established from a single inseminated female caught in the wild.

Morphological observations

The body size of adult flies is quite variable with changes in to culture conditions such as food and temperature. About 10 mature females were allowed to oviposit in glass vials (30 mm in diameter, 100 mm in height) at 20°C, each containing 10 ml of standard *Drosophila* medium (malt, yeast, corn meal, sucrose). When young flies emerged, they were transferred to new vials every three or four days. Thorax length, including the scutellum, and thorax width were measured by micrometer for twenty individuals (10 males and 10 females) of the GZ-1 and JP strains. Color patterns on the abdominal tergites were also checked in the GZ and JP strains and in their hybrids under the same rearing conditions as described above.

Karyotype analysis

For preparation of mitotic chromosomes, neuroblasts of 3rd-instar larvae were treated with a 0.1 mg/ml colchicine solution for 30 min and 1% of sodium citrate for 20 min, fixed with a methanol:acetic acid solution (3:1) for 2 hrs, stained with 4% Giemsa solution for at least 30 min, and then air-dried (Imai *et al*., 1977). About 100 nuclear plates were examined for each species or geographic strain. Photographs of metaphase chromosomes were taken with an analog camera (Olympus PM-6) and then converted to digital images with a film scanner (Nikon APS IX240).

Crossing experiments

When adult flies emerged, they were collected every 24 hrs, sorted to sex, and maintained in vials with *Drosophila* medium at 20°C under continuous illumination. All crossing experiments were carried out using 16- to 18-day-old flies, since both *D. tsigana* and *D. longiserrata* are slow breeders (Watabe *et al*., 1990). For the no-choice method, five males and the same number of alien or same-strain females were put together into a vial (30 mm in diameter, 100 mm tall). After 48 hrs, the females were taken out, dissected in Ringer's solution, and examined for sperm in the spermathecae and seminal receptacles. More than 50 (usually 150) females were checked in each crossing. The index of prezygotic isolation for each cross-pair was estimated by the formula of Coyne and Orr (1989): index=1– (% of heterogamic matings (*i.e.*, A♀ x B♂)) / (% of homogamic matings (*i.e.*, A♀ x A♂)). This formula gives a value of 1 when isolation is complete and a value of 0 for free crossings.

For mate-choice experiments, the choice-by-male method was applied. Five males and ten (five same-strain and five alien) 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 males, respectively. This formula gives a value of 1 for complete mating preference and 0 for lack of mating preference between two strains.

When hybrid adults were obtained, postmating isolation was checked by backcrossing the hybrids with the parental strains.

RESULTS

Morphological observations

Fig. 1 shows schematic color patterns on abdominal tergites of the Guizhou and Japanese strains of *D. tsigana*. GZ males are almost entirely black on the second to fourth tergites, whereas JP males have black caudal bands clearly interrupted in the middle on these tergites. In females, the

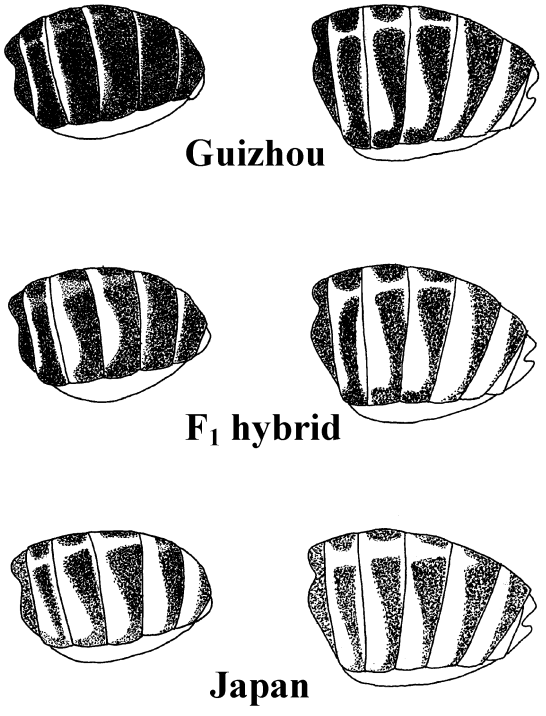

Fig. 1. Color patterns on the abdominal tergites (lateral view, slightly slanted) of different geographic strains of *Drosophila tsigana*. Top, Guizhou strains (GZ-1 and -2); bottom, Japanese strain; middle, F1 hybrid from crosses between Guizhou females and Japanese males. Left, males; right, females.

caudal bands on the tergites are much darker in the GZ than in the JP strain. A similar difference in abdominal color pattern was detected between European and Japanese populations of *D. tsigana*, which Watabe *et al.* (1990) regarded as conspecific. The abdominal color pattern of GZ *D. tsigana* is very similar to that of European *D. tsigana* from the Pyrenees Mountains. Although it is well known that body color of drosophilid flies is somewhat variable with culture temperature (Watabe, 1977), the body color of the *D. tsigana* strains studied was constant. Furthermore, we obtained F_1 adults from the crosses between GZ females and JP males (see Results, crossing experiments), and their color pattern was nearly identical to that of the GZ strains for both sexes.

Fig. 2 shows thorax length, including the scutellum, of the GZ and JP strains of *D. tsigana*. GZ *D. tsigana* was smaller in size than the JP strain, although the range in thorax length of males partially overlapped between these two geographic strains.

Karyotypes

Fig. 3A–C shows male metaphase configurations of the two strains of *D. tisgana* (GZ-1 and JP) and *D. longiserrata*. The karyotype of GZ *D. tsigana* comprised 10 chromosomes in diploid number, with 2 pairs of metacentric chromosomes (V-shaped, V), 2 pairs of acrocentric chromosomes (rod-shaped, R), and 1 pair of micro-chromosomes (dot-like, D). The X chromosome was acrocentric. This karyotype of 2n=10 with 2V+2R+1D is identical to that of Japanese *D. tsigana* (Fig. 3B; Watabe *et al.*, 1997), but slightly different from that of European *D. tsigana* with 2n=10 (1V+1J+2R+1D) (Burla and Gloor, 1952).

Drosophila longiserrata has the same number of chromosomes (2n=10) as *D. tsigana,* with a karyotype of 2V+1J+1R+1D (Fig. 3C). It is noteworthy that the X chromosomes of both *D. tsigana* and *D. longiserrata* are rod-shaped, the suggested "ancestral-like" form in the chromosomal evolution of the *melanica* species group (Stalker, 1966, 1972; Levitan, 1982; Clayton and Guest, 1986).

Crossing experiments

We did not observe any successful mating at all between GZ or JP of *D. tsigana* and *D. longiserrata* in either direction, indicating the presence of complete premating isolation. Table 1 gives the percentage ratios of successful mating between the two geographic populations of *D. tsigana*. In the no-choice tests, two iso-female strains (GZ-1 and -2) from Guizhou and one strain (JP) from Japan were used. The two parallel strains of GZ *D. tsigana* showed nearly the same results. Crosses between GZ females and JP males showed significantly higher ratios of successful copulation than the reciprocal crosses (χ^2=41.9 for GZ-1 vs JP and 56.6 for GZ-2 vs JP; p<0.01). According to the formula of Coyne and Orr (1989), the indices of premating isolation between GZ females and JP males were 0.321 (GZ-1♀ x JP♂) and 0.299 (GZ-2♀ x JP♂), which were much lower than those of the reciprocal direction, 0.868 (JP♀ x GZ-1♂) and 0.917 (JP♀ x GZ-2♂). Such asymmetric mating preferences have been frequently detected between closely related species or subspecies (Watanabe and Kawanishi, 1979; Coyne and Orr, 1989, 1997; Watabe *et al.*, 1990). Furthermore, JP *D. tsigana* more readily mated to flies of its own strain than did GZ under laboratory conditions.

Table 2 shows the results of sexual isolation between GZ and JP *D. tsigana* in the choice-by-male tests. In the

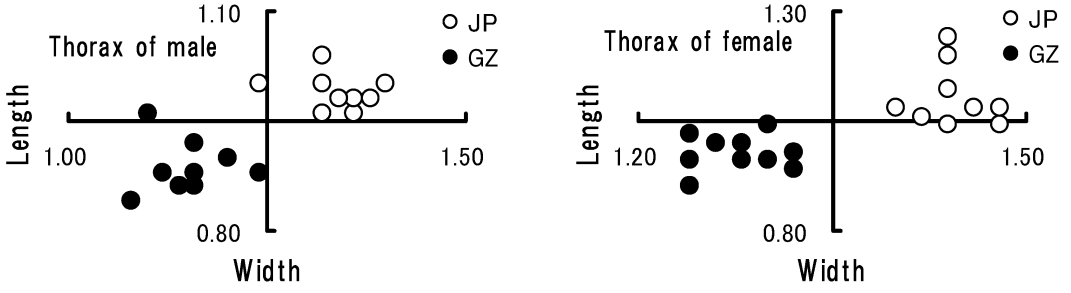

Fig. 2. Thorax length and width (in mm) in the Guizhou (GZ) and Japanese (JP) strains of *Drosophila tsigana*. Left, males; right, females.

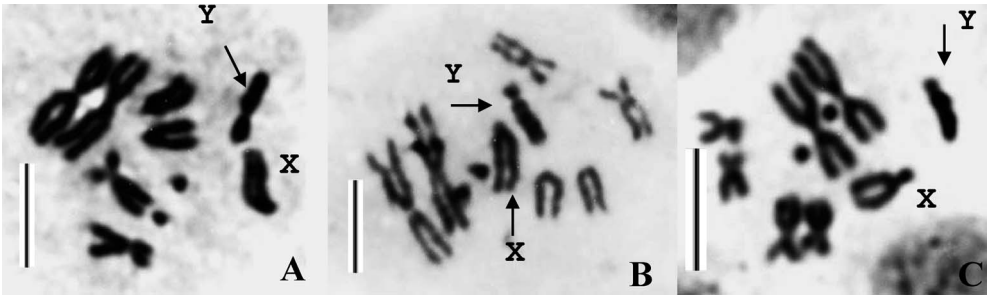

Fig. 3. Male metaphase chromosomes of *Drosophila tsigana* and *D. longiserrata*. **A)** Guizhou strain of *D. tsigana* (GZ-1); **B)** Japanese strain of *D. tsigana*; **C)** *D. longiserrata*. Scale bars indicate 10 μm.

Table 1. Percentages of inseminated females in crosses between two Guizhou (GZ-1, 2) and one Japanese (JP) strains of *Drosophila tsigana* by no choice method.

♀ \ ♂	JP	GZ-1	GZ-2
JP	89.1 (175)	11.8 (153) [0.868]	7.4 (149)[0.917]
GZ-1	46.3 (147) [0.321]	68.2 (151)	–
GZ-2	45.1 (193) [0.299]	–	64.3 (56)

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.

Table 2. Sexual isolation between Guizhou (GZ) and Japanese (JP) strains of *Drosophila tsigana* in crosses by the choice-by-male method.

Crosses		Homogamic		Heterogamic		I.I.
Female	Male	+* (%)	–* (%)	+ (%)	– (%)	
GZ JP	GZ	21 (56.8)	16 (43.2)	0 (0.0)	43 (100.0)	1.00
GZ JP	JP	37 (84.1)	7 (15.9)	25 (54.3)	21 (45.7)	0.22**

*+: inseminated, –: uninseminated. **Statistically significant (χ^2-test, p<0.01)

cross of GZ♀+JP♀/GZ♂, the isolation index was 1.00, indicating the complete mating preference of GZ males. In the cross of GZ♀+JP♀/JP♂, the value was 0.22 (χ^2=29.8, p<0.01), which meant that JP males also tended to mate with their own females, although the mating preference of JP males was not perfect.

We maintained the culture vials with JP females x GZ males for 60 days, but no hybrid offspring appeared. We examined 50 eggs deposited by these females, and no embryonic development was observed. This suggests perfect premating isolation between JP females and GZ males. In contrast, F_1 adults appeared on day 60 after crossing between GZ females and JP males. Although we maintained female and male hybrids together in a vial for 30 days, we did not obtain any F_2 flies. Some backcrosses were then conducted to check the fertility of F_1 males and females (Table 3). No adult flies emerged from the crosses between F_1 males and GZ females or in the reciprocal cross, whereas a few progeny appeared in reciprocal crosses between F_1 hybrids and JP *D. tsigana*. These results clearly indicate incomplete fertility in F_1 hybrids obtained from the cross between GZ females and JP males.

Table 3. Production of progeny in crosses between GZ, JP strains and their F1 (GZ♀ x JP♂) hybrids of *Drosophila tsigana*.

♀ \ ♂	(GZ ♀ x JP ♂) F_1	GZ	JP
(GZ ♀ x JP ♂) F_1	None	None	+
GZ	None	+++	++
JP	+	None	+++

+, ++ and +++ indicate relative abundance of progeny.

DISCUSSION

The differences we found in abdominal color patterns and the presence of reproductive isolation between the GZ and JP populations of *D. tsigana* demonstrate that these allopatric populations have greatly differentiated even to the species level, although no discriminating characters have been detected in the structures of the male and female genitalia. In our previous molecular trees, regardless of the gene used or tree-reconstruction methods, Guizhou *D. tsigana* always grouped with *D. longiserrata*, with a high level of support, whereas Japanese *D. tsigana* was distant from them (Wang *et al*., 2006). Therefore, "*D. tsigana*" from Guizhou should be regarded as a biologically valid species, distinct from *D. tsigana* distributed in Europe and East Asia, including Northeast China. Broad lowlands along the Yangtze River in mid-eastern China may act as a barrier separating the distributional ranges of these two species.

If our conclusion is correct, it raises a new question about the taxonomy of Guizhou *D. tsigana*. Toda (1988) reported a new species of the *melanica* group from Myanmar, *D. bisetata*, from a single male specimen, and stated that "morphological and genitalia characters of this species are very similar to those of *D. tsigana* and *D. longiserrata*, except for characteristically having 2 pairs of submedian (= paramedian) spines on the hypandrium in *D. bisetata* (a pair of such spines in the latter two species)". During the present study, we found a variant male with 2 paramedian spines on one side of the hypandrium and a single one on the other side in the iso-female line GZ-1. Except for this character, no difference in morphology was detected between Guizhou *D. tsigana* and *D. bisetata*. This suggests that Guizhou *D. tsigana* may be conspecific with *D. bisetata*, and that the holotype specimen of the latter, with 2 pairs of paramedian spines, is probably a variant.

Tan *et al*. (1949) described a member of the *melanica* group, *D. afer*, from Meitan, Guizhou, but did not refer to any structures of the genitalia. However, Toda (1988) stated that "there remains a doubt whether *D. afer* may nor may not be conspecific with any one of the three species, *D. tsigana, D. bisetata* and *D. longiserrata*, until *D. afer* is re-examined" when he described the latter two species from Myanmar. The karyotype of *D. longiserrata* from Guizhou, 2n=10 (2V, 1J, 1R, 1D), is identical to that of *D. afer* reported by Tan *et al*. (1949), and the two species are very similar in external morphology. These facts strongly suggest that *D. longiserrata* is conspecific with *D. afer*.

The origin of the *melanica* group is still open to question, although Throckmorton (1975) postulated an Asian origin without showing any data on the geography or phylogeny of the group. Both Guizhou *D. tsigana* (probably=*D. bisetata*) and *D. longiserata* (probably=*D. afer*) possess the "ancestral form" of rod-shaped X chromosomes, and two cool-temperate species of East Asia, *D. tsigana* itself and *D. moriwakii*, also retain the same type of sex chromosome. Six of the seven North American species of the *melanica* group have a derived form of V-shaped X chromosome (Stalker, 1972; Levitan, 1982). Based on this geographical and chromosomal information, it is reasonable to assume that the *melanica* species group might have emerged in southern China, probably the Yun-Gui Highlands and adjacent mountains, as a branch of the *virilis-repleta* radiation, spread to higher latitudes along green belts of East Asia, and then migrated to North America via Beringia and diversified in temperate deciduous forests there.

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