

Experimental hybridization between X0 and XY chromosome races in the grasshopper *Podisma sapporensis* (Orthoptera: Acrididae). II. Cytological analysis of embryos and adults of F₁ and F₂ generations

ELŻBIETA WARCHAŁOWSKA-ŚLIWA¹, ALEXANDER G. BUGROV², YOSHIKAZU SUGANO³, ANNA MARYANSKA-NADACHOWSKA¹ and SHIN-ICHI AKIMOTO³

¹Department of Experimental Zoology, Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Sławkowska 17, 31-016 Krakow, Poland; e-mail: warchalowska@isez.pan.krakow.pl; maryanska@isez.pan.krakow.pl

²Novosibirsk State University, 630090 Novosibirsk and Institute for Systematics and Ecology of Animals, Siberian Branch of Russian Academy of Sciences, 630091 Novosibirsk, Russia; e-mail: bugrov@fen.nsu.ru

³Systematic Entomology, Department of Ecology and Systematics, Graduate School of Agriculture, Hokkaido University, Sapporo, 060-8589 Japan; e-mail: yoshikazu.sugano@nifty.com; akimoto@res.agr.hokudai.ac.jp

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Abstract. Experimental hybridization of X0 and XY chromosome races of the brachypterous grasshopper *P. sapporensis* did not reveal pre-zygotic reproductive isolation. However, a partial zygotic barrier was found between the X0-standard race from Shimokawa and XY-standard chromosome race from Akan. Approximately 40% of embryos from females crossed with males from other chromosome races developed parthenogenetically, the remaining embryos were normal heterozygotes. Adult F₁ males and females from crosses of this type had properly developed testes and ovaries. Non-sister associations and other irregularities in meiosis were not observed in male meiosis. Crossing experiments demonstrated that hybrids between X0 and XY races occur to some extent. The absence of a hybrid zone between the X0 and XY chromosome races may be the result of selection against heterozygotes. Crosses between the XY-Tanno and X0-standard (Teine) subraces resulted in F₁ and F₂ generations in spite of the many chromosome differences between them such as a X-A translocation and fixed pericentric inversions in four pairs of autosomes. These results do not support the hypothesis that chromosomal differences play a key role in restricting gene flow between the X0 and XY races of *P. sapporensis*.

INTRODUCTION

The initial version of the chromosome speciation hypotheses postulated that chromosome changes are the primary cause of reproductive isolation. Small population size and, to a lesser degree, the selective advantage of the homozygotes on rearrangement are the most important factors increasing the fixation of chromosome change that negatively affect of the status heterozygotes (White et al., 1967; White, 1968).

There are several models of the role of chromosome changes in grasshopper evolution. One of the first was proposed by White et al. (1967) for the *viatica* species group of the genus *Vandiemena* (Eumastacidae). Later Shaw and colleagues (Shaw, 1976; Shaw & Wilkinson, 1980; Shaw et al., 1982; Coates & Shaw, 1982) studied another model based on chromosome races in the Australian grasshopper, *Caledia captiva*. Populations of this species consist of at least three main chromosome races that differ in fixed pericentric inversions as well as in the localization and size of the C-heterochromatic regions. All those races have the karyotype $2n\delta = 23$, $2n\eta = 24$ and X0 δ /XX η sex determination. The authors crossed different chromosome races and revealed the role of pericentric inversions in the creation of reproductive barriers. These experiments revealed the presence of postzygotic isolating barriers between two chromosome races that

were reflected in hybrid sterility and disruptions in meiosis due to cytogenetic and genetic differences in the parental forms. In the bivalents formed by heteromorphic chromosomes, the locations of chiasmata differed from in the parental forms and lead to the collapse of coadapted gene complexes and disruption of the ontogenesis of the progeny (Shaw & Wilkinson, 1978; Coates & Shaw, 1982, 1984).

Cytogenetic and genetic differences, and the presence of a marked and variable degree of postzygotic isolation between the *Caledia captiva* chromosome races, formed the basis of the concept that this species consists of a complex of sibling species (Shaw & Coates, 1983).

The following chromosome speciation model, first proposed by John and Hewitt (John & Hewitt, 1970; Hewitt & John, 1972) and then elaborated in detail by Hewitt and Barton (Barton, 1980; Barton & Hewitt, 1981), is based on cytogenetic research on two chromosome races of the apterous species *Podisma pedestris*. This grasshopper occurs from Siberia to Spain and has the characteristic karyotype of the majority of Acrididae ($2n\delta = 23$, FN – Fundamental Number = 23) and a X0 δ /XX η sex determination. In the north-western Alps there is another chromosome race ($2n\delta = 22$, FN = 23; sex determination is neo-XY δ /neo-XX η) in which the neo-X chromosome is formed by centric fusion of the X chromosome and a

middle-sized autosome (John & Hewitt, 1970; Hewitt & John, 1972). These populations form a narrow hybrid zone extending for 130 km (Hewitt, 1975).

Experimental crosses between X0 and neo-XY were conducted in order to determine the causes of the purity of the chromosome races. The results showed that the hybrid generation is less viable because of the disruption of coadapted gene complexes, which cause a decrease in the fertility of F₁ hybrid females (Barton, 1980; Barton & Hewitt, 1981). The authors presumed that the cause of low hybrid survival is due to genetic differences not changes in chromosomes (Barton, 1980; Barton & Hewitt, 1981). This model demonstrates selection against the heterozygotes. In nature they rarely meet each other due to natural barriers (in this case high mountain ranges) and there is low hybrid viability (Hewitt et al., 1987).

The brachypterous grasshopper, *Podisma sapporensis* Shiraki, occurs on Hokkaido (Japan), Sakhalin and Kunashiri islands (Russia), and is characterized by low mobility. The distribution of this species is often associated with the occurrence of host plants belonging to the genus *Petasites*, accordingly, the Japanese name of this grasshopper is fuki-batta ("fuki" – *Petasites*, "batta" – grasshoppers). It is a highly polymorphic species, morphologically (Tatsuta et al., 2000) and cytologically (Bugrov et al., 2001), and there are two main chromosome races, the X0 and neo-XY (Bugrov, 1995; Bugrov et al., 2000). In the central part of Hokkaido island the races are geographically isolated by the Daisetsu and Hidaka mountain ranges. A natural hybrid zone between the X0 and XY chromosome races has not been discovered so far in spite of the absence of geographical barriers in the northern part of Hokkaido, where populations from both races occur.

High polymorphism of pericentric inversions, B-chromosomes and C-banding pattern occurs in various chromosomes of both chromosome races (Warchałowska-Śliwa et al., 2001; Bugrov et al., 2001, 2003). In some populations chromosome changes are fixed in one or a few pairs of chromosomes, which enables the identification of discrete chromosome subraces (Bugrov et al., 2001).

The absence of a hybrid zone between the X0 and XY chromosome races may be due to selection against heterozygotes. To test this hypothesis, laboratory crosses between different subraces belonging to the X0 and XY races were undertaken. Strong zygotic reproductive isolation between the XY-standard race (Akan locality) and X0-standard race (Teine locality) in laboratory breeding experiments is recorded (Bugrov et al., 2004) (Fig. 1). In contrast, crosses between the XY-Tanno and X0-standard subraces gave a viable F₁ generation, in spite of the many chromosome differences between these subraces such as a X-A translocation and fixed pericentric inversions in four pairs of autosomes (Bugrov et al., 2004). These results do not support the hypothesis that chromosomal differences play a key role in restricting gene flow between X0 and XY races of *P. sapporensis*.

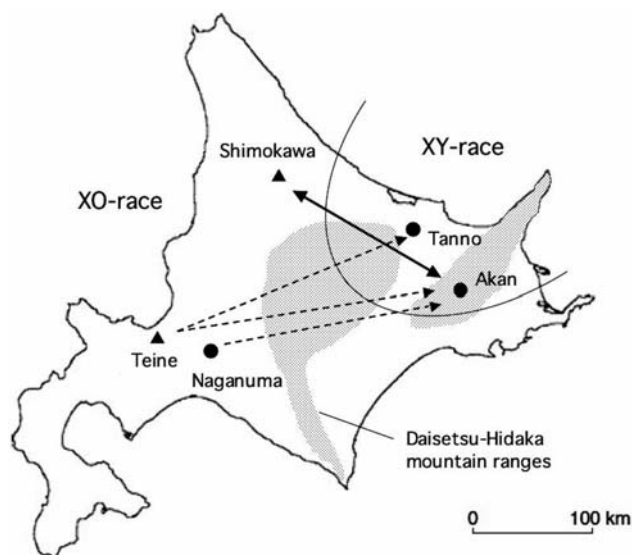


Fig. 1. Distribution of the X0- and neo-XY race of *Podisma sapporensis* on Hokkaido Island, northern Japan, and localities from which the grasshoppers were collected for the hybridization experiments. A solid line indicates the sources of the individuals crosses in the present study, and broken lines those of the crosses in the previous study in Bugrov et al. (2004). The direction of arrows indicate the locality from which females came.

The aim of the present study was to determine hybridization success of crosses between individuals from a population belonging to the X0-standard race from Shimokawa, potential contact zone between the X0 and XY races, and the XY-standard race (Akan) (Fig. 1). Additionally, meiosis was examined in a laboratory-bred F₂ generation from a cross between X0-standard and XY-Tanno chromosome races of *P. sapporensis*. As the zygotic barrier between X0 and XY populations has resulted in the parthenogenetic development of embryos (Bugrov et al., 2004), we report the results of a cytogenetic analysis of embryos obtained from virgin females.

MATERIAL AND METHODS

Collecting and laboratory crossing

In June 2005, nearly 450 male and female nymphs of *P. sapporensis* were collected in the north-eastern area of the distribution of the X0-standard subrace (near Shimokawa) and the XY-standard subrace (near Akan) (Fig. 1). The samples were transferred to the laboratory (Hokkaido University, Sapporo, Japan), where male and female nymphs were placed in separate plastic boxes. Every day fresh *Petasites* sp. leaves were given to the grasshoppers. Three or four days after the nymphs transformed into imagoes, experimental pairs were set up in individual cages. There were twenty experimental pairs (crosses) of the X0-standard (♀, XX) × XY-standard (♂, XY) chromosome and 20 of the XY-standard (♀, XY) × X0-standard (♂, X0) chromosome races.

Thirty last stage female nymphs from the Shimokawa population were placed in a separate box in order to obtain virgin females. Every day fresh *Petasites* sp. leaves were put in the cages. Three or five days after metamorphosis, they were placed in individual cages. Twenty four virgin females were obtained. After 12–23 days of adult life they began to lay pods of 8–20

TABLE 1. Comparison of the number of eggs per pod and eggs per pod with embryos produced by the control females and those of the experimental crosses.

Experiments	No. of eggs per pod (\pm SD; n_{ep} ; n_e)	% of eggs with embryos (n_e)	No. of eggs with embryos per pod (\pm SD)	No. of parthenoge- netic embryos (%)
Wild population X0 (Shimokawa)	15.05 (\pm 1.41; 20; 301) a	96.34 (290)	14.50 (\pm 0.92) a	–
Virgin female (Shimokawa)	14.95 (\pm 1.34; 20; 299) a	84.60 (253)	12.65 (\pm 0.74) c	100
Wild population XY (Akan)	14.45 (\pm 1.21; 20; 289) a	94.46 (273)	13.65 (\pm 1.10) b	–
X0 (♀, Shimokawa) \times XY (♂, Akan)	14.20 (\pm 1.29; 20; 284) a	63.38 (180)	9.00 (\pm 0.71) d	67 (37.2)
XY (♀, Akan) \times X0 (♂, Shimokawa)	14.35 (\pm 1.04; 20; 287) a	61.67 (177)	8.85 (\pm 0.87) d	75 (42.4)

Notes: n_{ep} – number of egg pods studied; n_e – total number of eggs. Turkey-Kramer's multiple comparison tests of no. of eggs per pod and no. of eggs per pod with embryos. Different letters represent significance at $P < 0.05$.

eggs in moist coarse sand. The first egg pod laid by each of the 20 females was used for the cytogenetic analysis of the embryos.

In a cross breeding experiment using X0 and XY individuals no pre-zygotic ethological reproductive barriers were observed. Pairs began to copulate after a minimum of 9 min and a maximum of 2 days. After 10–20 days of adult life, females began to lay pods of 9–19 eggs in moist coarse sand. The first egg pod laid by each female was used for a cytogenetic analysis of the embryos. As controls egg pods laid by pure X0-standard (Shimokawa) and XY-standard chromosome pairs were used. In the latter case (XY-standard) we used data obtained earlier by Bugrov et al. (2004). The remaining egg pods were stored in moist sand in a separate cell of a plastic container and kept at room temperature for 3 months. During this period, the embryos grew to fill the eggs, and then went into quiescent diapause. The eggs were then kept at 1°C for 6 months, simulating winter conditions, which broke the diapause. In the spring of 2006, each egg pod was put into moist sand in an individual plastic box at room temperature. After 7–12 days the eggs began to hatch. After another 3–5 days the eggs were inspected under a binocular microscope and classified into three groups: empty eggs (successful hatched), well developed eggs with an unhatched embryo, and eggs without embryos. In the laboratory (Novosibirsk State University, Russia, Siberia), *P. sapporensis* ate the leaves of *Tussilago sibirica* and *Arctium lappa* as a substitute for *Petasites* leaves.

Thirteen adult males and nine females of the F₁ generation of crosses between XY-Tanno and X0-standard population were reared in July 2003 in an insectary (Novosibirsk State University, Novosibirsk, Russia). Nine crosses between F₁ hybrid females and F₁ hybrid males were made. After about 5 days the pairs started to copulate. After about 10–17 days of adult life, females began to lay pods of 9–18 eggs, in moist coarse sand. Pods were laid at intervals of four to seven days. Following Barton (1980), after the first pod was laid the male from each cross was used for karyotypic analysis. The results of cytogenetic analysis of the F₁ males are in Bugrov et al. (2004). Each female laid one or two egg pods, rarely 3 egg pods. Usually, in the third egg pod there were less 7 eggs. Each egg pod was stored in a separate cell of a plastic container in moist sand and kept at room temperature for 3 months. Then the eggs were kept at 1°C for 6 months, simulating winter conditions, which broke the diapause as described above. In the spring of 2004; each egg pod was put into an individual plastic box in moist sand at room temperature. After 5–9 days the eggs began to hatch. After another 3–5 days the eggs were inspected under a binocular microscope and classified into three group: empty eggs (successful hatched), eggs with an unhatched embryo and eggs without embryos. As a control the data from pure X0-standard (Teine) and XY-Tanno chromosome races, published previously by Bugrov et al. (2004), was used.

Cytogenetic analysis of the embryos

Each egg pod from X0-standard (Shimokawa), XY-standard (Akan) and virgin X0-standard (Shimokawa) females were stored in moist sand in separate cells of a plastic container, and kept at room temperature. After 12–20 days of incubation, the first egg pod from each female was used to prepare slides for cytogenetic study using the C-banding technique following the method of Bugrov et al. (2004).

Cytogenetic analysis of the F₁ and F₂ hybrid males

Only seven adult F₁ males from the X0-standard (Shimokawa) \times XY-standard (Akan) cross and six F₂ males from the XY-Tanno \times X0-standard cross were obtained. All these males were used for cytogenetic analysis.

RESULTS

Cytogenetic analysis of the embryos from the X0-standard (Shimokawa) \times XY-standard (Akan) cross

The samples collected in the vicinity of Shimokawa belong to the pure X0-standard chromosome race. This chromosome race has the more usual chromosome complement of Acrididae, 23 acrocentric chromosomes in males (Fig. 2a) and 24 in females. The samples from the vicinity of Akan belong to the pure XY-standard chromosome race. The XY-standard chromosome race has 10 pairs of acrocentric chromosomes and two sex chromosomes, a metacentric neo-X and acrocentric neo-Y in the male (Fig. 2b) and two metacentric neo-X's in the female.

The hybrids, virgin and control females laid similar numbers of eggs per pod. The percentage of fertilized eggs laid by control females was significantly higher than of eggs with embryos laid by the hybrids (Table 1).

A total of 284 eggs from female XX (Shimokawa) \times male XY (Akan) crosses were examined; 180 of them contained embryos (Table 1). All these embryos were studied cytogenetically. Theoretically, this cross between a female gamete $n = 11A + X$ and male gametes $n = 10A + neo-X$ and $n = 10A + neo-Y$ must give rise to heterozygous female embryos with $2n_{\text{♀}} = 21A + X + neo-X$ and heterozygous male embryos with $2n_{\text{♂}} = 21 + X + neo-Y$. The majority of the embryos were heterozygotes, which corresponds to the theoretical expectations. Cytogenetic analysis of the remaining 67 embryos (37.2%) revealed that their tissues consisted of diploid cells with 24 chromosomes (Fig. 2c) or diploid cells mixed with haploid cells. In the latter case, diploid cells possessed 24 chromosomes, and all haploid cells had 12 chromosomes (Fig.

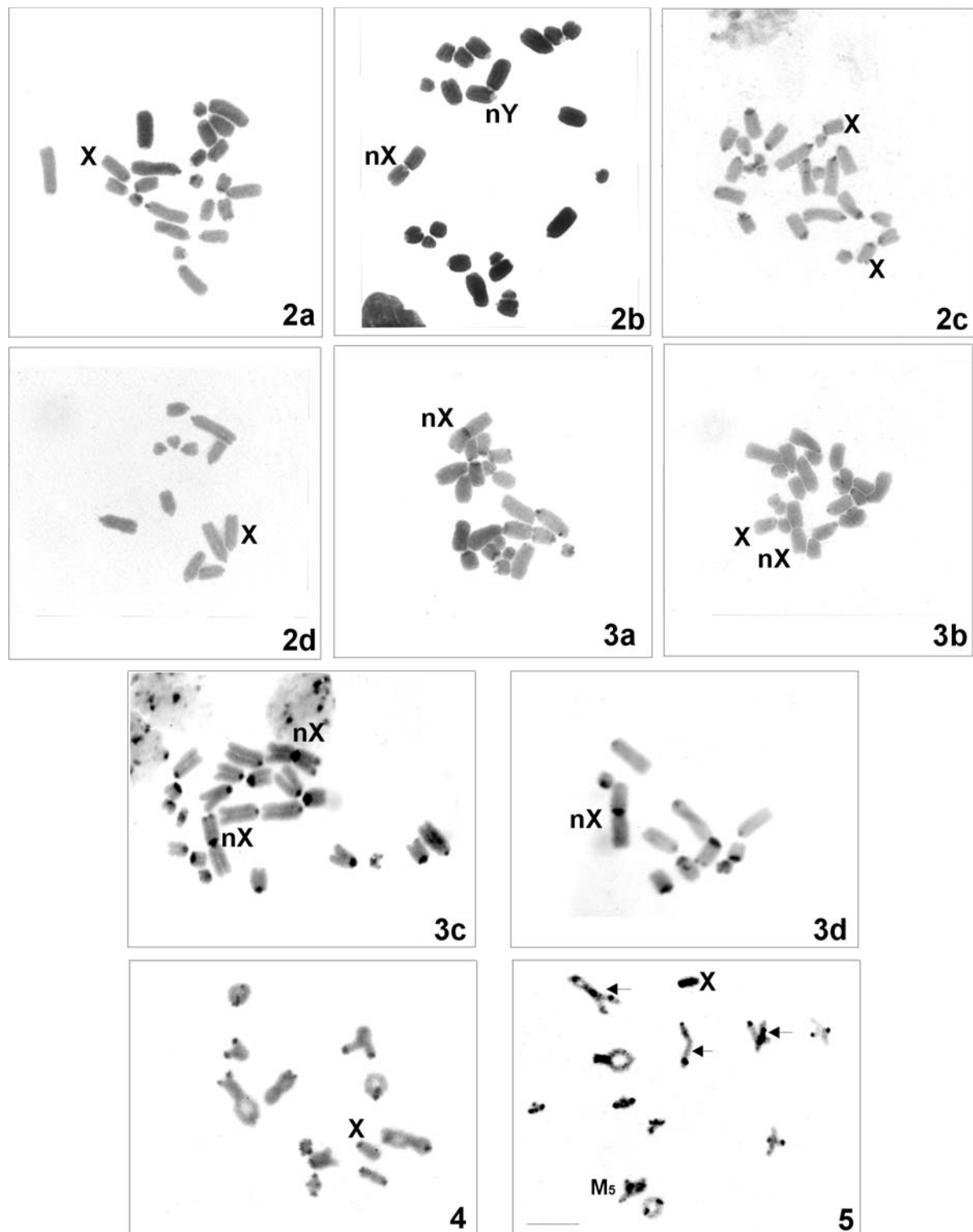


Fig 2. C-banded karyotypes of the embryos from (a) X0-standard (X chromosome = X); (b) XY-standard (neo-X = nX, neo-Y = nY) chromosome subraces; (c) diploid cell with 24 chromosomes; (d) haploid cell with 12 chromosomes. Fig 3. C-banded karyotype of F₁ embryos cells from X0-standard(♀) × XY-standard(♂) chromosome races: (a) normal heterozygotes, male, (b) normal heterozygotes, female, parthenogenetic diploid (c) and haploid (d) cells. Fig. 4. C-banded diakinesis of F₁ males from X0-standard × XY-standard crosses. Fig. 5. C-banded diakinesis of F₂ males from XY-Tanno(♀) × X0-standard(♂); normal heterozygotes and heteromorphous bivalents are indicated by arrows. The M₅ autosome is associated with the homologous neo-Y chromosome, belonging to the F₁ hybrid male. Bar = 10 μm.

2d). The ratio of embryos with only diploid cells to those with a mixture of diploid/haploid cells was approximately 1 : 16. Embryos consisting of only haploid cells were not

found. In haploid/diploid embryos; the vast majority of cells were diploid and had the maternal genome. The available evidence suggests that the females in this case

TABLE 2. Reproductive capacity of control females, those of experimental crosses between X0-standard (Shimokawa) \times XY-standard (Akan) populations and of X0-Standard (Shimokawa) virgin females.

Experiments	No. of eggs (no. of egg pods)	No. of eggs with embryos (%)	No. hatched	No. of adults
Pure population X0 (Shimokawa)	427 (32)	372 (87.1)	251	6♀, 15♂
Virgin female (Shimokawa)	368 (28)	245 (66.6)	38	—
X0 (♀, Shimokawa) \times XY (♂, Akan)	360 (29)	162 (45)	84	7♂
XY (♀, Akan) \times X0 (♂, Shimokawa)	334 (26)	184 (55.1)	112	—

laid unfertilized eggs and that they were able to develop parthenogenetically.

A total of 287 eggs from the female neoXX (Akan) \times male X0 (Shimokawa) cross were examined; 177 of them contained embryos (Table 1). All these were studied cytogenetically. Theoretically, in this cross the female gametes $n = 10A + \text{neo-X}$ and male gametes $n = 11A + X$ and $n = 11A + 0$ originate from a heterozygous female embryo with $2n♀ = 21A + X + \text{neo-X}(X-M_5)$ and heterozygous male embryo $2n♂ = 21 + \text{neo-X}$. The majority of the embryos corresponded to the theoretical expectations and were normal heterozygotes (Fig. 3a,b). Cytogenetic analysis of 75 embryos (42.4%) revealed that their tissues consisted of diploid cells with 22 chromosomes ($20AA + \text{neo-XX}$) (Fig. 3c) or diploid cells mixed with haploid cells. In the latter case the diploid cells had 22 chromosomes and haploid cells 11 chromosomes (Fig. 3d). Similar to another cross involving a female XX \times male XY, the proportion of embryos with only diploid cells to those with a mixture of diploid/haploid cells was very low. Embryos consisting of only haploid cells were not found.

A total of 299 eggs laid by virgin females were examined; 253 of these possessed embryos (Table 1). All these embryos were studied cytogenetically, with revealed that their tissues consisted of diploid cells with 24 chromosomes ($22AA + XX$) or diploid cells mixed with haploid cells.

Cytogenetic analysis of the F_1 male hybrids from the X0-standard (Shimokawa) \times XY-standard (Akan) cross experiment and the F_2 male hybrids from XY-Tanno \times X0-standard cross

The results for the eggs inspected after hatching are presented in Tables 2 and 3. In earlier experiments (Bugrov et al., 2004) and the current investigation many nymphs successfully hatched. Unfortunately the vast majority of the hybrids and control individuals died just after hatching, or failed to hatch in spite of completing their diapause development. Probably the nymphs of this species require the specific host plant *Petasites* sp. for

development, which was not available in Siberia. High mortality of the embryos during diapause may have been due to poor temperature, humidity and infection control.

Only seven adult F_1 males were obtained from the X0-standard (Shimokawa) \times XY-standard (Akan) cross. All these males had normally developed testes. At meiosis the large autosomes formed bivalents with 3 or 2 chiasmata, medium autosomes with 2 or 1 chiasmata, small bivalents with only one chiasma (Fig. 4). Non-sister associations and other irregularities in meiosis were not observed.

Only six F_2 male hybrids between XY-Tanno \times X0-standard population the follicle structure typical of this species. According to our earlier study the vast majority of the F_1 hybrids between XY-Tanno and X0-standard subraces are heterozygotes. The adult F_1 males had normally developed testes and meiosis, including all meiotic phases and each phase of spermatid and sperm formation. During male meiosis large autosomes formed normal 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, and formed 1 or 2 chiasmata (Bugrov et al., 2004).

In the $F_1♀ \times F_1♂$ cross variant the female gametes must be $n = 10A + \text{neo-X}$ and $n = 11 + X$. The F_1 male gametes develop of has been observed during meiosis (Bugrov et al., 2004). They have $n = 10A + \text{neo-X}$ and $n = 11A + \text{neo-Y}(M_5)$. Thus, females should have a diploid number of $2n♀ = 20A + \text{neoXX}$ and $2n♀ = 21 + X + \text{neoX}$. Two chromosome variants of the males may develop. The first is $2n♂ = 20AA + \text{neo-X} + \text{neo-Y}$ and the second $2n♂ = 21 + \text{neo-Y} + X$. The first of these corresponds to the male of the XY chromosome race and the second the X0 chromosome race, because the neo-Y chromosome and the M_5 autosome are homologous. All six males that were studied belong to the second type. During meiosis in these males large autosomes formed bivalents with 3 or 2 chiasmata, medium with 2 or 1 chiasmata, small with only one chiasma. The X is univalent (Fig. 5). The mean frequency of chiasmata per cell was 17.26 (SD = ± 1.29). Usually chiasmata did not form between regions of the

TABLE 3. Reproductive capacity of control females and those of experimental crosses between of XY-Tanno and XX-standard races.

Crosses	No. of eggs	No. of eggs with embryos	No. of eggs without embryos	No. hatched	No. of unhatched embryos	No. of adults
Pure XY-Tanno	257	229	28	123	106	12♀, 27♂
Pure XO-standard (Teine)	268	257	11	99	158	5♀, 21♂
F_1 XY-Tanno \times X0-standard (Teine)	390	374	16	65	309	9♀, 13♂
F_2 XY-Tanno \times X0-standard	227	188	39	48	140	3♂

chromosomes involved in pericentric inversions in heteromorphic bivalents. The M_5 autosome associated with the homologous neo-Y chromosome, belonging to the F_1 hybrid male. Non-sister associations and other irregularities in meiosis were not observed.

DISCUSSION

In all the examples of chromosomal speciation, populations were not absolutely isolated and gene flow may have occurred through narrow hybrid zones. These hybrid zones are a subject of a special study, which has contributed significantly to the understanding of the evolution of populations (Hewitt, 1975, 1990; Hewitt et al., 1987; Barton & Hewitt, 1989).

Podisma sapporensis combines the cytogenetic polymorphism characters of *C. captiva* (Shaw, 1976; Shaw & Wilkinson, 1980; Shaw et al., 1982; Coates & Shaw, 1982) and *P. pedestris* (John & Hewitt, 1970; Hewitt & John, 1972; Barton, 1980; Barton & Hewitt, 1981). After the discovery of the X0 and XY chromosome races in this species (Bugrov, 1995), efforts were directed towards determining the distribution of these races. Individuals from more than 70 localities on Sakhalin, Kunashir and Hokkaido islands were cytogenetically studied (Bugrov et al., 2000, 2001; Warchałowska-Śliwa et al., 2001, 2008). The X0 chromosome race of *P. sapporensis* that occurs in the western part of Hokkaido and the south of Sakhalin, consists of 4 chromosome subraces, which are homozygous in one or several chromosome rearrangements (Bugrov et al., 2001). The XY chromosome race is widespread in the eastern part of Hokkaido and south of Kunashiri, and includes two subraces. The XY-standard subrace (the X chromosome is metacentric as a result of a reciprocal translocation of the sex chromosome and fifth pair of chromosomes (M_5), the rest of the chromosomes are acrocentric) is widespread in the eastern part of Hokkaido while the XY-Tanno subrace (reciprocal translocation of the sex chromosome and fifth pair of chromosomes and also fixed inversions on three pairs of autosomes) is distributed very locally on the eastern part of the slope of Asahi volcano, near Tanno and Oketo (Bugrov et al., 2001).

In order to elucidate the causes of chromosome race purity, experimental crosses were conducted between the X0 and XY chromosome races and different chromosome subraces. The first crosses between the *P. sapporensis* chromosome forms belonging to the X0 and XY races revealed that the chromosome race purity results not only from geographic isolation but also the presence of other isolating mechanisms. Thus, first generation hybrid males from the crosses between the X0 race from Sakhalin island (X0-Sakhalin subrace) and XY-standard race from Kunashiri were sterile. Their testes consisted of a few strongly deformed follicles and show mitotic disturbances. (Tchernykh & Bugrov, 1997).

The isolating barriers between X0 and XY races in Hokkaido were investigated. In the case of pairs formed from individuals from isolated populations, namely, the X0 chromosome race from the vicinity of Sapporo (Mt

Teine, X0-standard subrace) and XY chromosome race from vicinity of Akan (XY-standard subrace), the vast majority of the embryos in the eggs laid by the females produced by this cross contained either haplo/diploid or diploid parthenogenetic cells, which suggested the presence of a strong zygotic barrier (Bugrov et al., 2004). The nature of this zygotic barrier is unknown. Probably it is a result of physiological and of genetic differences that accumulated in these populations during their long geographic isolation.

A zygotic barrier was recorded previously in experimental crosses between X0 and XY chromosome pairs of *P. pedestris*, though it did not involve more than 3% of the embryos (Barton, 1980).

The results of crossing X0 and XY chromosome races of *P. sapporensis* follows on the earlier experiments of Bugrov et al. (2004) but involved a population (Shimokawa) belonging to the X0-standard chromosome race from the probable contact zone between X0 and XY chromosome races in the north-eastern part of Hokkaido. The idea for this experiment was based on the results of crosses between X0 and XY chromosome races of *P. pedestris* (Barton & Hewitt, 1981). In this species, hybrid viability depends on the distance from the hybrid zone the parental specimens were collected.

A comparison of the results of current and previous experiments shows that the zygotic barrier in crosses between the X0-standard race from Shimokawa and XY-standard race (Akan) is significantly less expressed than in the X0-standard (Teine) \times XY-standard (Akan) cross (compare Table 1 of the present paper and Bugrov et al., 2004 – Table 1). In this cross more than 60% of embryos were normal heterozygotes; seven adult F_1 males had normally developed testicles and normal meiosis, but all were from the Shimokawa (X0 female) \times Akan (XY male) cross. No F_1 adult samples were obtained from the Akan (neo-XX female) \times Shimokawa (X0 male) cross (Table 2). Due to the high mortality in both experimental and control groups, there are insufficient results for statistical analysis as there was only one variant recorded among the hybrid male.

Because a significant proportion of parthenogenetic embryos was found in eggs laid by the different crosses between X0 and XY chromosome races, those produced by virgin females from vicinity of the Shimokawa were also studied. The proportion of eggs laid by virgin females that gave rise to embryos did not differ significantly from that in the control pairs of X0 (Shimokawa). All the embryos in the eggs laid by virgin females were either diplo/haploid or parthenogenetic diploids. In this connection it should be noted that in the eggs laid by females in the hybridization experiments, the proportion with the embryos is significantly lower than in those laid by females from wild populations and virgin females. It seems that although some part of the embryos in the eggs laid by the experimental females may overcome the zygotic barrier, their development is interrupted at an early stage before embryo formation.

In conclusion, there are zygotic barriers to crosses between X0-standard (Teine) (Bugrov et al., 2004) and X0-standard (Shimokawa) (present paper) with XY-standard (Akan) races. The strength of these barriers depends on the geographical location of the population relative to the probable contact zone between X0 and XY chromosome races. The results of crosses between X0-standard (Shimokawa) and XY-standard (Akan) individuals indicate in a principal that it is possible for X0 and XY races to hybridize. The production of some adult F₁ individuals in the laboratory supports this conclusion. Lack of polymorphism among X0/XY specimens in the Shimokawa region suggests natural selection against heterozygotes.

Unfortunately, the fitness and fertility of the hybrids is unknown because the control and hybrid generations suffered high mortality in the laboratory. Optimal conditions for the laboratory rearing of *P. sapporensis* are now being sought.

In contrast, the laboratory crosses between X0-standard (Teine) chromosome race and XY-Tanno chromosome subrace produced two generation of fertile hybrid progeny. It should be stressed that in this experiment the initial populations differed not only in a single fixed chromosome rearrangement, as in the case described above, but in five other rearrangements (X-M₅ translocation, pericentric inversions on four autosomes: Bugrov et al., 2001). However meiosis in the hybrid males of the first (Bugrov et al., 2004) and second (present paper) generations proceeded without substantial disruption. Only rarely, were multivalents formed and homology disjunction disruptions were fixed during the formation of spermatides. Also it should be mentioned that the chiasmata did not form in the proximal regions affected by inversion. It is possible that this prevents crossing-over in the hybrids between the different chromosome forms of *P. sapporensis* and the loss of the coadapted gene complexes, as described for *Caledia captiva* (Coates & Shaw, 1982).

The results obtained by hybridization of chromosome races of *P. sapporensis* contradict the initial hypothesis of chromosome speciation because in this case the rate of development of the isolating barrier does not depend on the extent of the differences in the chromosomes of the crossed populations. Probably in this case, as in *C. captiva* and *P. pedestris*, the isolating mechanism and low hybrid progeny survival are due to the genetic differences between the geographically isolated populations rather than chromosome changes.

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