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Establishing interspecific mosaic genome lines between *Drosophila ananassae* and *Drosophila pallidosa* by means of parthenogenesis

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Summary

Strong sexual isolation exists between the closely related species *Drosophila ananassae* and *D. pallidosa*, but there is no obvious post-mating isolation; both sexes of the hybrids and their descendants appear to be completely viable and fertile. Strains exhibiting parthenogenesis have been derived from wild populations of both species. We intercrossed such strains and established iso-female lines after the second generation of parthenogenesis. These lines are clones, carrying homozygous chromosomes that are interspecific recombinants. We established 266 such isogenic lines and determined their genetic constitution by using chromosomal and molecular markers. Strong pseudo-linkage was seen between loci on the left arm of chromosome 2 and on the right arm of chromosome 3; the frequency of inheriting the two chromosome regions from the same species was significantly larger than expected. One possible cause of pseudo-linkage is female meiotic bias, so that chromosomes of the same species origin tend to be distributed to the same gamete. But this possibility is ruled out; backcross analysis indicated that the two chromosome regions segregated independently in female hybrids. The remaining possibility is elimination of low-fitness flies carrying the two chromosome regions from different species. Thus, genetic incompatibility was detected in the species pair for which no hybrid breakdown had previously been indicated. The 'interspecific mosaic genome' lines reported here will be useful for future research to identify genes involved in speciation and phenotypic evolution.

1. Introduction

In order to elucidate the genetic mechanisms of speciation and species differentiation, interspecific hybrids and introgressions have been made in diverse organisms (for recent reviews see Sawamura & Tomaru, 2002; Coyne & Orr, 2004; Wu & Ting, 2004). The genus *Drosophila* is useful for such studies because detailed genetic analyses are possible. Recent examples are introgressions of *D. mauritiana* or *D. sechellia* genes into the *D. simulans* genome (e.g. Coyne & Charlesworth, 1989; True *et al.*, 1996; Wu *et al.*, 1996; Joly *et al.*, 1997; Naveira & Maside, 1998; Macdonald & Goldstein, 1999; Civetta *et al.*, 2002; Tao *et al.*, 2003). Fertile female hybrids are backcrossed to obtain the introgressions; sibling hybrid males are sterile in these crosses (David *et al.*, 1974; Lachaise *et al.*, 1986). In later generations, individuals carrying heterozygous introgressions are selected by means of genetic markers or introgressions are made homozygous by successive sib-matings. Enormous effort (e.g. selection in every generation) is necessary to establish and maintain such introgression lines. In the outgroup species, *D. melanogaster*, balancer chromosomes are available, and introgressions could be maintained easily if introgressions were made into this species. However, hybrids between *D. melanogaster* and the three sibling species are inviable or sterile (Sturtevant, 1920; David

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et al., 1974; Lachaise *et al*., 1986) and female fertility is very low even if it is genetically restored (Davis *et al*., 1996; Barbash & Ashburner, 2003). Therefore, introgressions have not been successful between *D. melanogaster* and these sibling species except for a few cases (Muller & Pontecorvo, 1940; Sawamura *et al*., 2000).

In the present analysis, we used a more closely related species pair, *D. ananassae* and *D. pallidosa*, which produce fertile female and male hybrids (Futch, 1966; Stone *et al*., 1966; Bock & Wheeler, 1972). The species belong to the *ananassae* subgroup of the *melanogaster* species group (Bock & Wheeler, 1972; Lemeunier *et al*., 1986). *D. ananassae* is cosmopolitan, distributed in the tropics and the subtropics, while *D. pallidosa* is endemic to the islands of the South Pacific (Tobari, 1993). Both species exist in Fiji, Tonga and Samoa, but it is unknown whether they speciated sympatrically or allopatrically (i.e. whether the current sympatry is secondary contact). Allopatric *D. ananassae* and *D. pallidosa* have light body colour, while *D. ananassae* sympatric with *D. pallidosa* has dark body colour (Futch, 1966, 1975, 1996; Tobari, 1993). Strong sexual isolation exists between the species (Spieth, 1966; Futch, 1973*a*; Oguma, 1993). That gene flow is restricted in the wild is evident from the low frequency of sharing of inversion and isozyme polymorphisms (Futch, 1966, 1973*a*; Johnson *et al*., 1966; Tomimura *et al*., 1993). Female sex pheromones (Nemoto *et al*., 1994; Doi *et al*., 1997) and male courtship songs (Yamada *et al*., 2002*a*, *b*) are species-specific. Excision experiments that removed wings (emitter organ) or antennae (receiver organ) have shown that discriminating the acoustic signal by females is important for the sexual isolation (Doi *et al*., 2001). The major gene determining the female preference has been mapped on the left arm of chromosome 2 (Doi *et al*., 2001).

D. ananassae has been an important model organism since classic studies in the 1930s (Moriwaki, 1934; Kaufmann, 1936; Kikkawa, 1938). In addition to the accumulation of decades of genetic information, the sequencing of the whole genome of *D. ananassae* has been achieved recently by the FlyBase Consortium and *Drosophila* Sequencing Centers (http://genome.ucsc.edu/) and the National Bio-Resource Project, Japan (http://hgp.gsc.riken.jp/fly/). Introgressions between *D. ananassae* and *D. pallidosa* will be useful for studying the genetics of differences between these species. However, because no general balancers suppressing crossing-over along entire chromosomes are known in the species, it will be difficult to isolate and maintain the potential introgressions. *D. ananassae* and *D. pallidosa* offer a genetic trick unavailable in many species of *Drosophila* that can avoid this difficulty: parthenogenesis. It has long been known that flies with genes allowing parthenogenesis exist in some wild populations of

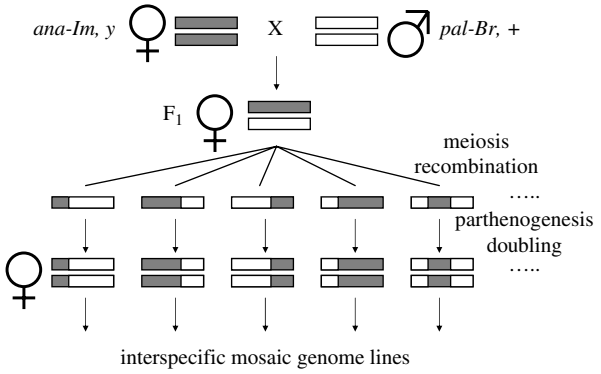

Fig. 1. Creating interspecific mosaic genome lines. Genomes are indicated by boxes (filled boxes from *ana-Im*, *y* and open boxes from *pal-Br*, +).

D. ananassae and its sibling species (Futch, 1972, 1973*b*; Matsuda & Tobari, 1999). Although various mechanisms of parthenogenesis are known in *Drosophila* (e.g. Stalker, 1954; Templeton, 1983; Fuyama, 1986), mechanisms similar to those in *D. mercatorum* (Carson *et al*., 1969) have been thought to act in *D. ananassae* and its siblings, namely recovery of diploidy by the fusion of two haploid nuclei or by the doubling of a single haploid nucleus after meiosis. Genetic analyses conducted thus far indicate that marker genes are made homozygous by parthenogenesis in *D. ananassae* and its siblings, which means that parthenogenesis is probably achieved mainly by the latter mechanism (Futch, 1972, 1973*b*, 1979, 1994; Matsuda & Tobari, 2004). The above mechanism of parthenogenesis acts in the female hybrids as well (Matsuda & Tobari, 2004), and we can establish homozygous lines possessing isogenic recombinant chromosomes in two generations (Fig. 1). By applying the above method, we established 266 'interspecific mosaic genome' lines.

To determine the genetic constitution of each line we examined polytene chromosomes of the mosaic genome lines. Further, we introduced molecular markers, the cytological positions of which have been identified by *in situ* hybridization (most of the locations were determined by ourselves). As shown in the present report, a strong pseudo-linkage between loci on the left arm of chromosome 2 and on the right arm of chromosome 3 was detected by statistical tests. In general, genes on different chromosomes must segregate at random, but genes from the same species on different chromosomes tended to segregate together. We investigated the cause of the pseudo-linkage and concluded that flies carrying the two chromosome regions from different species were eliminated in the process of establishing the lines, because of their low fitness. The lines established here are clones in which all the flies have the same genotype. This is unique for such studies, because obtaining flies with the same genetic constitution

en masse has been difficult using conventional backcrosses. We expect the mosaic genome lines reported here will be useful in future research on speciation and species differentiation.

2. Materials and methods

(i) *Fly strains*

D. ananassae and *D. pallidosa* strains with parthenogenetic potential (Futch, 1972, 1973*b*) were utilized. Both were derived from flies caught in Taputimu, Tutuila, American Samoa (probably by W. S. Stone and M. R. Wheeler in 1962). Parthenogenetic females were isolated from the original strains and the derived (Impaternate, *Im*) strains have been maintained without males; XO males occasionally appear but are sterile. Bridge (*Br*) strains consisting of both sexes have been established by crossing *Im* females with males from the original strains. *Im* strains can be re-established in any generation by isolating virgin females from the *Br* strains. We used *yellow* (*y*) on the left arm of the X chromosome (XL) of *D. ananassae* as a visible marker. This is a spontaneous mutant allele (y^d) found in the original Taputimu strain (Futch, 1973*c*). A recessive gene *parthenogenesis* (*parth*) on the left arm of chromosome 2 (2L) is responsible for the parthenogenesis (Matsuda & Tobari, 2004). We used the *Im* strain of *D. ananassae* with the *y* mutation (*ana-Im*, *y*) and the *Br* strain of *D. pallidosa* (*pal-Br*, +) as breeding parents. They were the gift of Dr D. G. Futch.

(ii) *Constructing interspecific mosaic genome lines*

To obtain interspecific hybrids, five virgin females of *ana-Im*, *y* and 10 males of *pal-Br*, + were placed together (two trials done). As *D. ananassae* females reject *D. pallidosa* males in the presence of courtship songs (Doi *et al*., 2001), the wings of the males were cut off with scissors under CO_2 anesthetization. Although *ana-Im*, *y* females produce y females parthenogenetically, female hybrids are discernible by their y^+ phenotype (*y*/+ genotype). Female hybrids were separated singly and the next generation was obtained parthenogenetically. As crossing-over takes place during meiosis of the female hybrids (Matsuda & Tobari, 2004), each fly of the next generation has a unique genetic constitution. Most of the flies are thought to be isogenic because of the mechanism of parthenogenesis, as described in Section 1. Females were separated singly and 266 parthenogenetic lines were established from the second-generation flies (Fig. 1).

(iii) *Chromosome analyses*

The chromosome arrangement is different between the *ana-Im*, *y* and *pal-Br*, + strains (Fig. 2). Crossing-over within or near the inversions is thought to be suppressed in female hybrids. We examined salivary gland chromosomes of the third instar larvae from 64 mosaic genome lines and determined the species origin of each chromosome region.

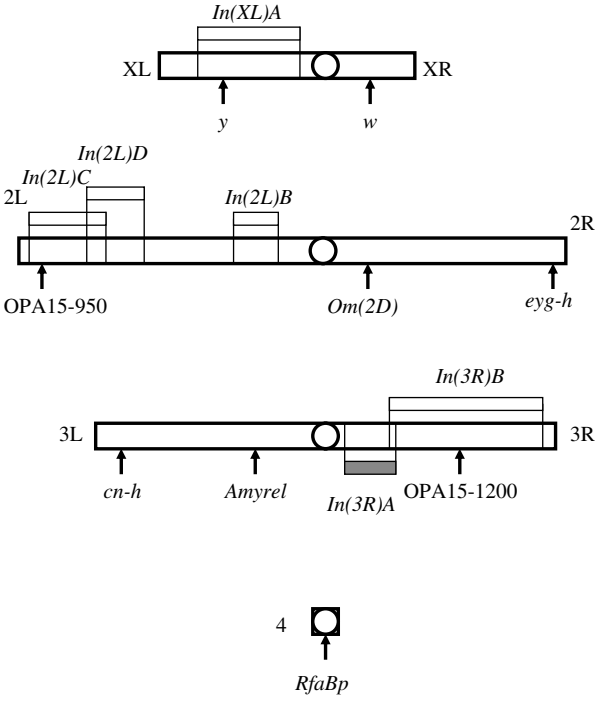

Fig. 2. Location of markers (arrows) and inversions (bars; filled bars from *ana-Im*, *y* and open bars from *pal-Br*, +). Chromosome arms are depicted according to cytology (circle indicates the centromere). The inversion of *ana-Im*, *y* is *In(3R)A* [83C; 87B]. The inversions of *pal-Br*, + are *In(XL)A* [4A; 10D], *In(2L)C* [22A; 28A], *In(2L)D* [26C; 30D], *In(2L)B* [37D; 41B] and *In(3R)B* [87A; 98D], where inversions are described as *In*(*chromosome arm*)*name* [left breakpoint; right breakpoint] (Tobari *et al*., 1993).

(iv) *Molecular marker analyses*

Genomic DNA was extracted by using the DNeasy tissue kit (Qiagen). To examine the genetic constitution of each mosaic genome line, we searched for molecular markers that differed between the *ana-Im*, *y* and *pal-Br*, + strains.

(a) *Random amplified polymorphic DNA (RAPD) analysis*

Genomic DNA was amplified for 70 lines by using Ready-To-Go RAPD Analysis Beads (Amersham Biosciences) with randomly chosen 10-mers (Qiagen Operon) as primers (20 primers tested), and we electrophoretically separated the amplified fragments with 2% agarose gel. After electrophoresis, two of the diagnostic bands (OPA15-950 and OPA15-1200; Table 1) were excised from the agarose gel with a razor and purified with GFX PCR DNA and Gel

Table 1. *Molecular markers used in the present analysis*

Locus	PCR primers	Temp.	Enzyme
w	TCGCAGAAGGGCAAGA ACCAGGTGGCCTTGT	60	–
Om(2D)	AGGAGTGGCACACGCACTTC ATGACAGCTCGCTGACGGTT	65	*Hind*III
eyg-h	CGTGACCTTGGCTCGCTTAC CTCCCAGGCGAAGATCGTTG	55	*Hae*III
cn-h	AATGATACGGCCGGCACTTGATGGA TGGGCAACCACGTGGATTTGTACGA	50	*Msp*I
Amyrel	TCGCTTCCAGGTGCAGCAGTG GTTCCCCAGCTCTGCAGCC	70	*Pvu*II
RfaBp	GGAAATCTGTCTGGGGCAAA GCTTTGACAGGTCCGATCAG	60	*Afa*I
RAPD OPA15	TTCCGAACCC	36	–

Temp., annealing temperature (°C); 30 cycles of amplification were done for PCR (exceptionally 45 cycles for RAPD analysis). Enzyme, the enzyme for detecting RFLP; – indicates no enzymes used (presence/absence of the band is diagnostic). RAPD OPA15 means OPA15-950 and OPA15-1200.

Band Purification Kit (Amersham Pharmacia). The DNA fragments were cloned with the pGEM-T Easy Vector System (Promega) and their sequences determined.

(b) *Restriction fragment length polymorphism (RFLP) analysis after polymerase chain reaction (PCR) amplification*

Primer sequences, annealing temperature and restriction enzymes to determine the origin of each locus are described in Table 1. As few genomic sequences of *D. ananassae* were known at the beginning of the project, some primers were designed in the gene region conserved between *D. melanogaster* and *D. pseudoobscura*, the sequences of which were obtained through FlyBase (Drysdale *et al.*, 2005; http://flybase.org). PCR was performed with Ex Taq (TaKaRa BIO) and the amplified DNA digested by restriction enzymes purchased from the same company. That the amplified DNA was of the intended chromosome region was confirmed by sequence similarity.

(v) In situ *hybridization*

The cytological position of each molecular marker (Fig. 2) was determined by *in situ* hybridization to the salivary gland chromosomes of *D. ananassae*. The DNA fragments cloned in the vector were labelled with digoxigenin-11-dUTP (PCR DIG Labeling Mix, Roche). The *in situ* hybridization was conducted with a modification of the procedures described in Biémont *et al.* (2004). The signals were detected by DIG High Prime DNA Labeling and Detection Starter Kit I (Roche).

(vi) *Markers (abbreviation, chromosome arm, cytological position) utilized*

The following markers were used:

yellow (*y*, XL, 5D): A visible marker utilized for the initial cross.

white (*w*, XR, 17C): Primers designed according to the *D. melanogaster*/*D. pseudoobscura* sequences. Amplified in *ana-Im*, *y* but not in *pal-Br*, + under the present PCR condition.

OPA15-950 (−, 2L, 22D): A 950 bp band appears in *ana-Im*, *y* but not in *pal-Br*, + when OPA-15 primer used in the RAPD analysis. Homologous to a region of 3R (92D) of *D. melanogaster* (syntenic to 2L of *D. ananassae*).

Optic morphology(2D) (*Om(2D)*, 2mid, 48B): Primers designed based on the sequence of *D. ananassae* (Yoshida *et al.*, 1994). The cytological position is according to Matsubayashi *et al.* (1992), where 'mid' indicates the region close to the base of the chromosome arms (the same abbreviation is used below).

eyegone-homologue (*eyg-h*, 2R, 62D): Primers designed according to the *D. melanogaster*/*D. pseudoobscura* sequences. Probably corresponds to *groove* (*gv*), not *eyg*, of *D. ananassae*, based on its location (Moriwaki & Tobari, 1993).

cinnabar-homologue (*cn-h*, 3L, 66A): Primers designed according to the *D. melanogaster*/*D. pseudoobscura* sequences. Probably corresponds to *bright* (*bri*), not *cn*, of *D. ananassae*, based on its location (Moriwaki & Tobari, 1993). Two signals detected by *in situ* hybridization (66A and 70D); position determined based on the linkage map (Moriwaki & Tobari, 1993).

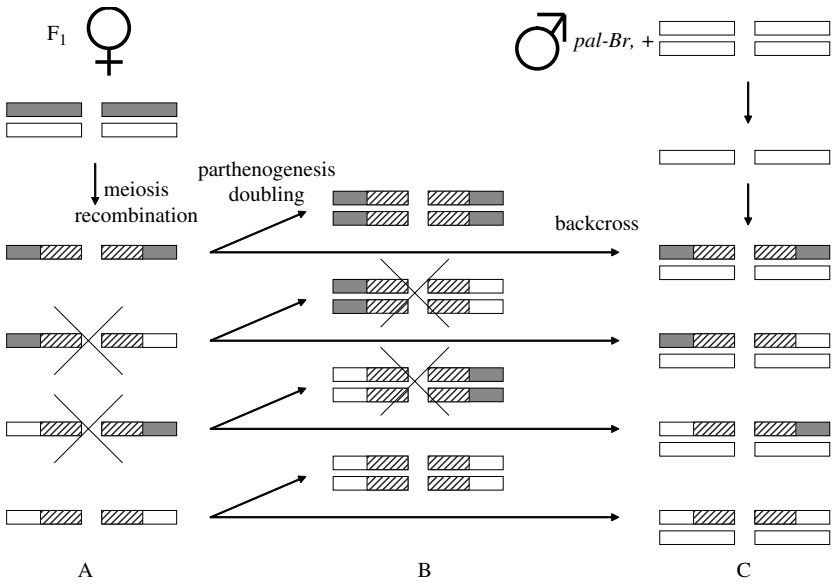

Fig. 3. Possible causes of pseudo-linkage (*A*, meiotic event; *B*, zygotic event) and the backcross analysis to discriminate the two hypotheses (*C*). Genomes are indicated by boxes (filled boxes from *ana-Im*, *y* and open boxes from *pal-Br*, +).

Amylase-related protein (*Amyrel*, 3mid, 76C): Primers (M. L. Cariou, pers. comm.) designed using the *D. ananassae* sequence (Da Lage *et al*., 1998).

OPA15-1200 (−, 3R, 92B): A 1142 bp band appears in *ana-Im*, *y* but not in *pal-Br*, + when OPA-15 primer is used in the RAPD analysis. Hmologous to a region of 2L (29D) of *D. melanogaster* (syntenic to 3R of *D. ananassae*).

Retinoid- and fatty-acid binding protein (*RfaBp*, 4, −): Primers designed based on the sequence obtained in the Expressed Sequence Tag (EST) analysis of *D. ananassae* (Oguma *et al*., 2005). Homologous to *RfaBp* of *D. melanogaster* (4, 102D3-4); chromosome 4 is syntenic between the species. A signal is detected on the chromocentre by *in situ* hybridization, consistent with chromosome 4 being under-replicated in polytene chromosomes of *D. ananassae* (Tobari *et al*., 1993).

(vii) *Statistical tests*

In the second-generation females (and the mosaic genome lines established from them), the allele derived from *D. ananassae* (a) and that derived from *D. pallidosa* (p) theoretically should segregate in a 1:1 ratio. We tested whether the observed values were significantly different from the expected by using the chi-square test. If two loci are independent, the frequency of the combinations of their alleles in the mosaic genome lines should be the product of the frequencies of each allele. To estimate linkage between the two loci (whether those are on the same chromosome or not) we calculated the recombination fraction (c) and coefficient of linkage disequilibrium (D). To make a comparison, linkage disequilibrium was normalized as $D' = D/D_{\max}$ (Lewontin, 1964).

(viii) *Backcross analysis*

There are two possible causes of pseudo-linkage. One is a meiotic event in which female hybrids produce fewer gametes carrying the combination of genes of different species origin (Fig. 3*A*). The other is a post-zygotic event in which daughters with homozygous genes from different species are eliminated (Fig. 3*B*). To discriminate these two possibilities we crossed two female hybrids individually to *pal-Br*, + males (Fig. 3*C*). The middle two genotypic classes must be under-represented in the meiotic event hypothesis, while four genotypic classes must be produced equally in the postzygotic event hypothesis. DNA was extracted from each male of the next generation (females were not used because some of them are produced parthenogenetically) and two RAPD markers (OPA15-950 and OPA15-1200) were examined. Because bands appear when the fly inherits the allele of the *ana-Im*, *y* strain, the segregation pattern in female hybrid meiosis is revealed.

3. Results

(i) *Establishment of interspecific mosaic genome lines*

The result of the crosses between *ana-Im*, *y* females and *pal-Br*, + males is shown in Table 2. Two phenotypic classes of females appeared. The y females are pure *ana-Im*, *y*, produced parthenogenetically. The y^+ females (y/y^+) and y males (y/Y) must be interspecific hybrids.

Table 2. *The crosses of* ana-Im, y *females and* pal-Br, + *males*

Trial no.	Females		Males
	y [*ana-Im*, *y*]	y$^+$ [Hybrid]	y [Hybrid]
1	59	28 (21)	26
2	>154[a]	25 (13)	42

The number of those producing the next generation is in parentheses.
[a] More appeared after the emergence of hybrids.

Table 3. *Total number of flies from 34 virgin female hybrids*

y females	y$^+$ females	y$^+$ males
473 (273[a])	466 (270[b] + 10[c])	1[d]

The number of those producing the next generation is in parentheses.
[a] Producing y females.
[b] Producing y$^+$ females.
[c] Producing y$^+$ and y females (and males).
[d] Presumably y^+/O.

The recessive *parth* genes of *D. ananassae* and *D. pallidosa* are thought to be homologous, and female hybrids of the strains result in parthenogenesis (Matsuda & Tobari, 2004). Among 53 female hybrids obtained, 34 produced offspring parthenogenetically when separated singly as virgins. On average, 28·5 ± 27·0 (standard deviation) flies appeared from a female (maximum 101). The next-generation flies from the virgin female hybrids are classified by their phenotype (Table 3). The numbers of y females and y$^+$ females were close ($\chi^2 = 0{\cdot}052$, $P > 0{\cdot}8$), which is consistent with the postulated mechanism of parthenogenesis (see Section 1). An exceptional y^+ male (presumably XO) appeared.

When the second-generation females were separated singly, 57·7% of y and 60·1% of y$^+$ produced offspring parthenogenetically. All the y females bred true, while among 280 y$^+$ females producing offspring, 270 bred true. The other 10 yielded an unexpected result: y$^+$ and y (including males in some cases) were segregated in the next generation. This implies that, at a low frequency, parthenogenesis is completed by the fusion of two haploid nuclei (Matsuda & Tobari, 2004). The reason that sons appeared from some females is unknown; we cannot rule out the possibility that those females were triploid. We established interspecific mosaic genome lines by transferring each clutch to new vials. The lines derived from the 10 exceptional females were excluded from the analyses below.

Table 4. *Segregation of markers in the hybrid mosaic genome lines*

Marker (chromosome)	a	p	χ^2 value
y (XL)	156	110	7·955**
w (XR)	130	136	0·135[ns]
OPA15-950 (2L)	86	180	33·218***
Om(2D) (2mid)	98	168	18·421***
eyg-h (2R)	109	157	8·662**
cn-h (3L)	105	161	11·789***
Amyrel (3mid)	152	114	5·429*
OPA15-1200 (3R)	116	150	4·346[ns]
RfaBp (4)	127	139	0·541[ns]

[ns] $P > 0{\cdot}05$, * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$.

Few offspring appeared in some of the lines and these lines were lost in the early stages of establishment. The lines that survived for several months could be maintained without a problem later. We had 266 lines in month 30 (corresponding to 49·0% of the second-generation fertile females) and these were subjected to the chromosome and molecular marker analyses.

(ii) *Preliminary linkage data*

To determine the genetic constitution of each line we first conducted RAPD analysis for 70 lines. Twenty primers were tested and 54 diagnostic bands appeared. We also examined polytene chromosomes for 64 lines of them and found complete linkage of some markers and inversions. One cluster consists of three RAPD markers and the visible marker, *y*, and is linked to *In(XL)A*. No recombination took place between 15 RAPD markers and *In(2L)C-In(2L)D + In(2L)B* and between 13 RAPD markers and *In(3R)B*. The markers are thought to be on tightly linked blocks, protected from recombination by the inversions. Two of the markers, OPA15-950 (2L) and OPA15-1200 (3R), were chosen for the analyses below.

(iii) *Segregation ratio on each locus*

We statistically tested whether the allele derived from *D. ananassae* (a) and that derived from *D. pallidosa* (p) segregate in a 1:1 ratio for nine marker loci (Table 4). Three on XR, 3R and 4 showed the result expected. Significant deviation was seen for the other six loci; especially significant were those on 2L, 2mid and 3L. The frequency of p is larger for four loci, and vice versa for two loci.

(iv) *Linkage and pseudo-linkage between loci*

The results of the calculations are shown in Table 5. First, we examined linkage between loci on the same

Table 5. *Linkage and pseudo-linkage of markers in the hybrid mosaic genome lines*

Chromosomes	Combination of markers (a; a)	(a; p)	(p; a)	(p; p)	χ^2_L	c	D **D'**
Linkage							
XL-XR							
O	80	76	50	60	0·737^{ns}	0·474	0·014
E	76·2	79·8	53·8	56·2			**0·070**
2L-2mid							
O	54	32	45	135	47·158***	0·289	0·083
E	31·7	54·3	66·3	113·7			**0·405**
2mid-2R							
O	46	53	63	104	4·346*	0·436	0·020
E	40·2	57·8	68·8	99·2			**0·094**
2L-2R							
O	43	43	66	114	8·662**	0·410	0·029
E	35·2	50·8	73·8	106·2			**0·153**
3L-3mid							
O	64	41	88	73	0·241^{ns}	0·485	0·015
E	60·0	45·0	92·0	69·0			**0·089**
3mid-3R							
O	91	61	25	89	33·218***	0·323	0·093
E	66·3	85·7	49·7	64·3			**0·497**
3L-3R							
O	43	62	73	88	0·060^{ns}	0·508	−0·010
E	45·8	59·2	70·2	90·8			**−0·061**
Pseudo-linkage (other pairs are not statistically significant)							
2L-3R							
O	72	14	44	136	84·586***	0·218	0·130
E	37·5	48·5	78·5	101·5			**0·711**
2mid-3R							
O	59	40	57	110	19·489***	0·365	0·060
E	42·7	55·3	73·3	94·7			**0·286**
2R-3R							
O	55	54	61	96	4·872*	0·432	0·028
E	47·5	61·5	68·5	88·5			**0·121**
2L-3mid							
O	61	25	91	89	4·346*	0·436	0·045
E	49·1	36·9	102·9	77·1			**0·322**
XL-3mid							
O	99	57	53	57	7·955**	0·414	0·037
E	89·1	66·9	62·9	47·1			**0·157**
2mid-4							
O	56	43	71	96	5·429*	0·429	0·033
E	46·8	51·2	80·2	87·8			**0·171**
2R-3L							
O	31	78	74	83	5·429*	0·571	−0·045
E	43·0	66·0	62·0	95·0			**−0·280**

^{ns} $P>0·05$, * $P<0·05$, ** $P<0·01$, *** $P<0·001$. χ^2_L, the χ^2 value for linkage; c, recombination fraction; D, coefficient of linkage disequilibrium (the normalized D value, D', is in bold); O, number observed; E, number expected.

chromosome. XL-XR and 3L-3mid are only loosely linked (i.e. independent; $c=0·5$; $D=0$). Strong linkage was seen between 2L-2mid and 3mid-3R ($c=0·298$, 0·323, respectively; $D>>0$). This is because the markers are located close to each other or crossing-over is suppressed by inversions in these chromosome regions. There was also weaker linkage in 2mid-2R (and 2L-2R).

Second, we examine pseudo-linkage between loci on the different chromosomes. Among 29 combinations, significant deviation from independence was seen for seven (significant even after Bonferroni correction for 2L-3R and 2mid-3R; $P<0·001/29$). D was negative in 2R-3L (i.e. repulsion; $c>0·5$) and positive in the others (i.e. coupling; $c<0·5$). The deviation was largest in 2L-3R ($c=0·218$, $D=0·130$, $D'=0·711$); the observed numbers of (a; p) and (p; a) combinations are lower than expected, especially the former. Pseudo-linkage in 2mid-3R, 2R-3R and 2L-3mid is thought to be secondary, caused by that

Table 6. *Segregation in female hybrids backcrossed to* pal-Br, + *males*

Chromosomes	Combination of markers (a; a)	(a; p)	(p; a)	(p; p)	χ^2_L	c	D / D'
2L-3R							
O	55	62	41	42	0·180^{ns}	0·515	−0·006
E	56·2	60·8	39·8	43·2			**−0·027**

^{ns} $P > 0{\cdot}05$. χ^2_L, the χ^2 value for linkage; c, recombination fraction; D, coefficient of linkage disequilibrium (normalized D value, D', is in bold); O, number observed; E, number expected.

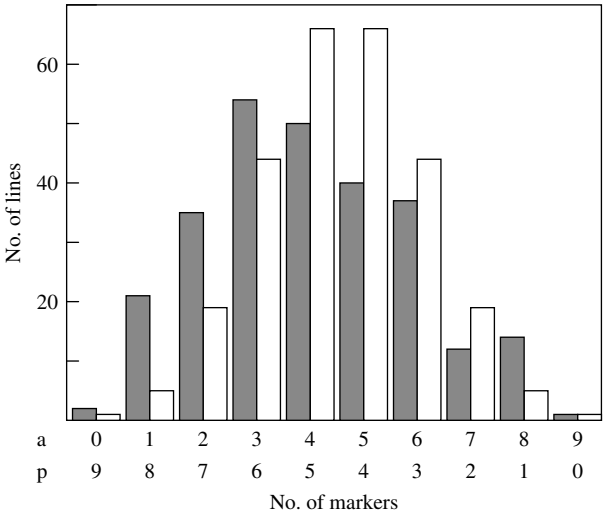

Fig. 4. Numbers of loci occupied by alleles from *ana-Im*, *y* (a) and *pal-Br*, + (p) in mosaic genome lines (filled bars). The hypothetical random distribution (open bars) is also indicated for comparison.

in 2L-3R, because linkage exists in 2L-2mid, 2L-2R and 3mid-3R (see above). There was also (weaker) pseudo-linkage for XL-3mid and 2mid-4. Thus, the possibility that multiple loci are occupied by the allele of the same species becomes high, and the distribution showing how many loci are occupied by a or p in each line deviates from random expectation (Fig. 4). The distribution is shifted to the side carrying more p alleles than expected, as the frequency of p is larger than that of four of the six deviated loci (see Section 3.iii).

(v) *The cause of pseudo-linkage*

In the backcross analysis (Fig. 3*C*), there were no deviations from independent segregation of 2L and 3R genes, irrespective of the two tested females and of the X chromosome constitution (whether inheriting y or y^+) of the backcross hybrids (total shown in Table 6). Therefore, we can rule out the meiotic event hypothesis (Fig. 3*A*), and conclude the results are explained by the zygotic event hypothesis (Fig. 3*B*).

4. Discussion

(i) *Recovery distortion*

Recovery distortion was detected at six loci among the nine examined in the present analyses. There are two possible causes: (1) meiotic drive in female hybrids; (2) selection operating on fitness differences between alleles of the marker or linked loci. We determined the segregation ratio for the *y* locus twice. In the second generation the ratio of $y : y^+$ was 473:466 (or 273:280 if only fertile females were counted), which indicates 1:1 segregation (Table 3). However, at the time of line establishment the ratio was 156:110, indicating significant deviation (Table 4). Recovery distortion is not caused by possibility (1) at least on this locus. According to the mating scheme (Fig. 1), the established lines must carry the *D. ananassae* cytoplasm. This might have caused the recovery distortion, but the deviation is in the opposite direction (more *D. pallidosa* alleles at four loci).

(ii) *Pseudo-linkage*

Strong pseudo-linkage was detected between the OPA15-950 and OPA15-1200 loci on different chromosomes (2L and 3R, respectively). As the backcross analysis ruled out the possibility of a meiotic origin, the remaining possible explanation involves fitness differences between genotypes. Flies with genetic incompatibility (Dobzhansky, 1937; Muller, 1940) may be selected out in the process of establishing lines. It is worth noting here that few offspring appeared in some of the mosaic genome lines and these lines were lost in the early stages of establishment. The mechanisms of inviability and sterility caused by such gene interactions have been well documented (Wu & Palopoli, 1994; Hutter, 1997; Hollocher, 1998; Gadau *et al.*, 1999; Sawamura, 2000; Wu & Ting, 2004; Orr *et al.*, 2004). In the interspecific mosaic genome lines, some loci are a/a homozygous and others p/p homozygous. If recessive–recessive incompatible gene pairs exist

(they are usually asymmetrical), the phenotype (low fitness) must be expressed in particular lines. As has been pointed out previously (Wu & Palopoli, 1994; Johnson, 2000; Orr & Presgraves, 2000), such genotypes do not appear in F_1 or backcross hybrids. Because both female and male *D. ananassae*/*D. pallidosa* hybrids are fertile and their descendants can be maintained without problems (e.g. Futch, 1966, 1973*a*; Stone *et al*., 1966; Doi *et al*., 2001; Yamada *et al*., 2002*a*), such hybrid breakdown has not previously been described in this species pair. The present result suggests the possibility that specific combinations of genes from the species may result in low fitness. Similar cases have also been documented in other species hybrids: e.g. in Nasonia (Weston *et al*., 1999). Care is needed in drawing linkage maps by using interspecies crosses.

(iii) *Inversion and speciation*

The regions of 2L and 3R where pseudo-linkage was detected have inversions that suppress crossing-over between the strains. Genes inside or near inversions behave as super-genes because of historical co-adaptation (Dobzhansky, 1970; Krimbas & Powell, 1992). And it is supposed that speciation genes are accumulated at a higher rate in the non-recombined region of the genomes if the species have had gene flow (Noor *et al*., 2001; Rieseberg, 2001; Navarro & Barton, 2003; Brown *et al*., 2004; Butlin, 2005). Thus, it may be that 2L and 3R have more genes causing lower fitness of hybrids. It is also possible that incompatible loci are distributed randomly in the genomes. In this case we assume the effect was detected on 2L and 3R by chance, because more genes are inherited together in these chromosome regions. Dissecting the causes of recovery distortion and pseudo-linkage is left for future work, although conventional recombination mapping of the responsible genes seems difficult.

(iv) *Advantage of the established materials*

In the present study we established 266 mosaic genome lines and determined the genetic constitution of each line. Because there are many individuals with exactly the same genotype in each line (i.e. clones), characters which can only be dealt with *en masse* (e.g. female mating preference) can be tested. Thus, the lines reported here will be useful for dissecting the genetic mechanisms of sexual isolation between the species. The mosaic genome lines can also be used to identify genes responsible for incompatibility between species and species-specific characters. There are perforce only females in the mosaic genome lines, but males with part of their genomes heterozygous can be made by backcrossing the females to one of the parental species. Thus, studying male traits (e.g. courtship song parameters) is also possible. We hope the mosaic genome lines reported here will be useful for future research on speciation and species differentiation. The established materials are available from Kyorin University upon request (http://kyotofly.kit.jp/kyorin/).

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