Experimental Hybridisation between X0 and XY Chromosome Races in the Grasshopper *Podisma sapporensis* Shir. (Orthoptera, Acrididae). I. Cytological Analysis of Embryos and F1 Hybrids*

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The results of experimental hybridisation between some chromosome subraces belonging to the X0 and XY chromosome races of the brachypterous grasshopper *P. sapporensis* are presented. Pre-zygotic reproductive isolation mechanisms in experimental pairs were not confirmed. In crossings of XY-standard x X0-standard and XY-standard x X0-Naganuma chromosome subraces, a zygotic barrier has been found. All embryos of XY-standard x X0-standard crosses and the vast majority of embryos of XY-standard x X0-Naganuma crosses were obtained from female diploid or haploid/diploid cells as a result of parthenogenesis. In very rare cases, when the zygotic barriers had been surmounted, normal embryo heterozygotes and a F1 hybrid generation were obtained in XY-standard x X0-Naganuma crosses. On the contrary, crosses between the XY-Tanno and X0-standard subraces gave viable offspring in spite of many chromosome differences such as a X-A translocation and fixed pericentric inversions in four pairs of autosomes. The results obtained do not support the hypothesis that chromosomal differences play a key role in restricting gene flow between X0 and XY races of *P. sapporensis*. The presence of crossing barriers explains the phenomena of the purity of the X0 and XY chromosomes races.

Key words: Grasshoppers, *Podisma sapporensis*, chromosome races, experimental crossing, zygotic barrier.

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There is great interest among evolutionary biologists for determininge the role of chromosome changes in speciation. The discussion on chromosome speciation focuses on the stasipatric model of speciation (WHITE *et al*. 1967; WHITE 1968)**.** One of the important features of the stasipatric model is that certain chromosomal changes play a key role in the initial stages of speciation. These changes are capable of generating isolating mechanisms as a result of the reduced fitness of chromosomal heterozygotes arising from abnormalities in meiosis. WHITE (1968) proposed that the chromosomal changes become fixed in small semi-isolated or isolated populations.

The concept of chromosomal speciation stimulated the development of cytogenetic studies in different groups of animals. In grasshoppers, studies have shown that karyotypic differences are only weakly selected markers for more fundamental changes in the genome (BARTON 1980; SHAW & WILKINSON 1980; BARTON & HEWITT 1981; COATES & SHAW 1982; SHAW *et al.* 1998). In spite of the discussion surrounding the concept of chromosome speciation in grasshoppers, there are very few models on this problem. The grasshopper *Podisma sapporensis* Shir. may be one in this respect.

P. sapporensis Shir. is distributed in Hokkaido, Sakhalin and Kunashiri islands. In this species two main chromosome races can be distinguished. The population from Sakhalin Is. (Krylion peninsula) and the western group of populations in Hokkaido, belonging to the X0 race, have a diploid number of chromosomes 2n=23 in the male and 2n=24 in the female (sex determination X0 male/XX female). The population from Kunashiri Is. and the eastern group of populations in Hokkaido Is., belonging to the XY race, differ from the western one as a result of a Robertsonian translocation between the originally acrocentric X-chromosome and M_5 autosome in homozygous state, having resulted in the forming of chromosome sex determination neo-XY in males and neo-XX in females (2n=22) (BUGROV 1995; BUGROV *et al.* 2000). In Hokkaido these races are geographically isolated by a mountain system consisting of the Daisetsu and Hidaka ranges, occupying the central part of Hokkaido island. Natural hybrid zones between the races have not been discovered so far.

Various levels of polymorphism in the pericentric inversions, B-chromosomes, and C-banding variation exist in different chromosomes in populations of both chromosome races (WARCHAŁOWSKA-ŚLIWA *et al.* 2001; BUGROV *et al.* 2001; BUGROV *et al.*, 2003). In some populations chromosome changes are fixed in one or some pairs of chromosomes, which enables the identification of discrete chromosome subraces (BUGROV *et al.* 2001). The high level of chromosome differentiation among populations in *P. sapporensis* suggests that this species may consist of several biological species, since karyotypic diversification often leads to reproductive isolation owing to hybrid incompatibility (JOHN & LEWIS 1965; WHITE *et al.* 1967, WHITE 1969; JOHN & WEISSMAN 1977; MRONGOVIUS 1979; KING 1993). In accordance with the biological species concept, crossing experiments between chromosome races and subraces of *P. sapporensis* can verify this suggestion.

The aims of the present study were a) to determine the success in formation of hybrid embryos between the X0 and XY chromosome races, and b)

to examine the laboratory-bred F1 generation between X0 and XY chromosome races in *P. sapporensis.*

Material and Methods

Collection and laboratory crossing

In early June 2000, nearly 120 male and female nymphs and 60 adult males of *P. sapporensis* were collected in the areas of distribution of the X0-standard, X0-Naganuma, XY-standard and XY-Tanno chromosome subraces. They were kept in the laboratory's insectaria at 20-22°C during the day and at 15-18°C during the night. In the laboratory the male and female nymphs were placed in separate insect plastic boxes according to their chromosome subrace. Every day fresh *Petasites* leaves were put in the cages for food. As the nymphs matured, experimental pairs were set up in individual cages. Ten experimental (crosses) pairs of XY-standard (♀) x X0-standard (♂), 8 pairs of XY-standard (♀) x X0-Naganuma (♂), and 23 pairs of XY-Tanno (♀) x X0-standard (♂) were made. At the same time 10 control pairs of each combination: X0-standard x X0-standard, X0-Naganuma x X0-Naganuma, XY-standard x XY-standard, and XY-Tanno x XY-Tanno, were made. After about 5 days the pairs started to copulate. After 10-20 days of adult life, females began to lay pods of 9-17 or so eggs, in moist coarse sand. Usually each female laid 2-3 egg pods.

Cytogenetic analysis of the embryos

Each egg pod was stored in a separate cell of a plastic container with moist sand and kept at 20-22°C by day and 15-18°C by night. After 15-20 days of incubation, 3-5 eggs from each egg pod were used for cytogenetic analysis. For this they were placed in a solution of 0.05% colchicine in insect saline and the tops of the non-micropylar ends were removed. They were then incubated at 30 C° for 1.5-2 h. Next the embryos were dissected from the eggs into a 0.9% sodium citrate solution for 20-30 min at room temperature prior to fixation in 3:1 alcohol/acetic acid. Air-dried preparations were made on precleaned slides by macerating the embryos in a drop of 60% acetic acid. They were then stained with C-banding by treatment with 0.2 N HCL for 15-20 min, immersed in a saturated solution of Ba(OH)$_2$ at 61°C for 3-5 min, rinsed in water, immersed in 2xSSC at 61° C for 60 min, rinsed, air-dried, and stained with 2% Giemsa.

Cytogenetic analysis of the F1 hybrid males

The rest of the eggs in the pods were stored in the separate cells of plastic boxes with moist sand at 20-22°C by day and 15-18°C by night for 2 months. The egg pods were then kept at 1-4°C for 6 months, simulating winter conditions and breaking diapause. In the spring each egg pod was put into an individual plastic box at room temperature (18-24°C) to hatch. After hatching each egg pod was removed and eggs were inspected under a binocular microscope. The number of eggs in the egg pod and the number of eggs with embryos were determined. The data are summarized in Table 1.

In the laboratory (Novosibirsk State University, Russia, Siberia) *P. sapporensis* ate the leaves of *Tussilago sibirica* and *Arctium lappa*. As the insects matured, single pairs were set up as before, in order to obtain the F2 generation. After laying the first egg pod, the male was used for cytogenetic analysis. For this it was injected with a 0.1% colchicine for 1.5-2.0 hours. The testes were fixed in alcohol/acetic acid (3:1), and kept in 70% ethanol. Air dried preparations were made by squashing the testis follicles in 45% acetic acid and freezing them in dry ice. They were stained using C-banding methods similar to those for the embryos.

Results

Cytogenetic analysis of the experimental populations

The grasshoppers collected in the areas distribution of chromosome races and subraces described earlier (BUGROV *et al*., 2001) were studied cytologically. The samples collected in the vicinities of Sapporo (Mt Teine) were represented by the X0-standard subrace. This chromosome subrace corresponds to the more usual chromosome complement of Acrididae, consisting of 23 acrocentric chromosomes in males and 24 in females (Fig. 1a). The samples from the vicinities of Naganuma belonged to X0-Naganuma chromosome subrace. The X0-Naganuma chromosome subrace differs from the X0-standard subrace in respect of the fixed pericentric inversion in the M_6 chromosome and a high frequency of heterozygotes and homozygotes for the inversion in M_5 (Fig. 1b).

The XY race was represented by XY-standard and XY-Tanno subraces. The XY-standard chromosome subrace has 10 pairs of acrocentric chromosomes and two sex chromosomes (the metacentric neo-X and the acrocentric neo-Y in the male and the two metacentric neo-X's in the female) (Fig. 1c). The XY-Tanno chromosome subrace clearly differs from the populations with standard XY karyotype in showing a high frequency of homozygotes for inversions in some large and medium sized pairs of autosomes (Fig. 1d).

Cytogenetic analysis of the F1 embryos and F1 males of XY-Tanno x X0-standard crosses

Ethological barriers to mating between individuals from the XY-Tanno (female) and X0-standard (male) were not found. The number of pods laid and the numbers of eggs per pod did not differ significantly between the hybrid and control crosses. The percentage of fertilized eggs of control crosses was significantly higher than eggs with embryos in experimental ones (Table 1).

Eighty seven embryos from the XY-Tanno x X0-standard crosses were cytogenetically examined. The vast majority of the embryos studied cytologically (89,7%) were heterozygotes and contained genomes of both parents (Fig. 2a). Four embryos (4.12%) consisted of diploid and haploid female cells (Fig. 2b,c).

Table 1

Comparison of eggs per pod and eggs with embryos produced among cross combinations

Crossing pairs	No. eggs per pod ($\pm$SD)	% eggs with embryos (n)	No. eggs with embryos per pod ($\pm$SD)
X0-standard x X0-standard	13.67 (0.41)	96.4 (268)	12.94 (0.39)
X0-Naganuma x X0-Naganuma	13.88 (0.33)	93.6 (283)	12.76 (0.45)
XY-standard x XY-standard	14.07 (0.40)	98.1 (288)	12.46 (0.28)
XY-Tanno x XY-Tanno	12.90 (0.31)	89.80 (257)	12.24 (0.34)
XY-standard x X0-standard	13.40 (0.45)	63.88 (264)	7.56 (0.54)
XY-standard x X0-Naganuma	14.01 (0.35)	58.5 (185)	6.68 (0.48)
XY-Tanno x X0-standard	13.24 (0.4)	94.68 (390)	12.54 (0.36)

The adult F1 males from this type crossing had normally developed testes. At meiosis large autosomes form bivalents with 3 or 2 chiasmata; medium autosomes – 2 or 1 chiasmata; small bivalents – only one chiasma. The mean frequency of chiasmata per cell is x=15,05 (±1.79). Chiasmata did not usually form between regions of the chromosomes involved in pericentric inversions in heteromorphic bivalents. The X_R-arm of the neo-X chromosome belonging to the XY-Tanno female associated with homologous M_5 chromosome, belonging to the X0-standard males, formed 1 or 2 chiasmata (Fig. 3a).

Sporadic non-sister associations between two or three autosome bivalents (Fig. 3b) were observed.
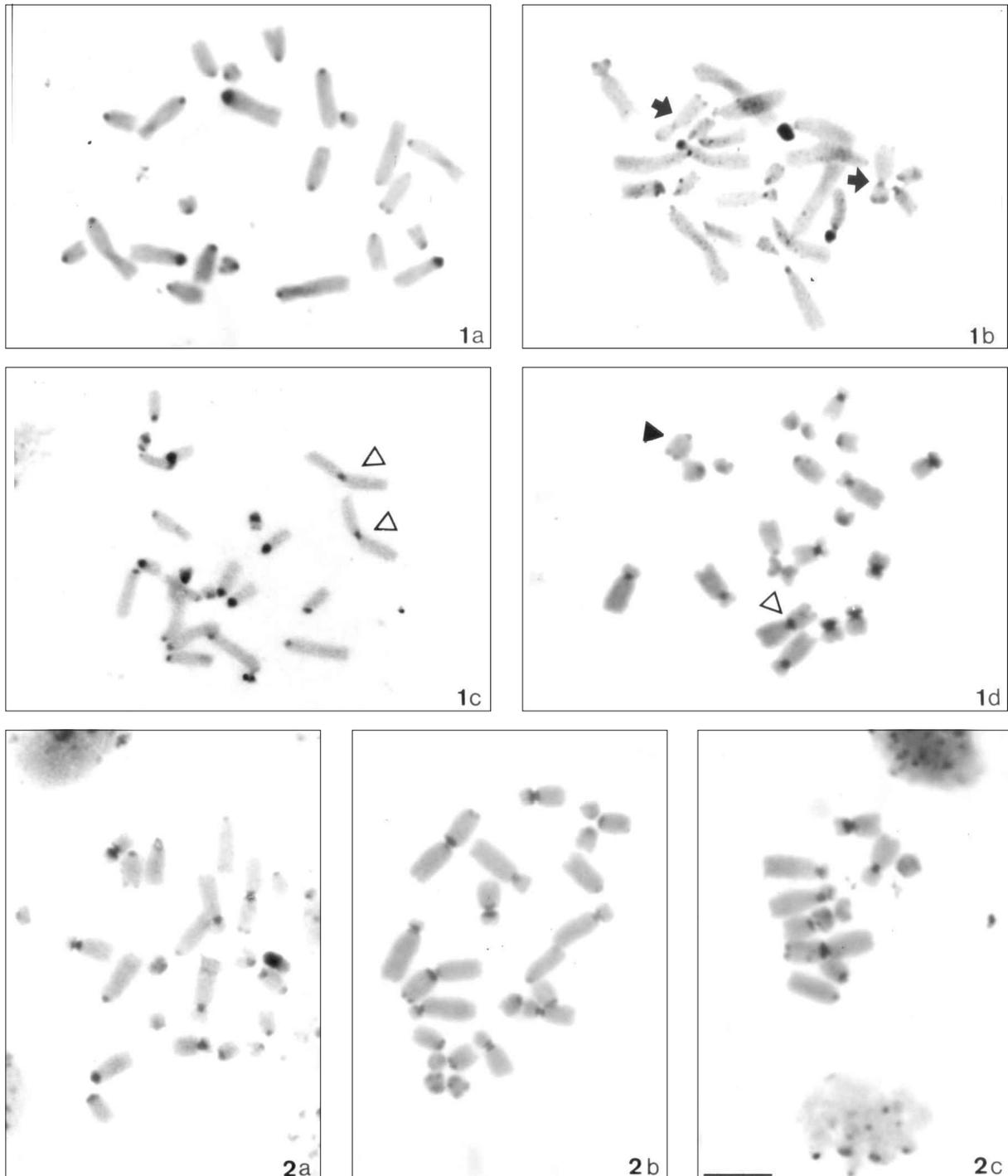
Figs 1 & 2. Fig. 1a-d. C-banded karyotypes of the chromosome subraces: (a) X0-standard (Teine): (b) X0-Naganuma, M_6 arrows; (c) XY-standard, neo-X (Δ); (d) XY-Tanno, inverted chromosomes arrows, neo-X - (Δ) and neo-Y - (▲). Fig. 2a-c. C-banded karyotypes of the embryos from XY-Ta x X0-standard crosses: (a) mitotic metaphase of the hybrid embryo; (b) and (c) mitotic metaphase of parthenogenetic diploid (b) and haploid (c) cells of the same embryo. Bar = 10 µm.
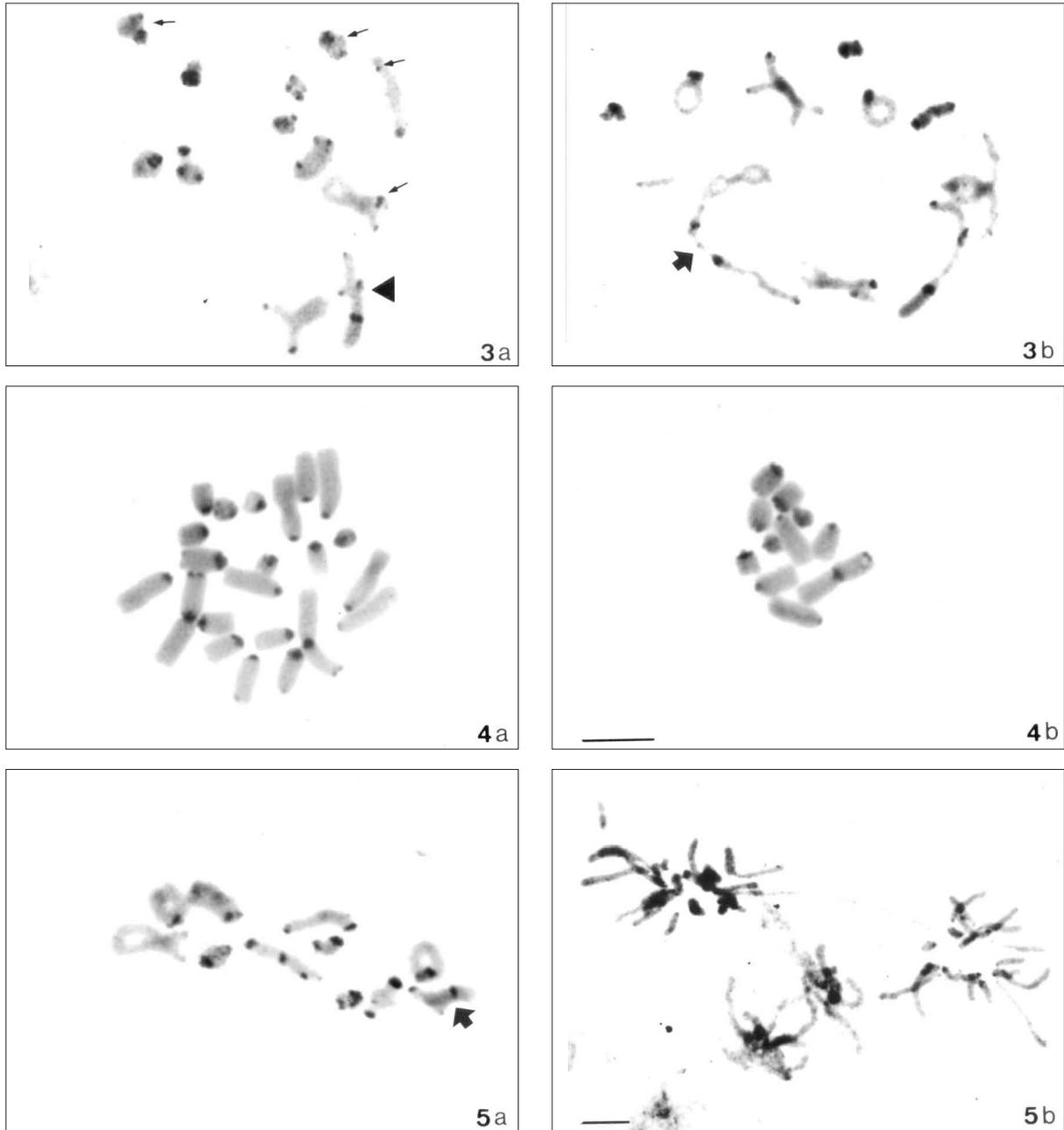
Figs 3-5. Fig. 3a,b. C-banded meiotic cells of F1 males from XY-Ta x X0-standard crosses: (a) normal heterozygotes, heteromorphic bivalents arrows, neo-XY (▲); (b) sporadic associates between L and M chromosomes (arrow). Fig. 4a,b. C-banded karyotypes of the embryos from XY-standard x X0-standard crosses: (a) and (b) parthenogenetic diploid (a) and haploid (b) cells of the same embryo. Fig. 5a-b. C-banded meiotic cells of F1 males from XY-standard x X0-Naganuma crosses: (a) normal heterozygotes, associates between X_R arm and M_5 arrow; (b) sporadic "bridge" in cell division. Bar = 10 μm.

Cytogenetic analysis of the F1 embryos and F1 males of XY-standard x X0-standard and XY-standard x X0-Naganuma

The number of pods laid and the numbers of eggs per pod did not differ significantly between the hybrid and control crosses. The percentage of fertilized eggs of control crosses was significantly higher than that of experimental ones (Table 1).

One hundred twenty five embryos were cytogenetically examined. All of the embryos from the XY-standard x X0-standard crosses and the vast majority (96.2%) of the embryos from the XY-standard x X0-Naganuma crosses were from female diploid or haploid cells (Fig. 4a,b). Only 3.8% of the embryos from the XY-standard x X0-Naganuma crosses were heterozygotes. The adult F1 males from this cross type had normally developed testes. All these males were heterozygotes, containing genomes of both parents. During meiosis large autosomes formed bivalents with 3 or 2 chiasmata, medium – 2 or 1 chiasmata, small bivalents – only one chiasma. The mean frequency of

chiasmata per cell was x=16,44 ($\pm$1.13). The X_R-arm of the neo-X chromosome belonging to the XY-standard female was well associated with the homologous M_5 chromosome belonging to the X0-standard male or XO-Naganuma male (Fig. 5a). Non-sister associations and other irregularities in meiosis were not observed.

Discussion

These crosses have shown that there is considerable incompatibility between chromosome races. This was revealed more clearly in the case of the XY-standard x X0-standard and XY-standard x X0-Naganuma crosses. These chromosome subraces differ by a X-A translocation (XY-standard x X0-standard) and a X-A translocation plus a fixed inversion in M_6 (XY-standard x X0-Naganuma) (Fig. 1a,b,c). The vast majority of embryos in these types of crosses were parthenogenetic.

It seems probable that unfertilised females of all orthopterans are capable of laying eggs (HEWITT 1979). Usually in Acrididae the proportion of unfertilised eggs which undergo development is very small (WHITE 1973). The XY-standard x X0-standard and XY-standard x X0-Naganuma experimental hybridisation may suggest that copulations in these cases stimulate the development of a relatively large quantity of non-fertilized parthenogenetic eggs. In *P. sapporensis*, as in some other Acrididae species in which the development of parthenogenetic eggs has been studied (KING & SLIFER 1934; BERGERARD & SEUGE 1959; SMITH 1969), a parthenogenetic generation from parthenogenetic embryos was not obtained. WHITE (1973) explained embryonic death in parthenogenetic eggs in many Acrididae species as a consequence of diploid/haploid mosaicism. This explanation corresponds well with the present research model, in which the parthenogenetic embryos were presumably diploid/haploid. The results of the cross experiment between the XY-standard x X0-standard and XY-standard x X0-Naganuma chromosome subraces in *P. sapporensis* suggests that zygotic but not post-zygotic barriers exist between crossed chromosome subraces belonging to the X0 and XY chromosome races. In very rare cases (in XY-standard x X0-Naganuma crosses), when the zygotic barrier had been surmounted, normal embryo heterozygotes and a F1 hybrid generation were obtained.

On the contrary, crosses between of the XY-Tanno and X0-standard subraces gave viable offspring in spite of many chromosome differences such as a X-A translocation and four fixed pericentric inversions (Fig. 1a,d). During meiosis in F1 males, homologous chromosomes are associated in spite of structural changes. Significant chromosome differences between XY-Tanno and X0-standard subraces probably have less effect on incompatibility of the gametes compared to the more fundamental genetic or physiological differences which lead to significant incompatibility in the case of the XY-standard x X0-standard and XY-standard x X0-Naganuma crosses. The accumulation of cytogenetic, genetic and other differences between various populations of *P. sapporensis* is probably connected with long-term isolation of the populations of this wingless grasshopper.

Nevertheless, the laboratory cross experiments have shown that the existence of a natural hybrid zone between the X0-Standard subrace and the XY-Tanno subrace is possible. The distribution pattern of these subraces suggests that a hybrid zone may be located in the area of the northern slope of Asahi volcano.

The presence of isolation barriers between X0 and XY races in the current research is in well accordance with earlier crossing experiments between the X0-Sakhalin and XY-standard (Kunashiri) subraces. In the latter case the hybrid embryos displayed mitosis disturbances which had lead to a high frequency of embryonic and offspring mortality and also to sterility of the F1 hybrid males (TCHENYKH & BUGROV 1998).

The results obtained in the present study do not support the opinion that chromosomal differences play a key role in restricting gene flow between X0 and XY races of *P. sapporensis,* because the level of isolation does not depend on the level of chromosome differences. Nevertheless, the current and earlier (TCHERNIKH & BUGROV 1998) crossing experiments support the presence of zygotic and post-zygotic barriers between X0 and XY chromosome races. The presence of barriers in crossing explains the phenomenon of the purity of these chromosomes races.

The reciprocal and other crosses between chromosome races and subraces in *P. sapporensis* will be helpful in resolving the taxonomic status of these populations.

Acknowledgements

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