Studies on sex ratio distortion in the *Ostrinia furnacalis* species group

（アワノメイガ種群における性比異常に関する研究）

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PhD thesis

Studies on sex ratio distortion in the *Ostrinia furnacalis* species group

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General Introduction

0.1 Genetic elements that can cause sex ratio distortion

In contrast to autosomes, which are inherited equally from both parents, cytoplasmic elements and sex chromosomes tend to be inherited asymmetrically from parents to offspring. Fisher (1930) predicted that 1:1 sex ratio is evolutionarily stable, but this is with regard to the interest of autosomes, and is not true for either sex chromosomes (Hamilton, 1967) or cytoplasmic elements (Cosmides and Tooby, 1981). Cytoplasmic elements and sex chromosomes that bias their hosts' sex ratio toward the sex they are transmitted have a potential to spread in sexually reproducing organisms. In fact, such sex-ratio-distorting elements have been found in a number of animals, and are considered to be one of the 'selfish genetic elements' (ref. Werren *et al.*, 1988).

0.2 The criteria for establishing existence of cytoplasmic sex ratio distorters

Several procedures should be employed to demonstrate the cytoplasmic nature of the sex ratio distorters (Hurst, 1993). First, inheritance pattern of female-biased sex ratio (SR) should be analysed to verify the maternal inheritance. These should involve backcrossing which replace the nuclear DNA of affected lineage with that of an unaffected lineage. If the SR persists, it must be cytoplasmic. In female-heterogametic organisms, however, the effect of W chromosome cannot be ruled out. A second procedure involves experimental manipulation of the sex ratio distorter involved. Ideally, the Koch's postulates should be fulfilled, i.e. the distorter should be removed from an affected individual, it should be grown in culture, and it should cause the SR when replaced into an unaffected individual. Since many of cytoplasmic distorters are unable to be cultured outside of their hosts, the Koch's postulates are seldom fulfilled. An easier but less thorough analysis is injection of the homogenate of the tissue from an affected individual into an unaffected individual. A more circumstantial evidence of cytoplasmic sex ratio distortion comes from the recovery from the sex ratio bias after elimination of the distorters by administration of high temperature and/or antibiotics. Lastly, detection and/or visualisation of distorters that are present in every affected lineage but never in unaffected lineages is indirect but highly suggestive evidence of cytoplasmic sex ratio distortion. Practically, a set of procedures, e.g. the maternal inheritance, cure from sex ratio distortion by elimination of distorters by antibiotics/temperature and the detection of distorters by PCR/electron microscope, have most often been selected to establish the existence of cytoplasmic sex ratio distorters.

0.3 Female-biased sex ratio due to cytoplasmic elements

In general, cytoplasmic elements in animals are transmitted exclusively from the mother. Therefore, biasing the sex ratio toward female is advantageous for cytoplasmic elements. SR induced by cytoplasmic elements, referred to as cytoplasmic sex ratio distorters, has been repeatedly found in a wide variety of arthropods (reviewed by Hurst, 1993; Hurst *et al.*, 1997a) (Table 0.1). There are three known mechanisms for the sex ratio distortion. Selective male death (male-killing) has been known in five insect orders: Diptera, Coleoptera, Hemiptera, Lepidoptera and Hymenoptera (reviewed by Hurst *et al.*, 1997a). A variety of bacteria and a protist of the genus *Amblyospora* induce male-killing in different hosts. Female production by virgin females (referred to as thelytokous parthenogenesis) has been known in parasitic wasps, gall wasps and a thrip (Stouthamer *et al.*, 1990; Schilthuizen and Stouthamer, 1998; Arakaki *et al.*, 2001). At present, the bacterium *Wolbachia* is the only known cytoplasmic element that can induce thelytokous parthenogenesis. Feminization of genetic males has been known in crustaceans such as woodlice *Armadillidium* spp. and a shrimp *Gammarus duebeni* (Rousset *et al.*, 1992; Dunn *et al.*, 1993). In *Armadillidium*, *Wolbachia* and another unidentified cytoplasmic element induce feminization of genetic males, while a protist of the genus *Octosporea* induces feminization of *Gammarus duebeni* males. Feminization of haploid males due to infection with Flavobacteria has been recently found in a mite *Brevipalpus phoenicis* (Weeks *et al.*, 2001).

Female-biased sex ratio has been known in many species of Lepidoptera (Table 0.2). Most cases where mechanisms are reported are male-killing during embryonic stage (e.g. Hurst *et al.*, 1999a; Jiggins *et al.*, 2000). To date, *Wolbachia* and *Spiroplasma* have been reported as the causal agent of male-killing in Lepidoptera. Besides, thelytokous parthenogenesis, including gynogenesis, has been found from different families of Lepidoptera (Lokki *et al.*, 1975; Mitter and Futuyma, 1977; Menken and Wiebosch-Steeman, 1988; Gorbunov and Kishida, 1995). Causal agents of the thelytokous parthenogenesis in Lepidoptera have not been identified.

Table 0.1 Female-biased sex ratio due to cytoplasmic elements which have been identified

Mechanism	Organism	Causal agent	Reference
Early male killing	Insecta: Coleoptera: Coccinelidae: *Adalia bipunctata*	Procaryota: α-Proteobacteria: *Rickettsia*	(1)
	Insecta: Coleoptera: Coccinelidae: *Adalia bipunctata*	Procaryota: α-Proteobacteria: *Wolbachia*	(2)
	Insecta: Coleoptera: Coccinelidae: *Adalia bipunctata*	Procaryota: Mollicutes: *Spiroplasma*	(3)
	Insecta: Coleoptera: Coccinelidae: *Adalia decempunctata*	Procaryota: α-Proteobacteria: *Rickettsia*	(4)
	Insecta: Coleoptera: Coccinelidae: *Coleomegilla maculata*	Procaryota: Flavobacteria	(5)
	Insecta: Coleoptera: Coccinelidae: *Adonia variegata*	Procaryota: Flavobacteria	(6)
	Insecta: Coleoptera: Tenebrionidae: *Tribolium madens*	Procaryota: α-Proteobacteria: *Wolbachia*	(7)
	Insecta: Coleoptera: Buprestidae: *Brachys tessellatus*	Procaryota: α-Proteobacteria: *Rickettsia*	(8)
	Insecta: Diptera: Drosophilidae: *Drosophila willistoni*	Procaryota: Mollicutes: *Spiroplasma*	(9)
	Insecta: Diptera: Drosophilidae: *Drosophila bifasciata*	Procaryota: α-Proteobacteria: *Wolbachia*	(10)
	Insecta: Lepidoptera: Danaidae: *Danaus chrisippus*	Procaryota: Mollicutes: *Spiroplasma*	(11)
	Insecta: Lepidoptera: Acraeidae: *Acraea encedon*	Procaryota: α-Proteobacteria: *Wolbachia*	(12)
	Insecta: Lepidoptera: Acraeidae: *Acraea encedana*	Procaryota: α-Proteobacteria: *Wolbachia*	(13)
	Insecta: Hymenoptera: Pteromalidae: *Nasonia vitripennis*	Procaryota: γ-Proteobacteria: *Arsenophonus*	(14)
Late male killing	Insecta: Diptera: Culicidae: *Aedes* spp.	Eucaryota: Microspora: *Amblyospora*	(15)
Thelytokous parthenogenesis	Insecta: Hymenoptera: Trichogrammatidae: *Trichogramma* spp.	Procaryota: α-Proteobacteria: *Wolbachia*	(16)
	Insecta: Hymenoptera: Pteromalinidae: *Muscidifurax uniraptor*	Procaryota: α-Proteobacteria: *Wolbachia*	(17)
	Insecta: Hymenoptera: Aphelinidae: *Aphytis* spp.	Procaryota: α-Proteobacteria: *Wolbachia*	(18)
	Insecta: Hymenoptera: Aphelinidae: *Encarsia formosa*	Procaryota: α-Proteobacteria: *Wolbachia*	(19)
	Insecta: Hymenoptera: Encyrtidae: *Apoanagyrus diversicornis*	Procaryota: α-Proteobacteria: *Wolbachia*	(20)
	Insecta: Hymenoptera: Cynipoidea: *Diplolepis rosae*	Procaryota: α-Proteobacteria: *Wolbachia*	(21)
	Insecta: Thysanoptera: Aeolothripidae: *Franklinothrips vespiformis*	Procaryota: α-Proteobacteria: *Wolbachia*	(22)
Feminization of genetic males	Crustacea: Isopoda: Oniscidea: *Armadillidium vulgare*	Procaryota: α-Proteobacteria: *Wolbachia*	(23)
	Crustacea: Amphipoda: Gammaridae: *Gammarus duebeni*	Eucaryota: Microspora: *Octosporea*	(24)
	Arachnida: Acari: Tenuipalpidae: *Brevipalpus phoenicis*	Procaryota: Flavobacteria	(25)

For each name of organism, Class: Order: Family: Species are shown in this order. (1) Werren *et al.* (1994), (2) Hurst *et al.* (1999), (3) Hurst *et al.* (1999), (4) Schulenburg et al. (2001), (5) Hurst *et al.* (1997b), (6) Hurst et al. (1999c), (7) Fialho and Stevens (2000), (8) Lawson et al. (2001), (9) Hackett *et al.* (1986) (10) Hurst *et al.* (2000), (11) Jiggins *et al.* (2000b), (12) Hurst *et al.* (1999), (13) Jiggins *et al.* (2000a), (14) Gherna *et al.* (1991), (15) Andreadis *et al.* (1988), (16) Stouthamer (1997), (17) Stouthamer *et al.* (1993), (18) Zchori-Fein *et al.* (1995), (19) Zchori-Fein *et al.* (1992), Werren *et al.* (1995a), (20) Pijls *et al.* (1996), (21) Schiltuizen and Stouthamer (1998), (22) Arakaki *et al.* (2001), (23) Rigaud *et al.* (1997), (24) Dunn *et al.* (1993), (25) Weeks *et al.* (2001).

Table 0.2 Incidences of female-biased sex ratio in Lepidoptera

Mechanism	Family	Species	References
Early male killing	Acraeidae	*Acraea encedon*	(1)
	Acraeidae	*Acraea encedana*	(2)
	Danaidae	*Danaus chrysippus*	(3)
	Lymantriidae	*Lymantria dispar*	(4)
	Pieridae	*Pieris napi*	(5)
	Nymphalidae	*Hypolimnas bolina*	(6)
	Noctuidae	*Spodoptera littoralis*	(7)
	Tortricidae	*Epiphyas postvittana*	(8)
	Gracillaridae	*Phyllonorycter sorbicola*	(9)
	Arctiidae	*Estigmene acrea*	(10)
	Pyralidae	*Cadra*(=*Ephestia*) *cautella*	(11)
	Notodontidae	*Pygaera*(=*Clostera*) *pigra*	(12)
Parthenogenesis	Bombycidae	*Bombyx mori*	(13)
	Geometridae	*Alsophila pometaria*	(14)
	Psychidae	*Solenobia triquetrella*	(15)
	Nepticulidae	*Ectoedemia argyropeza*	(16)
	Saturniidae	*Neoris huttoni schenki*	(17)
Suspected as W drive	Geometridae	*Abraxus grossulariata*	(18)
	Satyridae	*Maniola jurtina*	(19)
	Lasiocampidae	*Philudoria potatoria*	(20)

(1) Hurst *et al.* (1999), (2) Jiggins *et al.* (2000a), (3) Jiggins *et al.* (2000b), (4) Higashiura *et al.* (1999), (5) Bowden (1987), (6) Simmonds (1930), Clarke *et al.* (1975), (7) Brimacombe (1980), (8) Geier and Briese (1977), Geier *et al.* (1978), (9) Ujiye (1981), (10) Earle and MacFarlane (1968), (11) Takahashi and Kuwahara (1970), (12) Federley (1936), (13) Astaurov (1936), (14) Mitter and Futuyma (1977), (15) Lokki *et al.* (1975), (16) Menken and Wiebosch-Steeman (1988), (17) Gorbunov and Kishida (1995), (18) Doncaster (1913, 1914), (19) Scali and Masetti (1973), (20) Majerus (1981).

0.4 *Wolbachia pipientis*: cytoplasmic bacterium that can cause reproductive alterations in arthropods

Bacteria of the genus *Wolbachia* are cytoplasmic parasites in arthropods. *Wolbachia* belongs to the alpha Proteobacteria, and is closely related to *Rickettsia*. *Wolbachia* bacteria are common, infecting 26 of 154 Panamanian neotropical insect species (16.9%) and 28 of 145 temperate North American insect species (19.3%) (Werren *et al.*, 1995a; Werren and Windsor, 2000). A recent study suggested that the percentage might be even higher (Jeyaprakash and Hoy, 2000). *Wolbachia* is attracting evolutionary biologists in that it manipulates the hosts' reproduction in a variety of ways for their own good, and hence is a good model of selfish genetic elements (reviewed by Werren *et al.*, 1988; Werren, 1997; Stouthamer *et al.*, 1999).

Some *Wolbachia* strains force their hosts to produce progenies with female-biased sex ratio by inducing male-killing, thelytokous parthenogenesis or feminization (Hurst *et al.*, 1999a; Fialho and Stevens, 2000; Stouthamer, 1997; Rigaud, 1997). Other *Wolbachia* strains decrease the fitness of uninfected females by inducing cytoplasmic incompatibility: typically, an uninfected female produces a drastically small number of progenies if mated with an infected male (Hoffmann and Turelli, 1997). *Wolbachia*-induced sperm precedence that enhances the effect of cytoplasmic incompatibility has been also known (Wade and Chang, 1995). Recently, a novel type of *Wolbachia* infection that is prerequisite for oogenesis of a parasitic wasp *Asobara tabida* has been reported

(Dedeine *et al.*, 2001). All of these reproductive manipulations have potential to increase *Wolbachia* prevalence.

To characterize *Wolbachia* strains, 16S *rRNA* gene sequences were initially used. However, slow evolutionary rate of this gene has not made it possible to adequately resolve a fine-scale phylogeny of *Wolbachia* strains. Subsequently, a faster-evolving cell-cycle gene (*ftsZ*) was used to improve the phylogenetic resolution, and revealed that there are two major clades (A and B) within the genus *Wolbachia* (Werren *et al.*, 1995b). Recently, a surface protein gene of *Wolbachia* (*wsp*) was cloned and sequenced (Braig *et al.*, 1998). This gene is evolving at a much faster rate than any other previously reported *Wolbachia* genes, and at present, it is assumed to be the most appropriate gene for phylogenetic analyses of *Wolbachia* (Zhou *et al.*, 1998).

Major phenotypes of reproductive alterations caused by *Wolbachia* (cytoplasmic incompatibility, thelytokous parthenogenesis, feminization and male-killing) are not concordant with the phylogeny of *Wolbachia* gene (Figure 0.1). This led us to assume that *Wolbachia* has been repeatedly transmitted horizontally in the recent past (Werren and O'Neill, 1997).

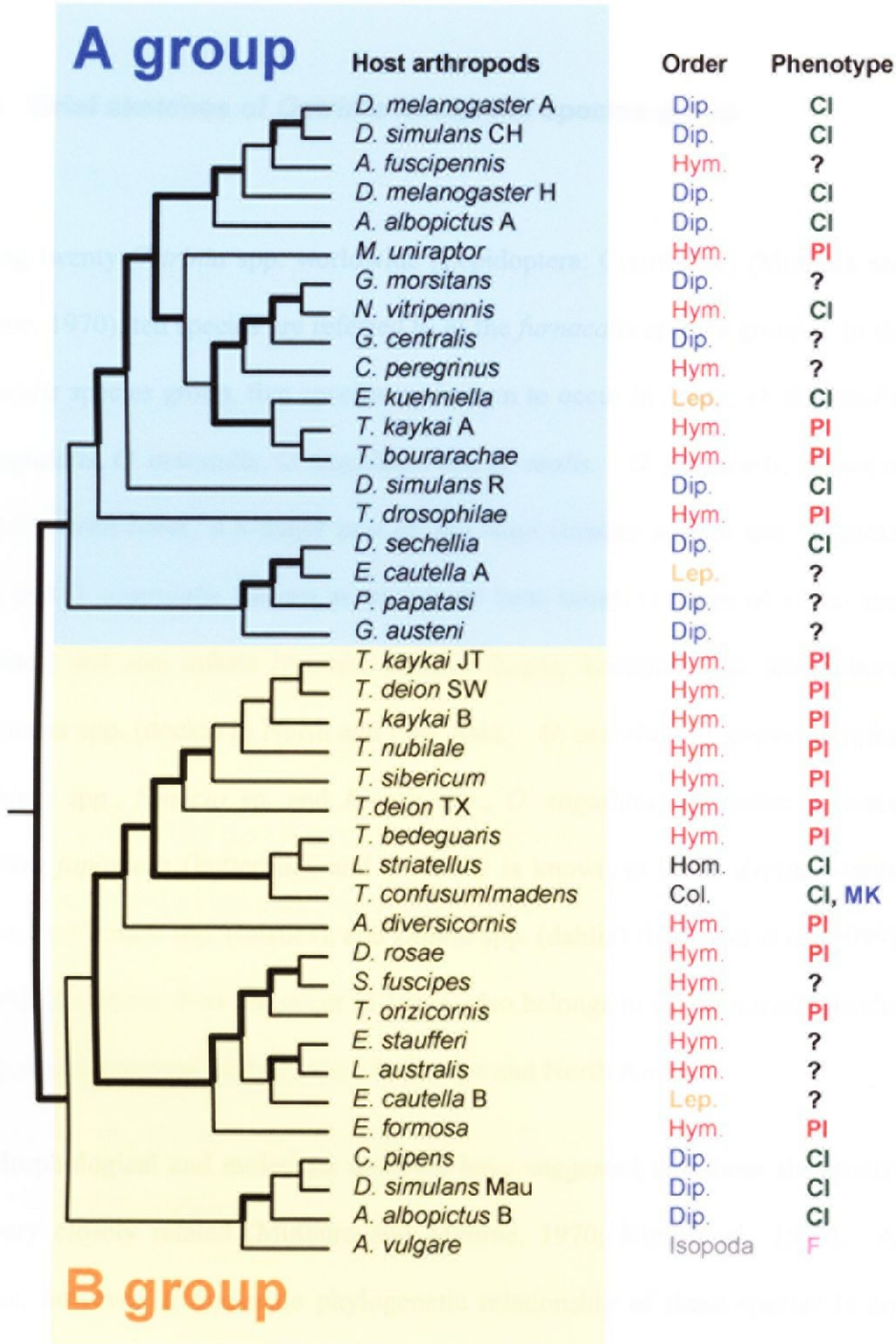

Figure 0.1 Cladogram of *Wolbachia* based on the *wsp* sequences. MK, PI, F and CI are abbreviations of male-killing, parthenogenesis induction, feminization and cytoplasmic incompatibility, respectively. Name of the host arthropod species represents *Wolbachia* lineage. Branches with bootstrap value more than 80% are shown with bold line. Topology was extracted from Neighbour-Joining tree constructed by van Meer *et al.* (1999).

0.5 Brief sketches of *Ostrinia furnacalis* species group

Among twenty *Ostrinia* spp. worldwide (Lepidoptera: Crambidae) (Mutuura and Munroe, 1970), ten species are referred to as the *furnacalis* species group. In the *furnacalis* species group, five species are known to occur in Japan; *O. furnacalis*, *O. scapulalis*, *O. orientalis*, *O. zaguliaevi* and *O. zealis*. *O. furnacalis*, known as the Asian corn borer, is a major pest of *Zea mays* (maize) in East and Southeast Asia, and *O. scapulalis*, known as the adzuki bean borer, is a pest of *Vigna* spp. (legumes) and also infests *Humulus lupulus* (hops), *Xanthium* spp. (cockleburs) and *Rumex* spp. (docks) in North and East Asia. *O. orientalis* is known to infest *Xanthium* spp., *Senecio* sp. and *Rumex* spp., *O. zaguliaevi* is known to infest *Petacites japonicus* (butterbur), and *O. zealis* is known to infest *Arctium lappa* (burdock), *Cirsium* spp. (thistles), and *Dahlia* spp. (dahlia) (Ishikawa *et al.*, 1999). *O. nubilalis*, which does not occur in Japan, also belongs to the *furnacalis* species group, and is a serious pest of maize in Europe and North America.

Morphological and molecular analyses have suggested that these six species are very closely related (Mutuura and Munroe, 1970; Kim *et al.*, 1999). At present, however, a fine-scale phylogenetic relationship of these species is not clear.

0.6 Aims of the present study

The aim of the present study was to precisely describe the forces moulding the sex ratio in *Ostrinia* species.

First, I examined the sex ratio of progenies produced from wild *O. furnacalis* females. I investigated the field prevalence, the mode of inheritance, the mechanism and the causal agent of the sex ratio distortion (Chapter 1).

Second, I examined whether the same and/or different type(s) of sex ratio distortion occurs in *O. scapulalis* (Chapter 2).

Third, I surveyed the occurrence of sex ratio distortion in the other species of the *furnacalis* species group in Japan (Chapter 3).

Finally, I examined the effect of partial elimination of the causal agent of one type of female-biased sex ratio on sexuality of their progenies in *O. scapulalis* (Chapter 4).

In general discussion, I argued the significance of sex ratio distortions found in *Ostrinia* species and suggested their potential use for the research of sex determination in insects.

Chapter 1

Wolbachia-induced feminization in the Asian corn borer, *Ostrinia furnacalis*

1.1 Introduction

Female-biased sex ratios have been repeatedly found in arthropods. Most of them involve parasitic microorganisms in cytoplasm. Since cytoplasmic elements are inherited exclusively from females, female-biased sex ratio is advantageous for these microorganisms.

Much attention has been increasingly paid to *Wolbachia* (see general introduction for detail). *Wolbachia* is a group of bacteria that infects wide varieties of arthropods and vertically transmitted via cytoplasm. *Wolbachia* can bias their hosts' sex ratio by inducing male-killing (e.g. Hurst *et al.*, 1999), thelytokous parthenogenesis (e.g. Stouthamer *et al.*, 1993) or feminization of genetic males (e.g. Rousset *et al.*, 1992). In addition, *Wolbachia* can reduce the

fitness of uninfected females by inducing cytoplasmic incompatibility in many arthropod species (e.g. Breeuwer *et al.*, 1992; O'Neill *et al.*, 1992).

During the course of rearing experiments, I found an all-female family among those produced from wild *O. furnacalis* females collected at Matsudo, Chiba-pref. Later, I found that Miyahara (1984) is the first who reported the occurrence of female-biased sex ratio in the Asian corn borer *Ostrinia furnacalis*. However, the mechanism and causal agent of the sex ratio trait have not been elucidated. Furthermore, since Miyahara (1984) examined sex ratio in each insect rearing case i.e. that of progenies produced from multiple females, frequencies of the causal agent in the populations were not known. It is also not known whether there was only one sex-ratio-distorting element in *O. furnacalis* or not.

The aim of this chapter is to clarify the frequencies of female-biased sex ratio in the natural populations of *O. furnacalis*, and to identify its mechanism and causal agent.

1.2 Materials and methods

1.2.1 Insects

Female adults of *Ostrinia furnacalis* were collected at Matsudo (Chiba pref., Japan) in the summers of 1996-2000. The individual females were allowed to oviposit in the laboratory. Most of the collected females laid fertile eggs within a few days. Larvae were reared by broods on an artificial diet (Silk Mate 2M, Nihon-Nosan, Yokohama, Japan). At the pupal stage, insects were sexed based on the abdominal tip morphology. A piece of cotton soaked with 3% sucrose was provided for adult moths. Insects were reared under the conditions of 23°C and 15L/9D. Twenty females and twenty males were put in a mating cage, and two days later, the females were separately allowed to oviposit. After oviposition, ovaries of females were dissected and stored in STE buffer (100 mM NaCl/ 10 mM Tris-HCl, pH 8.0/ 1 mM EDTA, pH 8.0; O'Neill *et al.*, 1992) at – 20°C until extraction of DNA.

1.2.2 Maternal inheritance of female-biased sex ratio

When a wild female produced a family with female-biased sex ratio (SR) ($P < 0.01$, chi-squared test), daughters were used to found a matriline (Figure 1.1). Matrilines were maintained by crossing females with males from normal lines.

A normal line was a pool of matrilines with the parental sex ratio not significantly distorted from 1:1.

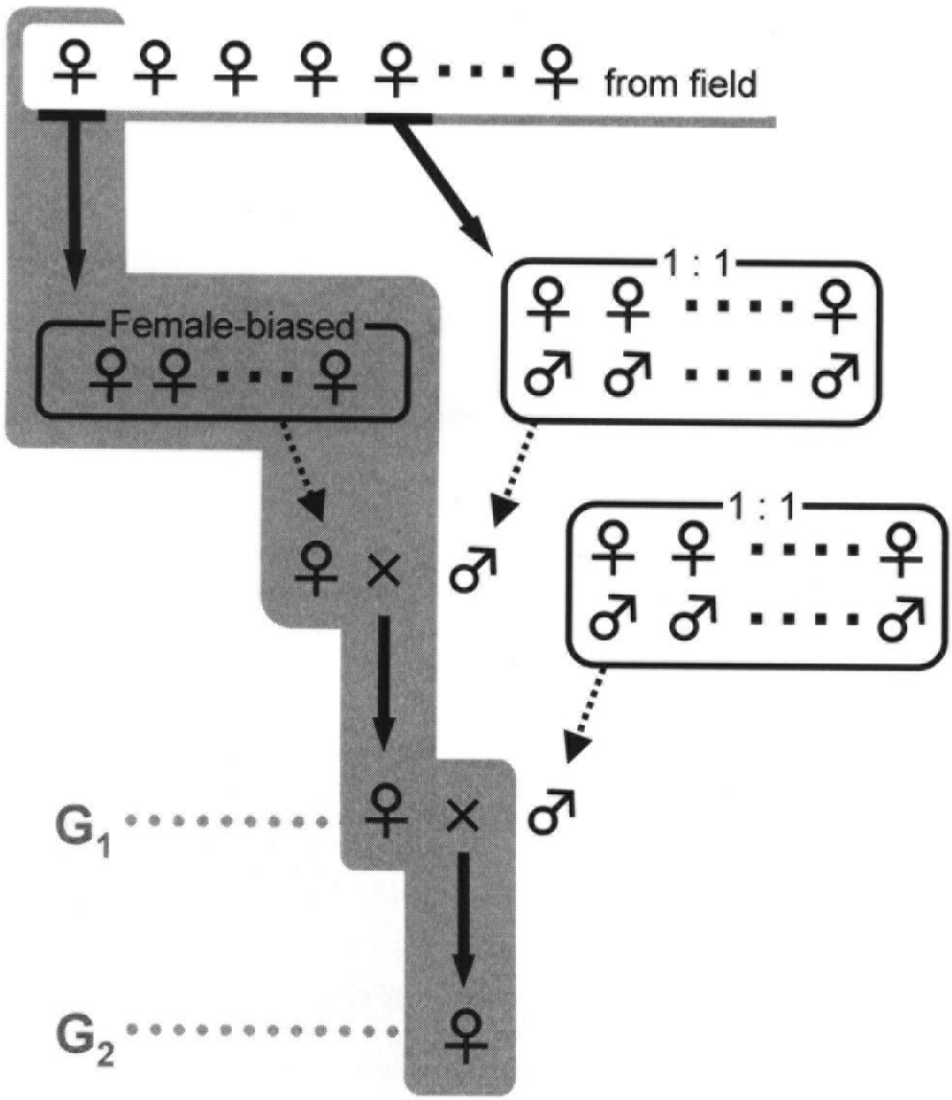

Figure 1.1 Procedure for establishing an SR matriline derived from a wild female. Female adults collected in the field, most of which are gravid, are individually allowed to oviposit. In each generation, single female of an SR family was crossed with males of a 1:1 sex ratio family. The generation of the progenies produced from wild females are referred to as parental generation (P), and subsequent generations are referred to as G_1, G_2 ...

1.2.3 Tetracycline treatment

Tetracycline hydrochloride was mixed into the larval diet at 0.06% (w/w) and fed to larvae from the neonate stage. The tetracycline treatment was used to check whether the SR trait was due to bacterial infection. SR trait due to bacteria infection has been reported in many arthropod species (see general introduction).

1.2.4 PCR assay of *Wolbachia* infection

One of the pair of ovaries in a female adult was ground in 100 μl of STE buffer with 2 μl of proteinase K (20 mg/ml), 1 μl of 2-mercaptoethanol and 10 μl of 10% SDS. The homogenate was incubated at 37°C for at least 30 min and at 95°C for 5 min. The lysate was extracted with phenol-chloroform (1:1) and chloroform once each, and DNA was precipitated with ethanol. The DNA pellet was dissolved in 50 μl of TE buffer (10 mM Tris-HCl, pH 8.0/ 1 mM EDTA, pH 8.0).

Polymerase chain reactions (PCR) specific to *Wolbachia* 16S *rRNA* gene were conducted in 10 μl reaction volumes including 1 μl of DNA samples following the method described by O'Neill *et al.* (1992).

1.2.5 Sequencing

PCR amplification of *wsp*, a surface protein gene of *Wolbachia*, was performed (Zhou *et al.*, 1998), using 2 μl of template DNA in reaction volumes of 50 μl.

PCR amplification of *ftsZ*, a cell-division-related gene of *Wolbachia*, was performed using primers *ftsZ*f1 and *ftsZ*r1 (Werren *et al.*, 1995b), and 2 μl of template DNA in reaction volumes of 50 μl.

The PCR products were purified from agarose gels and directly sequenced for both strands using the BigDye™ terminator cycle sequencing kit (Applied Biosystems). The products of sequence reaction were run on an automated DNA sequencer (ABI377, Applied Biosystems).

1.2.6 Phylogenetic analysis

The *wsp* sequence of *Wolbachia* infecting *O. furnacalis* was initially aligned with 29 *wsp* sequences of other strains of B-group *Wolbachia* and two outgroup sequences from A-group *Wolbachia* (*Wolbachia* strains in *Drosophila melanogaster* and *Drosophila sechellia*) using the program package CLUSTAL W ver1.5 (Thompson *et al.*, 1994), and then the alignment was manually modified based on the estimated amino acid sequences using the sequence alignment editor BioEdit (Hall, 1999). The third hypervariable region (positions 518-565) was excluded from the analyses (Zhou *et al.*, 1998).

The *ftsZ* sequence of *Wolbachia* infecting *O. furnacalis* was initially aligned with 20 *ftsZ* sequences of other strains of B-group *Wolbachia* and two outgroup sequences from A-group *Wolbachia* (*Wolbachia* strains in *Drosophila melanogaster* and *D. sechellia*) using the program package CLUSTAL W ver1.5

(Thompson *et al.*, 1994).

The molecular phylogenetic trees were estimated by the maximum likelihood method using the program package PAUP* (Swofford, 1996). I employed the general time reversible (GTR) model (e.g. Lanave *et al.*, 1984) with discrete-gamma approximation (four rate categories). All model parameters were estimated from the data using a starting tree topology that had previously been inferred from the unweighted maximum parsimony method. These parameter estimates and the starting tree topology were then employed for a heuristic search based on tree bisection and reconnection (TBR). Bootstrap analyses were done with 100 replications each.

1.3 Results

1.3.1 *Wolbachia* infection and female-biased sex ratio

Wolbachia infection was found in 13 of 104 field-collected females (Figure 1.2). Through 1996 to 2000, the frequencies of infected females were low (0-43%). Twelve of the 13 infected females produced all-female offspring (strong SR), while no uninfected females produced offspring with the strong SR (Table 1.1; Figure 1.2). One infected female (MD771) produced a brood with a sex ratio not significantly distorted (22 females and 10 males, $P > 0.01$, chi-squared test). Six wild females that were not infected produced families with significantly female-biased sex ratios ($P < 0.01$ by chi-squared test), but these families included males at more than 20% (weak SR, Table 1.1, Figure 1.2) in contrast to the strong SR.

The strong SR was maternally inherited in all of the seven matrilines examined, while the weak SR in both of the two matrilines examined disappeared in subsequent generations (Table 1.1). I could not obtain offspring from the MD771 female.

Thus, *Wolbachia* infection was shown to be strongly associated with the all-female production in *O. furnacalis* females.

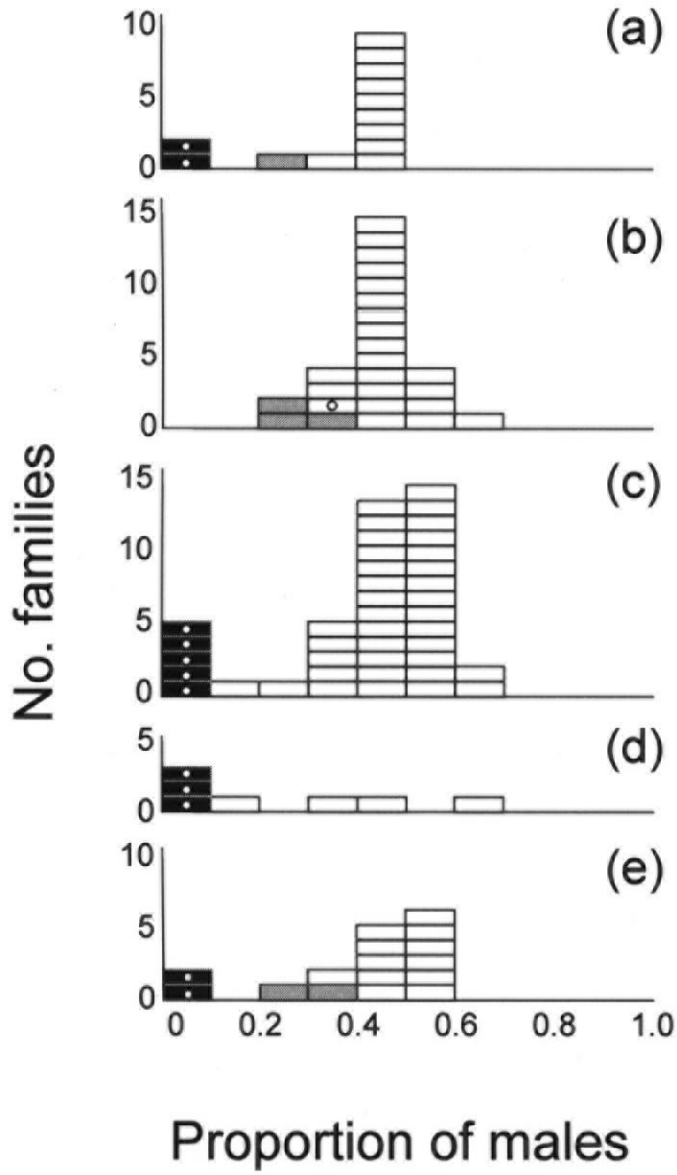

Figure 1.2 Distribution of sex ratio in progenies produced by wild females of *O. furnacalis* collected at Matsudo in (a) 1996, (b) 1997, (c) 1998, (d) 1999 and (e) 2000. Boxes with white circles indicate *Wolbachia*-infected broods. Black and grey boxes indicate strong and weak SR broods, respectively. Two broods with size less than ten in (c) and (d) each were excluded from the figure.

Table 1.1 Incidence of *Wolbachia* infection and sex ratios of strong and weak SR matrilines of *O. furnacalis* with egg hatch rates in parental families

Line	*Wolbachia* infection	No. Parental		Egg hatch Rate	No. F_1		No. F_2		No. F_3	
		Female	Male		Female	Male	Female	Male	Female	Male
Strong SR matrilines										
M9	+	89	0	n.e.	184	27	15	0		
							9	0		
					22	7	81	0		
M11	+	29	0	n.e.	56	0	37	8		
MD804	+	38	0	0.97 (123)	43	0	65	0	43	0
									55	0
									36	0
									65	0
MD807	+	32	0	0.96 (97)	35	0				
					34	0				
MD825	+	31	0	0.81 (392)	43	0				
MD826	+	25	0	n.e.	20	0				
					31	0				
MD846	+	42	0	0.95 (186)	45	0	45	0	10	1
MD903	+	48	0	n.e.	51	0				
MD910	+	58	0	n.e.	82	3	3	10		
							2	2		
MD920	+	28	0	n.e.	44	0				
					43	0				

Table 1.1 (Continued.)

Line	*Wolbachia* infection	No. Parental Female	No. Parental Male	Egg hatch Rate	No. F_1 Female	No. F_1 Male	No. F_2 Female	No. F_2 Male	No. F_3 Female	No. F_3 Male
MD030	+	14	0	n.e.	48	0	43	0	26	0
					51	0	29	0	25	0
									32	0
									21	0
									18	0
									33	0
MD049	+	47	0	n.e.	30	0	43	0	20	0
					56	0	49	0	12	0
									7	0
									9	0
Weak SR matrilines										
M13	-	22	6	n.e.						
MD743	-	94	48	n.e.						
MD745	-	39	11	n.e.	13	8				
MD747	-	44	16	n.e.						
MD003	-	59	29	1.00 (204)	16	12	34	33		
							3	11		
MD0037	-	31	10	n.e.						

Wild females were checked for *Wolbachia* infection by PCR. Egg hatch rates are given with total numbers of eggs in parentheses. n.e., not examined.

1.3.2 The mechanism of strong SR

The average egg hatch rate for four families with strong SR was 0.923 (Table 1.1), indicating that the *Wolbachia*-associated SR was not due to early (embryonic) male-killing. All the larvae fed with tetracycline developed to females in all of seven matrilines of strong SR, but these female adults produced all-male progenies (Table 1.2). These findings indicate that the *Wolbachia* infection caused the strong SR through feminization of genotypic males in *O. furnacalis*. Namely, *Wolbachia* feminizes individuals carrying ZZ sex chromosomes (male genome), and in the absence of *Wolbachia* such feminized individuals solely produce ZZ eggs that develop into male adults (Figure 1.3).

Table 1.2 Sex ratios of broods produced from tetracycline-treated strong SR females

Line	Replication	Female	Male
M11	a	0	24
	b	0	59
	c	0	58
	d	0	24
	e	0	50
	f	0	8
MD804		0	91
MD826		0	70
MD846		0	46
MD920		0	51
MD030		0	29
MD049	a	0	51
	b	0	49

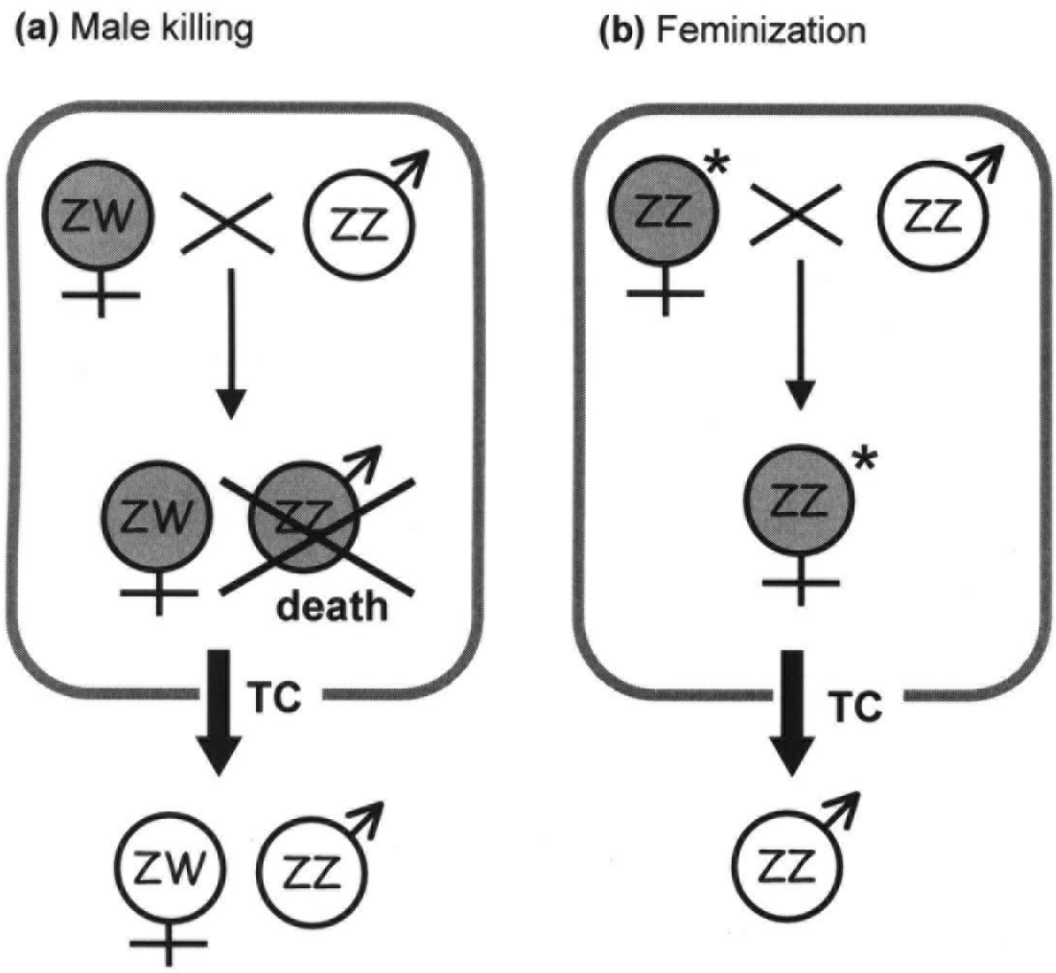

Figure 1.3 Illustration showing that feminization of genetic males explains all-male production from tetracycline (TC) treated SR females. If male-killing is the mechanism of the SR trait (a), tetracycline treatment will result in production of both males and females with equal ratio. On the other hand, if feminization of genetic male (genotype ZZ) is the mechanism of the SR trait (b), tetracycline-treated, *Wolbachia*-free females (genotype ZZ) will produce only ZZ eggs after crossing with normal males. Being free from *Wolbachia*, these eggs will normally develop to male adults.

1.3.3 Molecular phylogenetic affiliation of the *Wolbachia* strain in *O. furnacalis*

Nucleotide sequences of a portion (555 bp) of the *wsp* gene of *Wolbachia* in the 13 infected *O. furnacalis* females were determined. All the 13 sequences were identical. Nucleotide sequences of a portion (1025 bp) of the *ftsZ* gene of *Wolbachia* in the seven infected *O. furnacalis* females were also determined. All the seven sequences were identical. These findings suggest the infection of an identical strain of *Wolbachia*.

Molecular phylogenetic analyses have revealed the occurrence of two major clades (A and B) in *Wolbachia* strains infected with arthropods (Werren *et al.*, 1995b; van Meer *et al.*, 1999; see also Vandekerckhove *et al.*, 1999). The present analyses of *ftsZ* and *wsp* sequences showed that the *Wolbachia* strain in *O. furnacalis* is a member of the B group (Figure 1.4; Figure 1.5). Although *ftsZ* gene sequences could not clarify the phylogenetic relationship between the *Wolbachia* strain in *O. furnacalis* and those harbouring other insect species (Figure 1.4), *wsp* gene sequences have proved to be useful for the present purpose (Figure 1.5). Within the B group, the feminizer *Wolbachia* in *O. furnacalis* did not have a sister relationship to the feminizing *Wolbachia* strains in isopods, *Armadillidium vulgare*, *Oniscus asellus* and *Porcellio scaber* (Figure 1.5).

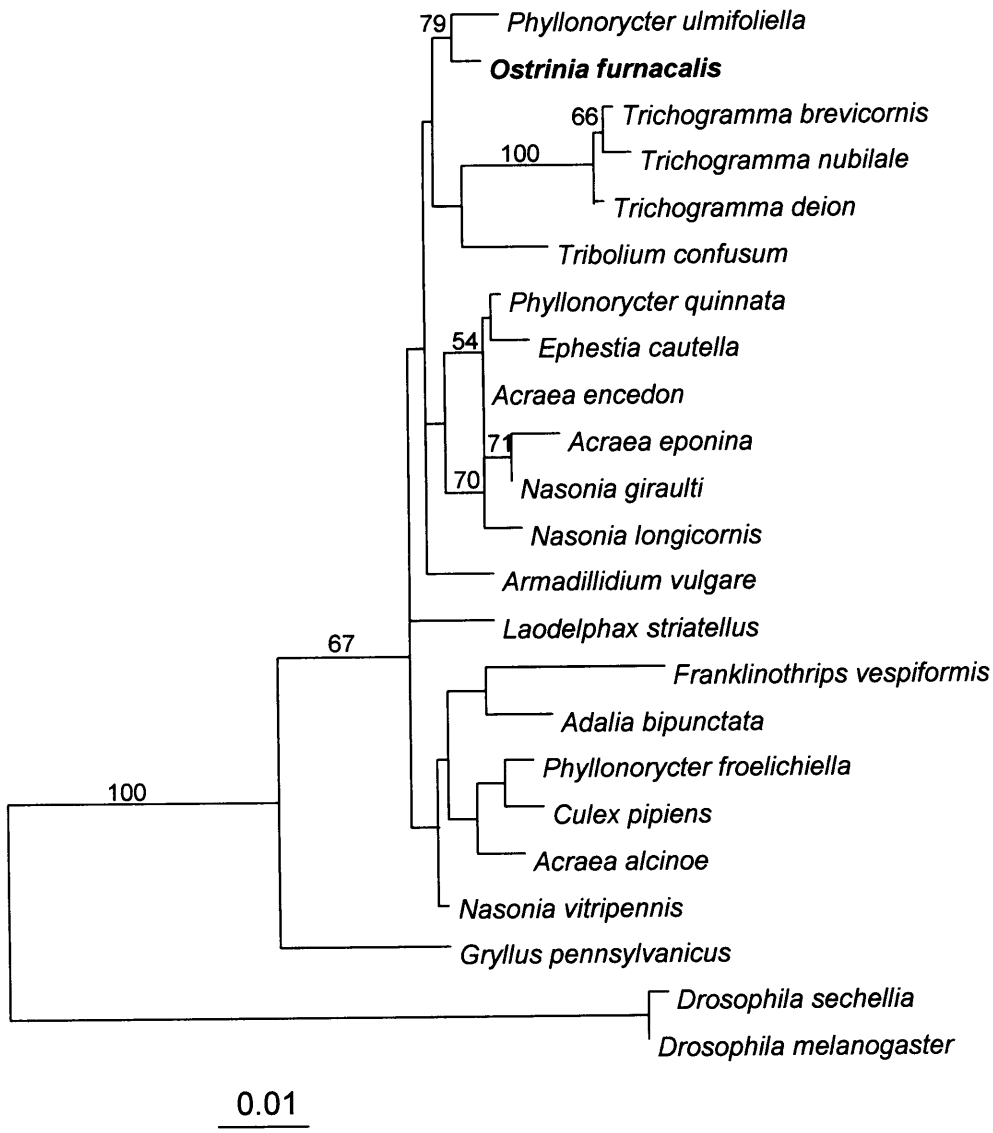

Figure 1.4 Phylogenetic tree of B-group *Wolbachia* based on *ftsZ* gene sequence data with two outgroups of A-group *Wolbachia* from *Drosophila sechellia* and *D. melanogaster*. *Wolbachia* strains are given as their host species names. The tree was constructed by the maximum likelihood methods using PAUP* (Swofford, 1996). The tree has log likelihood of - 2737.74. The results of 100 bootstrap replicates are shown above the branches. GenBank accession numbers of included published sequences are as follows:

AJ005885, U28198, U74479, U28199, U97352, AJ005887, U28207, AJ271199, AJ271200, U28203, U28204, AJ223243, AB039038, AB045315, AJ130717, AJ005883, U28209, AJ271202, U28205, U83099, U28179, U28189.

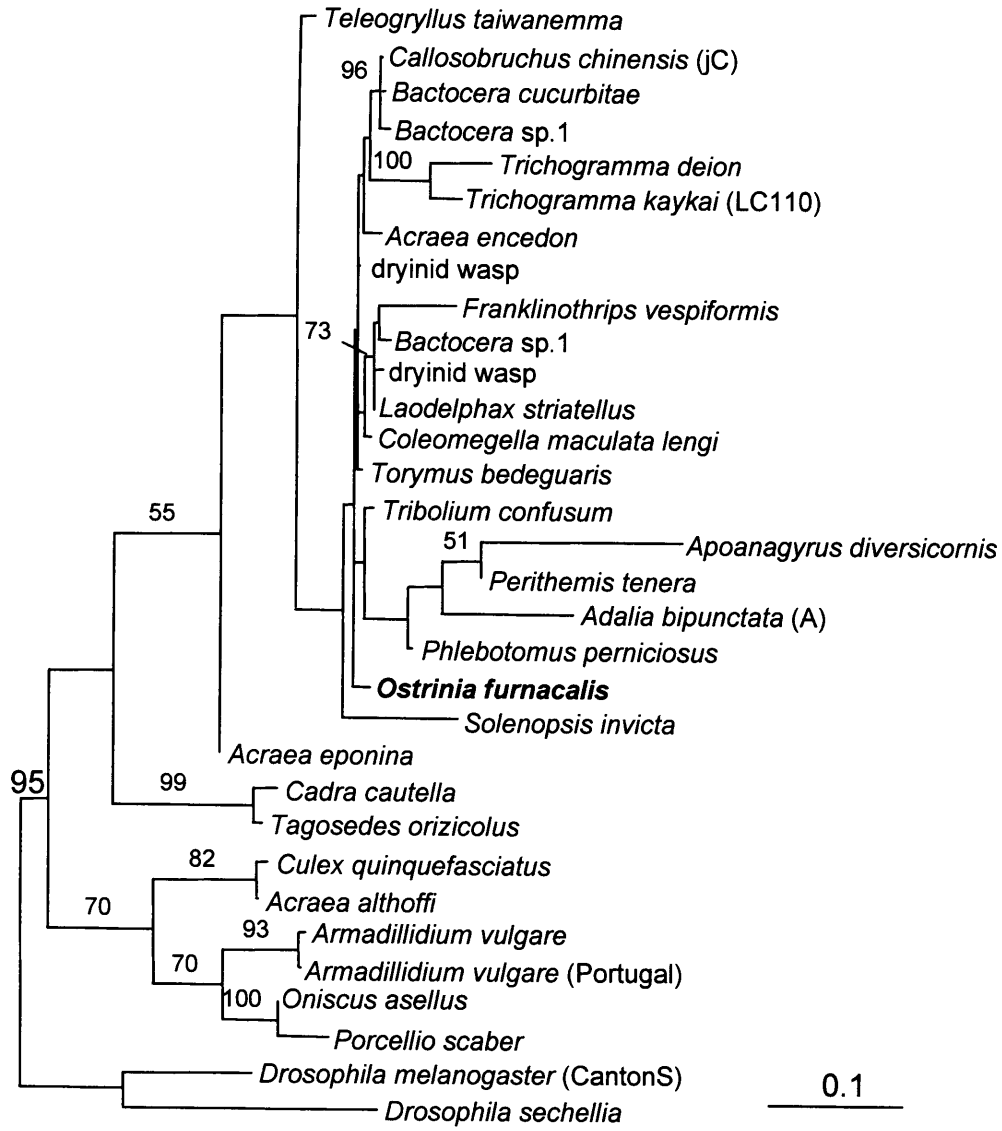

Figure 1.5 Phylogenetic tree of B-group *Wolbachia* based on *wsp* gene sequence data with two outgroups of A-group *Wolbachia* from *Drosophila sechellia* and *D. melanogaster*. *Wolbachia* strains are given as their host species names. The tree was constructed by the maximum likelihood methods using PAUP* (Swofford, 1996). The tree has log likelihood of - 4136.75. The results of 100 bootstrap replicates are shown above the branches. GenBank accession numbers of included published sequences are as follows:

AB035514, AB038326, AB039284, AB045314, AB046720, AF020060, AF020065, AF020073, AF020076, AF020080, AF020083, AF020084, AF020085, AF071915, AF071916, AF071917, AF071927, AF217720, AF217725, AF237884, AF243436, AF295346, AF295347, AF295348, AJ130714, AJ130716, AJ269474, AJ269475, AJ269476, AJ271194, AJ271197.

1.4 Discussion

In lepidopteran insects, thelygenies due to early (embryonic) male-killing have been known, some of which are caused by *Wolbachia* infection (Hurst and Majerus, 1993; Higashiura *et al.*, 1999; Hurst *et al.*, 1999a; Jiggins *et al.*, 2000a, 2000b). In *O. furnacalis*, however, two lines of evidence indicate that *Wolbachia* infection causes feminization of genetic males in *O. furnacalis*. First, *Wolbachia* infection was strongly correlated to the strong SR: this trait was found in 12 of 13 wild *Wolbachia*-infected females, but not in 93 uninfected females. Second, the result of antibiotic elimination of *Wolbachia* infection indicated that feminization of genetic males underlay the strong SR. Lepidopteran insects generally have either ZW/ZZ or ZO/ZZ sex chromosomes (Traut and Marec, 1996), and this was also assumed for *O. furnacalis*. If feminization of genetic males is occurring, a female parent should be genotypically a male (ZZ), and produces exclusively ZZ eggs. Thus, production of all-male offspring due to elimination of causal bacteria indicates the occurrence of feminization of genetic males. If a male-killing bacterium was causing the SR, a 1:1 sex ratio should have resulted from the tetracycline treatment (Figure 1.3). This is the first study to report the occurrence of *Wolbachia*-induced feminization in insects, which has been known only from several isopod species (Rigaud *et al.*, 1997).

Among the 13 *Wolbachia*-infected females of *O. furnacalis*, one female (MD771) produced non-SR progeny (22 females and 10 males). Three

explanations are possible for this exceptional case. Firstly, the MD771 female may have harboured a *Wolbachia* strain different from the other 12 infections, although it was not distinguishable in terms of the *wsp* and the *ftsZ* sequence. Secondly, the MD771 female may have failed to transmit the infection to a part of her eggs. Lastly, the density of *Wolbachia* in MD771 females may have been low, leading to failure in feminizing some of the progeny. In addition, the latter two explanations may be also relevant to other cases of non-SR broods that were found in F_2 offspring from the MD910 female (Table 1.1).

Wolbachia-induced feminization has been found in isopods such as *Armadillidium vulgare* (Rigaud *et al.*, 1997 for a review). Two phenotypic differences were found between the feminizations in *A. vulgare* and *O. furnacalis*. First, *Wolbachia*-infected lines of *A. vulgare* occasionally produce intersexes, which has not been found in *O. furnacalis*. Second, young females of *Wolbachia*-infected *A. vulgare*, when reared at 30°C for curing the infection, progressively acquired the male phenotype within the treated generation. In *O. furnacalis*, however, tetracycline did not change the sex of individuals in the treated generation, and all-male offspring was produced in the subsequent generation.

These distinct phenotypic characteristics of feminization are most likely to be relevant to differences in sex determination and/or differentiation processes between *A. vulgare* and *O. furnacalis*. In *A. vulgare*, a 'male gene' was suggested to control development of the androgenic gland that produces androgenic hormone. The androgenic hormone triggers differentiation to the

male after the fourth moult. It was suggested that *Wolbachia* affects the activity of the male gene (Rigaud *et al.*, 1997).

As for insects, the sex determination process has been well elucidated only in *Drosophila melanogaster* (for a recent review, Schütt and Nöthiger, 2000). The sex in *Drosophila* is determined at the embryonic stage, and is not controlled by diffusing substances such as sex hormones in the later developmental stages. This mechanism is also believed to apply for insects other than Diptera (e.g. Hoy, 1994; but see also De Loof and Huybrechts, 1998). In congruence with this widely accepted dogma, tetracycline-treated larvae of *Wolbachia*-infected *O. furnacalis* developed into female adults, suggesting that the feminizing action of *Wolbachia* operates at the embryonic stage. Although the molecular mechanism of sex determination in Lepidoptera has not been elucidated, the silkworm *Bombyx mori* has been shown to possess a homolog of the *dsx*, which is one of the *Drosophila* sex determination genes (Ohbayashi *et al.*, 2001). The *dsx* homolog in *B. mori*, as well as the *dsx* in *Drosophila*, is subjected to sex-specific splicing. The target(s) of the feminizing action in *O. furnacalis* may be some molecule(s) that play a role in sex determination during the embryonic stage.

On the maximum likelihood tree of *Wolbachia* strains based on the *wsp* sequences, the feminizing *Wolbachia* in *Ostrinia* does not have a sister relationship with that in *A. vulgare* while the *Wolbachia* strains in isopods are monophyletic. This suggests that the evolutionary origins of feminization are independent in *A. vulgare* and *O. furnacalis*, if no recombination between *Wolbachia* strains is assumed (but see Werren and Bartos, 2001).

Chapter 2

Two kinds of sex ratio distortion in the adzuki bean borer, *Ostrinia scapulalis*

2.1 Introduction

Female-biased sex ratio in a brood (an SR trait) has been found in various arthropods (see General introduction). Several cytoplasmic bacteria causing SR condition have been found (Hurst *et al.*, 1997a). Among these bacteria, *Wolbachia* is of particular interest since it is found in diverse groups of arthropods and causes a variety of reproductive alterations (see General introduction).

Molecular phylogenetic studies of *Wolbachia* strains have suggested that *Wolbachia* has been horizontally transmitted between different host species repeatedly (Stouthamer *et al.*, 1999). Some groups of closely related species of insects, and even some single species, harbour *Wolbachia* strains of different lineage. On the other hand, the phylogenetic studies of *Wolbachia* have also

revealed that *Wolbachia* strains inducing the same effect on their hosts are often distantly related to each other, and that the closely related *Wolbachia* strains sometimes bring about different effects on their hosts. For example, two *Wolbachia* strains infecting *Tribolium confusum* and *T. madens* are indistinguishable by gene sequences, but cause cytoplasmic incompatibility and male-killing, respectively (Fialho and Stevens, 2000). Due to these characteristics of *Wolbachia* infection, investigating *Wolbachia* infections in diverse groups of closely related arthropod species has particular significance in understanding the evolution of *Wolbachia*-host interactions.

In Lepidoptera, maternally inherited SR traits have been recorded in a number of species. Some of these were suggested to be due to meiotic drive of the sex chromosome, however, recent studies suggested that caution must be taken to conclude that sex chromosome drive is the mechanism of the SR trait; It was recorded in the literature that certain matrilines of two butterflies, *Danaus chrysippus* and *Acraea encedon* produce all-female broods, and it had been suggested that the most likely explanation was the sex chromosome meiotic drive (Smith, 1975; Owen and Smith, 1991). However, in both of these species, it was revealed later that the SR trait is due to bacteria-induced male-killing (Jiggins *et al.*, 1998, 2000b).

In the present study, I surveyed the maternally inherited SR trait in the adzuki bean borer *Ostrinia scapulalis,* a species closely related to *O. furnacalis*. I report *Wolbachia*-associated and non-associated SR traits in *O. scapulalis*. The former SR trait is *Wolbachia*-induced feminization, and the feminizing *Wolbachia* in *O.*

scapulalis is identical or closely related to that in *O. furnacalis*. For the latter SR trait, evidence suggests that its mechanism is sex chromosome meiotic drive.

2.2 Materials and methods

2.2.1 Collecting and rearing insects

Female adults of *O. scapulalis* were collected at five locations in central Japan: Kuroishi in 1997, Takizawa in 2000, Furukawa in 1997, Matsudo in 1997, 1998, 1999 and 2000, and Yayoi in 1997 (Figure 2.1). The wild females were individually allowed to oviposit in the laboratory. Most of them laid fertile eggs within a few days. Larvae of *O. scapulalis* were collected from the bur-marigold, *Bidens frondosa*, at Sado in 1997 and from the dock, *Rumex* sp., at Furukawa in 1997 (Figure 2.1). The larvae were brought to the laboratory and raised to adults that were individually allowed to oviposit after mating.

Insects were reared at 23°C and a photoperiod of 15L/9D. Larvae were fed on an artificial diet (Silk Mate 2M, Nihon Nosan, Yokohama). Pupae were sexed based on the morphology of abdominal tip, and maintained separately by sex. The adults were provisioned with 3% sugar water. More than two days after crosses, females were individually allowed to oviposit. After oviposition, ovaries were dissected from the females and stored in STE buffer under –20°C until DNA extraction.

The numbers of eggs were counted under a binocular microscope. The egg hatch rates were estimated from the numbers of unhatched eggs.

For examining the survival rate of larvae, broods were separately reared in

groups of five larvae from the neonate stage. Number of surviving larvae was counted every two or three days until pupation.

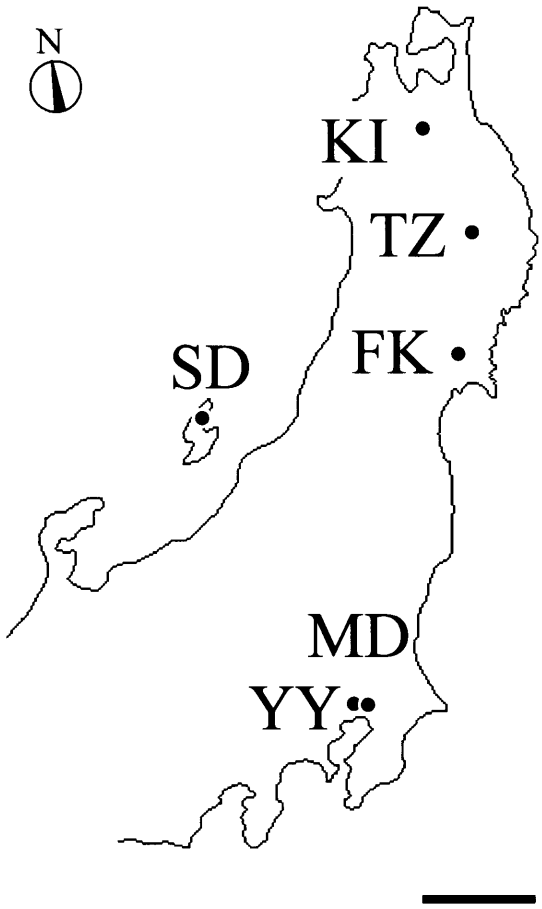

Figure 2.1 Collection sites of *Ostrinia scapulalis* in northern Honshu, Japan. KI, Kuroishi; TZ, Takizawa; SD, Sado island; FK, Furukawa; MD, Matsudo; and YY, Yayoi. The scale bar indicates 100 km.

2.2.2 Antibiotic treatment

Larvae were treated with antibiotics to check whether SR traits are due to bacterial infection. Either tetracycline hydrochloride or rifampicin was mixed into the larval diet: 0.06% (w/w) for tetracycline, and 0.1% (w/w) for rifampicin. The diets containing the antibiotics were fed to the larvae from the neonate stage until pupation.

2.2.3 Cross experiment using a visible marker

A mutant strain of *O. scapulalis* that expresses a melanic form of pupae and adults was used to check whether an SR trait is due to gynogenesis. This melanism is caused by a recessive allele on an autosomal gene (Ataka *et al.*, 2002). A female from an SR matriline was crossed with a melanic male. Their daughters were backcrossed with melanic males. If male sperm was used for fertilization, melanic individuals will appear in the backcross offspring.

2.2.4 DNA extraction

DNA was extracted from ovaries from female adults as described in Chapter 1.

2.2.5 PCR assays for infection with *Wolbachia* and other bacteria

Infection with *Wolbachia* was examined by a polymerase chain reaction (PCR) specific to *Wolbachia* 16S ribosomal RNA gene (16S *rRNA*) as described in Chapter 1.

Infection with bacteria other than *Wolbachia* was examined by PCR with primers fD1 and rP1 that amplify eubacterial 16S *rRNA* gene (Weisburg *et al.*, 1991). The PCR was conducted in 20 μl reaction volumes including 1 μl of template DNA.

2.2.6 Sequencing the *Wolbachia wsp* and *ftsZ* genes

PCR amplifications and sequencing of *wsp* gene encoding a surface protein and *ftsZ* gene, a cell-division-related gene, of *Wolbachia* were performed as described in Chapter 1.

2.3 Results

2.3.1 SR females in natural populations

The sex ratio in offspring was examined for a total of 372 wild females sampled from six locations. At least ten progenies were examined per family. Thirty-one wild females from four locations produced significantly female-biased progenies (summarized in Table 2.1; sex ratio data are shown in Tables 2.2 and 2.3). The PCR diagnosis of *Wolbachia* infection revealed that 17 of the 31 SR females were infected and that the other 14 were uninfected (Table 2.1). All of 302 non-SR females examined were negative for *Wolbachia* infection. Hereafter, the SR traits associated with and free from *Wolbachia* infection are referred to as SR-w^+ and SR-w^- traits, respectively. There was no significant difference in prevalence of both types of SR female among localities (for SR-w^+, $X^2 = 14.03$, d. f. = 5, $P > 0.01$; for SR-w^-, $X^2 = 4.77$, d. f. = 5, $P > 0.01$). At two localities (Furukawa and Matsudo) where wild females were sampled in multiple years (Table 2.1), no significant changes in the frequency of SR female were found among years (for SR-w^+ trait in Furukawa, $X^2 = 1.52$, d. f. = 2, $P > 0.05$; for SR-w^+ trait in Matsudo, $X^2 = 1.29$, d. f. = 3, $P > 0.05$; for SR-w^- trait in Furukawa, $X^2 = 0.34$, d. f. = 2, $P > 0.05$; and for SR-w^- trait in Matsudo, $X^2=0.23$, d. f. =3, $P > 0.05$). Six wild females produced significantly male-biased progenies although the biases were weak (male proportion of 0.68, n=65; 0.62, n=140; 0.64, n=111; 0.65, n=93; 0.71, n=85; and 0.72, n=39).

Table 2.1 Numbers of wild females categorized by the sex ratio of their progenies

Sex ratio	Collection site and year											Total
	KI	TZ	SD	FK			MD				YY	
	1997	2000	1997	1997	1998	1999	1997	1998	1999	2000	1997	
SR-w^{+}*	3	0	0	1	0	0	2	5	3	3	0	17
SR-w$^{+\dagger}$	0	3	0	1	1	2	2	2	2	1	0	14
Male-biased‡	0	3	1	0	0	0	1	0	0	1	0	6
Normal§	10	41	8	18	9	19	48	54	65	41	22	335
(Total)	13	47	9	20	10	21	53	61	70	46	22	372

*Wild females that produced progenies with significantly female-biased sex ratio ($P < 0.01$) with *Wolbachia* infection.

†Wild females that produced progenies with significantly female-biased sex ratio ($P < 0.01$) without *Wolbachia*.

‡Wild females that produced progenies with significantly male-biased sex ratio ($P < 0.01$).

§Wild females that produced progenies with sex ratio not significantly deviated from 1:1 ($P \geq 0.01$).

¶Collection sites are KI: Kuroishi, TZ: Takizawa, SD: Sado, FK: Furukawa, MD: Matsudo and YY: Yayoi (See Figure 2.1).

Table 2.2 Maternal inheritance of SR trait and the effect of tetracycline on sex ratio in SR-w^+ matrilines

Matriline	Parental sex ratio (n)*	No. SR families†			No. all-male families produced from cured females‡
		G_1	G_2	G_3	
KI721	0.06 (16)	2/2	2/2	1/2§	3/3
KI722	0.00 (43)	2/2	4/4	4/4	2/2
KI730	0.00 (11)	5/5	3/3	1/1	3/3
FK708	0.00 (49)	4/4	2/2	---¶	2/2
MD739	0.01 (156)	26/26	17/18§	5/5	13/13
MD785	0.00 (20)	3/3	3/3	---	3/3
MD816	0.00 (39)	3/3	2/2	---	2/2
MD836	0.00 (40)	1/1	1/1	---	1/1
MD855	0.00 (48)	2/2	1/1	---	1/1
MD863	0.00 (46)	2/2	---	---	---
MD8102	0.00 (43)	2/2	2/2	---	1/1
MD923	0.00 (64)	1/1	2/2	---	2/2
MD927	0.00 (63)	1/1	1/1	---	1/1
MD976	0.00 (59)	2/2	1/1	---	1/1
MD014	0.00 (80)	2/2	2/2	6/6	3/3
MD055	0.00 (24)	2/2	2/2	---	2/2
MD031	0.00 (71)	2/2	1/1	1/1	---

*Proportion of males produced from wild females, with the number of pupae in parenthesis.

†Numbers of SR families per family examined (each family consisted of at least 15 progenies).

‡Numbers of all-male families per family produced from tetracycline-treated females (each family consisted of at least 20 progenies).

§Two families, one is in G_3 of KI721 and the other is in G_2 of MD739, were not significantly deviated from 1:1 (12 females and 4 males; 22 females and 26 males).

¶Not examined.

Table 2.3 Maternal inheritance of SR trait in SR-w^- matrilines

Matriline	Parental sex ratio (n)*	No. SR families†		
		G_1	G_2	G_3
TZ039	0.00 (15)	3/3	0/3	---‡
TZ055	0.03 (63)	6/7	2/2	---
FK721	0.00 (58)	10/11	1/2	1/5
FK815	0.31 (105)	4/6	2/2	5/5
FK929	0.00 (58)	0/2	0/7	---
MD707	0.00 (7)	3/3	4/5	4/9
MD873	0.00 (35)	2/2	1/1	1/1
MD8100	0.00 (47)	2/2	1/1	1/1
MD063	0.02 (44)	2/2	4/4	---

*Proportion of males produced from wild females is given with numbers of pupae in parenthesis.

†Numbers of SR females per families examined.

‡Not examined.

2.3.2 SR-w$^+$ matrilines

Vertical transmission of SR-w$^+$ trait was examined. The SR condition was maternally inherited for initial two or three generations in all of seventeen SR-w$^+$ matrilines examined (Table 2.2, at least ten individuals examined per family). Exceptionally, two families were not significantly female-biased: 12 females and 4 males in one G_3 family in KI721 matriline, and 22 females and 26 males in one G_2 family in MD739 matriline ($P > 0.01$ by chi-squared test).

For three SR-w$^+$ matrilines (KI730, MD739 and MD014), observation of sex ratio was continued for further generations. SR condition was consistently transmitted to offspring in the three matrilines for more than ten generations (data not shown).

To examine the effect of eliminating *Wolbachia* infection on SR condition, a total of 40 females from fifteen SR-w$^+$ matrilines were treated with tetracycline during the larval period. All of the treated females produced only male progenies (Table 2.2), suggesting that the *Wolbachia* infection feminizes genetic males of *O. scapulalis* as in the case of *O. furnacalis* (Chapter 1).

2.3.3 *Wolbachia* sequences

Partial sequences of the *ftsZ* gene (1025 bp) and *wsp* gene (555 bp) were determined for ten and thirteen wild SR-w$^+$ females of *O. scapulalis*, respectively. Single sequences were shared for both genes among the infections in females

examined. The *ftsZ* and *wsp* sequences of *Wolbachia* in *O. scapulalis* were identical to respective sequences of the *Wolbachia* strain previously found in *O. furnacalis* (Chapter 1).

2.3.4 SR-w$^-$ matrilines

In nine SR-w$^-$ matrilines, vertical transmission of SR condition was examined for initial two or three generations (Table 2.3). SR condition was mostly transmitted to subsequent generations in the matrilines except for FK929 and TZ039, in which SR condition disappeared at G_1 and G_2, respectively. SR condition did not appear in a part of families in four matrilines (TZ055, FK721, FK815 and MD707).

I continued to examine the maternal inheritance of SR condition for further generations in four SR-w$^-$ matrilines (MD707, MD873, MD8100 and FK721). The pedigree tree of sex ratios in matriline MD707 are shown in Figure 2.2a. In summary, vertical transmission of the SR condition was incomplete: SR condition occasionally changed to nearly 1:1 conditions, and the nearly 1:1 conditions occasionally reverted to SR condition. For example, in matriline MD707, a female from an all-female (39:0) G_1 family produced all-female (73:0) offspring, but another female from the same family produced nearly 1:1 (52:47) offspring; two females from the 52:47 G_2 family produced nearly 1:1 (64:59 and 17:17) offspring families while another female from the same G_2 family produced an SR offspring family (30:6). Similarly, the other three matrilines examined (MD873,

MD8100 and FK721) sometimes failed to inherit SR condition, and sometimes nearly 1:1 conditions reverted to SR conditions (Figure 2.2b-d). In these four SR-w^- matrilines, the sex ratio in non-SR conditions was distributed around 0.5 (Figure 2.3). Thus, the sex ratio in SR-w^- matrilines appeared to alternate between two discrete conditions (nearly all-female and nearly 1:1), rather than to change continuously between the two extreme conditions.

The egg-hatch rate was examined in the four SR-w^- matrilines (MD707, MD873, MD8100 and FK721). No significant diffrences were found between SR and non-SR families (Table 2.4). The egg-hatch rates in SR families were higher than 0.5 on average. Therefore, male-killing at the embryonic stage is not likely to be the cause of the SR-w^- trait.

The larval survival rate was examined for three SR-w^- matrilines (MD707, MD873 and MD8100). The larval survivorship was similar among families irrespective of the sex ratio and matriline (Figure 2.4). Therefore, male-killing at the larval stage is also not likely to be the cause of the SR-w^- trait.

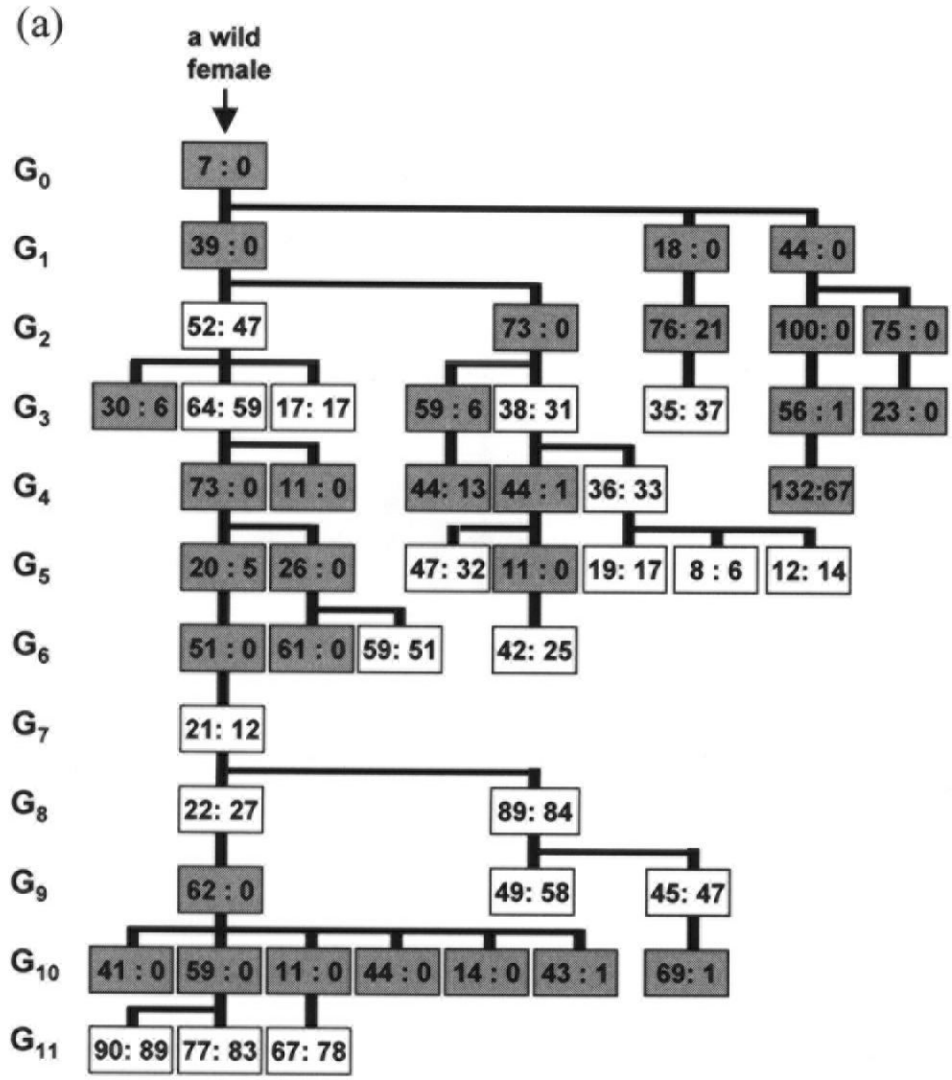

Figure 2.2 Incomplete vertical transmission of female-biased sex ratio in four SR-w$^-$ matrilines. Families less than ten individuals were excluded from the figures unless they were used in obtaining subsequent generations. (a) The sex ratio of families (number of females and males) in matriline MD707 from parental to 11th generations. Significantly female-biased ratios ($P < 0.01$) are shown in grey.

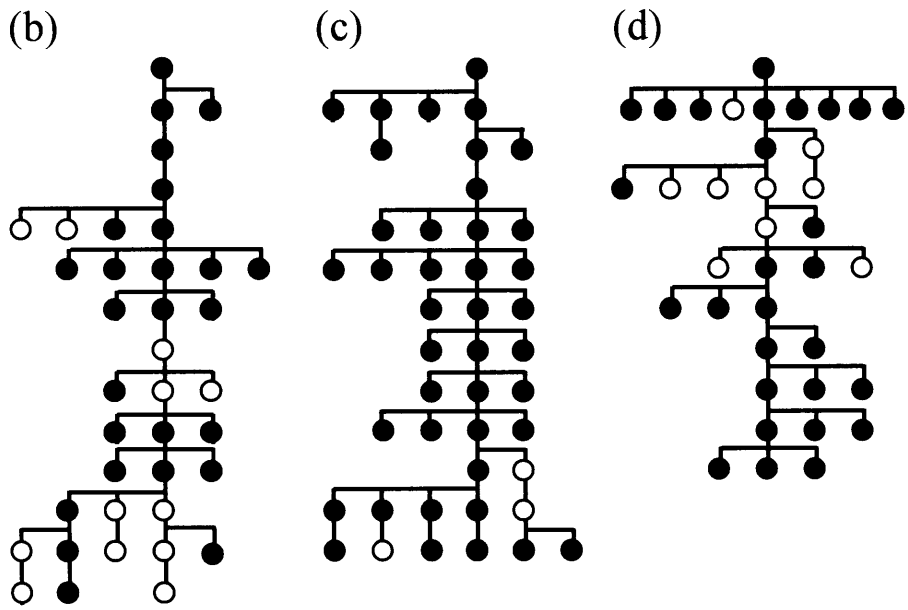

Figure 2.2 (Continued.) (b), (c) and (d) The sex ratio of families in matrilines MD873, MD8100 and FK721, respectively. Solid and open circles indicate significantly female-biased and unbiased families, respectively.

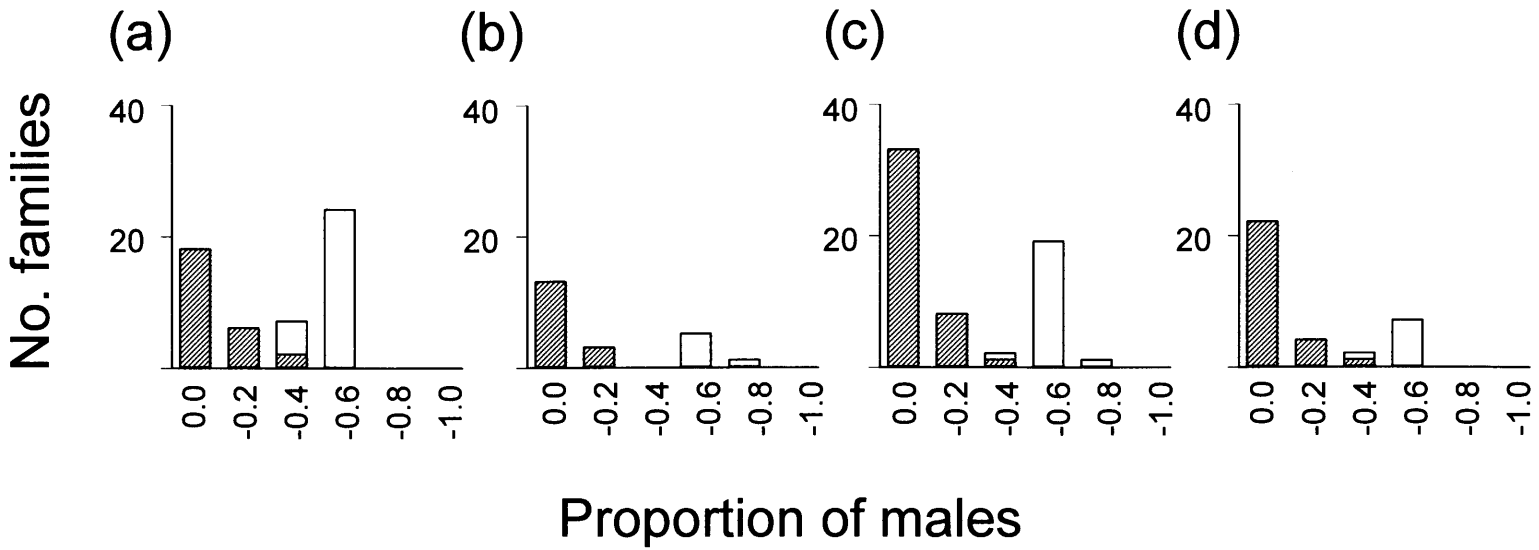

Figure 2.3 Distribution of the sex ratio among families in four SR-w$^-$ matrilines. Shaded bars represent significantly female-biased families ($P < 0.01$), and open bars represent families in which sex ratios are not significantly distorted from 1:1. Each family contains >30 individuals. (a) MD707, (b) MD873, (c) MD8100 and (d) FK721.

Table 2.4 Egg-hatch rate in five SR-w$^-$ matrilines

Matriline	Sex ratio	Avg.[‡]	SD[§]	N[¶]
MD707	SR*	0.73	0.23	36
	1:1[†]	0.86	0.17	38
MD873	SR	0.79	0.13	23
	1:1	0.84	0.22	22
MD8100	SR	0.84	0.16	45
	1:1	0.94	0.08	8
FK721	SR	0.86	0.11	24
	1:1	0.89	0.10	8

*Families with female-biased sex ratio ($P < 0.01$).

[†]Families with sex ratio not significantly deviated from 1:1 ($P \geq 0.01$).

[‡]Average egg hatch rates of families.

[§]Standard deviation.

[¶]Numbers of families examined.

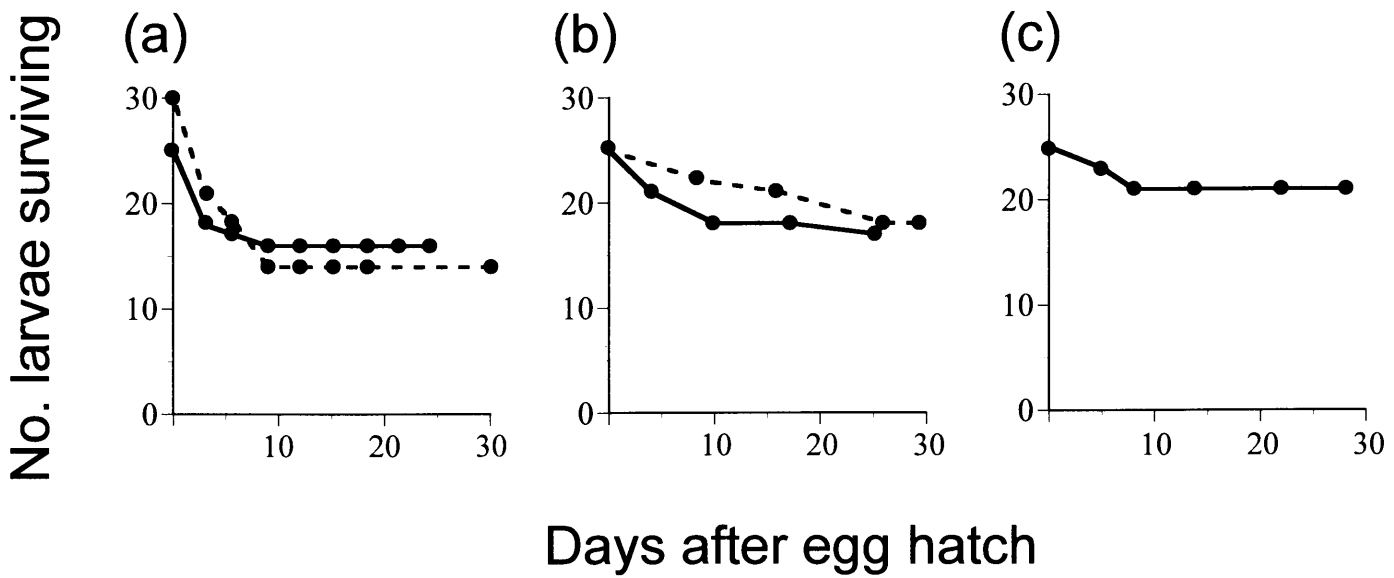

Figure 2.4 Numbers of surviving larvae along days after the egg hatch in three SR-w$^-$ matrilines. Solid lines indicate significantly female-biased families, and broken lines indicate families in which sex ratios are unbiased from 1:1. (a) MD707. The female-biased family contained pupae of 16 females, and the normal family contained pupae of six females and eight males. (b) MD873. The female-biased family contained pupae of 16 females and one male, and the normal family contained pupae of nine females and nine males. (c) MD8100. The female-biased family contained pupae of 20 females.

2.3.5 Use of male gene for fertilization in SR-w$^-$

To examine whether gynogenesis is the mechanism of SR-w$^-$ trait in *O. scapulalis*, crossing experiments using a melanic strain were conducted. One female from matriline MD707 and one male from the melanic strain were crossed. Their daughters were backcrossed with the melanic strain with four replicates. In all the four backcross families, melanic and normal forms appeared with nearly equal numbers irrespective of the sex ratio (Table 2.5). This indicates that male genes were used for fertilization, and accordingly the SR-w$^-$ trait is not due to gynogenesis.

Table 2.5 Phenotypes of the backcross offspring of an SR-w⁻ matriline (MD707) with a melanic strain

Family	Sex	Phenotype	
		Wild	Melanic
1	Female	32	32
	Male	0	0
2	Female	18	13
	Male	18	5
3	Female	29	34
	Male	0	0
4	Female	9	6
	Male	0	0

2.3.6 Examining bacterial infection for SR-w$^-$ matrilines

No obvious effect of tetracycline on the sex ratio was found in eight SR-w$^-$ matrilines examined (TZ039, TZ055, FK721, FK815, MD707, MD873, MD8100 and MD063, Table 2.6). In the former four matrilines, every treated female produced SR families. For the latter four matrilines, where larger numbers of females were treated, the treated females produced both SR families and non-SR families.

Similarly, no evident effect of the rifampicin treatment on the sex ratio was found in two matrilines examined (MD873 and MD8100). In MD873 matriline, the treatment resulted in one SR family and three non-SR families. In MD8100 matriline, all three families examined expressed SR condition. These results of antibiotic treatment do not support a hypothesis that the SR-w$^-$ trait is caused by bacterial infection.

This hypothesis was also tested by PCR assays using general primers for eubacterial 16S *rDNA*. Five females were randomly sampled from each of five SR-w$^-$ matrilines (MD707, MD873, MD8100, FK721 and FK815) and subjected to the 16S *rDNA* PCR assay for bacterial infection (Figure 2.5a). Bacterial 16S *rDNA* was not consistently amplified from the five matrilines; In FK721 and MD873 matrilines, all the five samples examined were PCR-negative. The other three matrilines included both positive and negative samples. To confirm the power of the present PCR assay, three females were randomly chosen from each of five SR-w$^+$ matrilines and subjected to the PCR assay (Figure 2.5b). These *Wolbachia*-infected samples consistently gave positive signals. Thus, the PCR

assays confirmed that the SR-w$^-$ trait is independent from bacterial infection.

Table 2.6 Effects of tetracycline on sex ratio in SR-w$^-$ matrilines

Matriline	No. families*	
	SR	1:1
TZ039	2	0
TZ055	1	0
FK721	1	0
FK815	2	0
MD707	8	9
MD873	5	2
MD8100	6	2
MD063	3	1

*Families are categorized by sex ratio (SR: $P < 0.01$, 1:1: $P \geq 0.01$ by Chi-squared test).

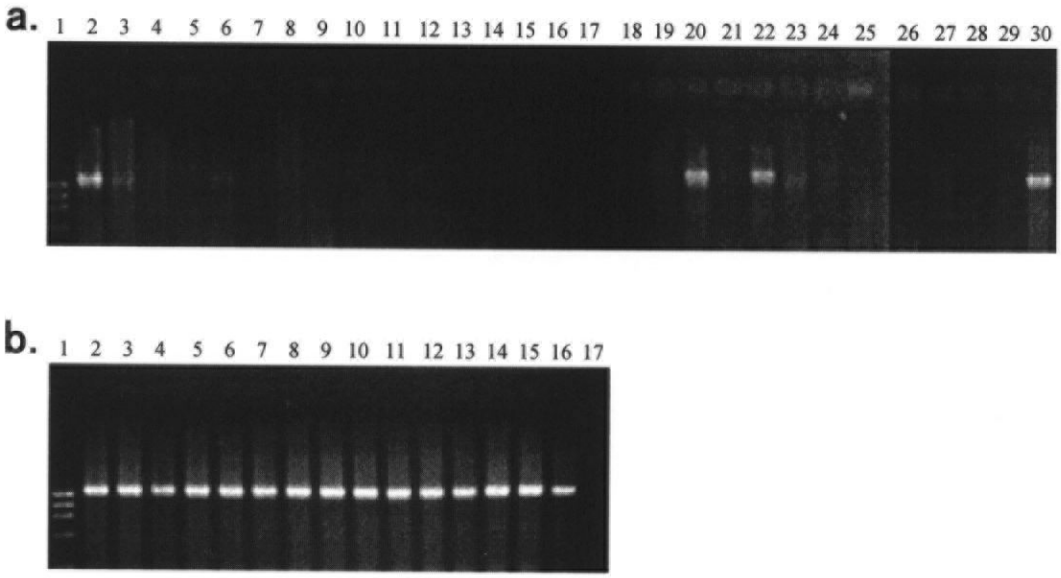

Figure 2.5 PCR assays using primers specific to eubacterial 16S ribosomal RNA gene. (a) Five females were chosen from each of five SR-w$^-$ matrilines as templates for the assay. Lane 1, øx174-*Hae*III digest; lanes 2-6, MD707 matriline; lanes 7-11, FK721 matriline; lanes 12-16, MD873 matriline; lanes 17-21, MD8100 matriline; lanes 22-26, FK815 matriline; lane 27, a negative control (a female from a normal strain); lane 28, STE buffer; lane 29, DW; lane 30, a positive control (*Wolbachia*-infected female). (b) Five females from a SR-w$^+$ matriline were subjected to the assay with three replications. Lane 1, øx174-*Hae*III digest; lanes 2-4, 5-7, 8-10, 11-13 and 14-16 represent different females, respectively; lane 17, DW.

2.4 Discussion

The study in this chapter revealed two types of maternally inherited SR trait in *O. scapulalis*: one associated with *Wolbachia* (SR-w^+) and the other not associated with *Wolbachia* (SR-w^-).

2.4.1 Two *Ostrinia* species appear to share the same feminizing *Wolbachia*

SR-w^+ trait in *O. scapulalis* was maternally inherited, and antibiotic treatment resulted in production of all-male progenies (Table 2.2). These results indicate that SR-w^+ is the feminization of genetic males due to cytoplasmic bacterial infection. Since the characteristics of feminization were not found in *O. scapulalis* matrilines not infected with *Wolbachia*, SR-w^+ trait is the feminization caused by *Wolbachia* infection.

In two SR-w^+ matrilines (KI721 and MD739), single families did not express SR condition (Table 2.2). In these two families, a number of males may have been produced through failure of feminization. The failure of feminization may be caused by unsuccessful *Wolbachia* transmission from mother to offspring or decrease in the cellular density of *Wolbachia*. The intensity of cytoplasmic incompatibility in *Drosophila simulans* is known to be influenced by the density of bacteria in cells (Hoffmann and Turelli, 1997).

The *Wolbachia*-induced feminization has been demonstrated in *O. furnacalis* (Chapter 1) and *O. scapulalis* (the present study). These two species are members of the *furnacalis* species group (Mutuura and Munroe, 1970; Ishikawa *et al.*, 1999). Six *Ostrinia* species inhabiting Japan in the *furnacalis* group. Molecular phylogenetic analysis has revealed that these six species are very closely related to each other (Kim *et al.*, 1999). The present finding suggests that the *Wolbachia*-induced feminization may be found in other species of the *furnacalis* species group.

Wolbachia strains in *O. scapulalis* and *O. furnacalis* were indistinguishable in terms of the *wsp* and *ftsZ* gene sequences. This suggests that the *Wolbachia* strains are identical or belong to a pair of sister lineage derived from a common ancestor, unless the sequence identity was resulted from molecular recombination between *Wolbachia* strains (cf. Werren and Bartos, 2001; Jiggins *et al.*, 2001a).

2.4.2 Female meiotic drive most likely underlies the SR-w$^-$

In the SR-w$^-$ matrilines, the sex ratio alternated between all-female and nearly 1:1 conditions (Table 2.2, Figure 2.3), indicating that their default sex ratio is 1:1. This suggests that feminization of genetic males is not the mechanism of SR-w$^-$ trait since the default condition in feminized matrilines is all-male (Chapter 1; the present study).

Three other mechanisms can cause SR condition in female-heterogametic insects such as Lepidoptera: male-killing, parthenogenesis, and non-random

segregation of sex chromosomes (Hurst, 1993). Male-killing is probably the most popular mechanism of SR condition in Lepidoptera (Hurst, 1993; Hurst and Majerus, 1993). Most cases of male-killing in Lepidoptera are expressed at the embryonic or early larval stage (early male-killing, e.g. Jiggins *et al.*, 1998, 2000a, 2000b). Male-killing expressed at a late larval period (late male-killing) was recently found in *Homona magnanima* (Tortricidae) (Morimoto *et al.*, 2001). The mechanism of SR-w$^-$ trait in *O. scapulalis* is not male-killing, because both the egg hatch rate and the larval survivorship in SR families were not different from those in non-SR families (Table 2.4 and Figure 2.4).

Parthenogenesis (including gynogenesis) has been reported in Lepidoptera (Lokki *et al.* 1975; Mitter and Futuyma, 1977; Menken and Wiebosch-Steeman, 1988; Gorbunov and Kishida, 1995). In SR-w$^-$ matrilines of *O. scapulalis*, unfertilized eggs did not hatch, and the crossing experiments indicated the use of paternal genome in fertilization (Table 2.5). Thus, parthenogenesis hypothesis is not likely to explain the present case.

Therefore, non-random segregation of sex chromosomes during female meiosis is most likely to underlie SR-w$^-$ trait in *O. scapulalis*. In lepidopteran insects, which have ZW-ZZ or ZO-ZZ sex chromosomes (Traut and Marec, 1996), SR condition can be achieved when Z chromosome is selectively disposed to polar bodies during the female meiosis. To date, non-random segregation of sex chromosomes during female meiosis, which will result in SR condition, has not been known. On the other hand, non-random segregation of autosomes during female meiosis has been reported from diverse taxonomic groups over mammals

and insects (reviewed by Pardo-Manuel de Villena and Sapienza, 2001). Non-random segregation during female meioses has been sometimes called as chromosomal meiotic drive (e.g. Lyttle, 1991).

In SR-w$^-$ matrilines of *O. scapulalis*, the vertical transmission of SR condition was occasionally unsuccessful, and it occasionally reverted to SR condition (Table 2.3; Figure 2.2). This can be explained by a nuclear factor(s) that suppresses the meiotic drive. If this is the case, the frequency of the SR-w$^-$ element in natural populations of *O. scapulalis* would be higher than 14/380, the observed frequency in wild SR-w$^-$ females.

The SR-w$^-$ trait in *O. scapulalis* was maternally inherited, albeit incomplete (Table 2.3; Figure 2.2). This finding indicates that the causal element of SR-w$^-$ trait resides in either cytoplasm or W chromosome. In an insect possessing ZW-ZZ system of sex chromosomes, a cytoplasmic element and W chromosome behave as a single linkage group, and thus they cannot be genetically separated (Hurst, 1993).

Most of cytoplasmic sex ratio distorters known to date in insects are bacteria. However, the PCR assay and the antibiotic treatment did not support the hypothesis that SR-w$^-$ is caused by a bacterium (Figure 2.5; Table 2.6). This finding is congruent with the fact that no cytoplasmic infectious elements have been found to cause non-random segregation of chromosomes. Rather, the factors known to cause non-random segregations are related to chromosomal features, such as chromosome fusion and fission (summarized by Pardo-Manuel de Villena and Sapienza, 2001). Some peculiar structural feature(s) of W

chromosome, if present in *O. scapulalis*, may be the causal factor of SR-w^- trait in *O. scapulalis*. Yet, it has not been studied whether *O. scapulalis* has ZW-ZZ or ZO-ZZ system.

In *A. vulgare*, two types of feminization have been found (Rigaud, 1997; Rigaud *et al.*, 1997). One type is induced by *Wolbachia* infection, and the other is caused by an unidentified genetic factor (f factor) other than *Wolbachia*. The f factor is speculated to be a part of *Wolbachia* genome transferred into host nuclear genome. In this connection, however, the evolutionary origin of SR-w^- trait in *O. scapulalis* is not likely to be related to the feminizer *Wolbachia* in *O. scapulalis*, since SR-w^+ and SR-w^- traits most likely have different mechanisms.

Chapter 3

Occurrence of *Wolbachia* infection and female-biased sex ratio distortion in other *Ostrinia* species

3.1 Introduction

Wolbachia, which is known to induce various reproductive alterations to its hosts (see general introduction), has been found from a wide variety of arthropods. Although evidence for the horizontal transmission of *Wolbachia* between host lineages is accumulating (Vavre *et al.*, 1999; Heath *et al.*, 1999; Huigens *et al.*, 2000; Noda *et al.*, 2001), little is known how *Wolbachia* strains have moved between lineages of their hosts.

I have demonstrated *Wolbachia*-induced feminization in two species of *Ostrinia furnacalis* species group: *O. furnacalis* and *O. scapulalis* (Chapter 1 and Chapter 2; see Rigaud, 1997 for feminizing *Wolbachia* in terrestrial isopods).

Analysis of the *wsp* and *ftsZ* gene sequences, has shown that *Wolbachia* strains found in *O. furnacalis* and *O. scapulalis* are likely to be the same or very closely related.

In this chapter, from the same interest as that discussed in Chapter 2, I examined the association between sex ratio traits and *Wolbachia* infection in other *Ostrinia* species in Japan, i.e., *O. orientalis*, *O. zaguliaevi* and *O. zealis*, and a species in Europe and North America, *O. nubilalis*.

3.2 Materials and methods

3.2.1 Insect collection

Insects were collected as adults or larvae. Collected larvae were reared to adults, and the females obtained were crossed with males of the same species to produce G_0 generation. Species were identified by host plant species and/or sex pheromone analysis. Most of the collected female adults were gravid and laid fertile eggs (G_0 generation).

As to *O. orientalis*, three and nineteen larvae were collected at Sado and Sugadaira, respectively, and two female adults were collected at Matsudo (Figure 3.1).

As to *O. zaguliaevi*, five, nine, one and one larvae were collected at Hoshioki, Misumai, Takinosawa and Akaigawa, respectively, and three female adults were collected at Appi (Figure 3.1).

As to *O. zealis*, four and six female adults were collected at Appi and Kawaji, respectively (Figure 3.1).

As to *O. nubilalis*, 62 females were obtained from the larvae collected at Kuban Station, southwest of Russia. Four, four, eight and four female adults were collected at Dobrovnik, Nova Gorica, Ozeljan and Črni log in Slovenia.

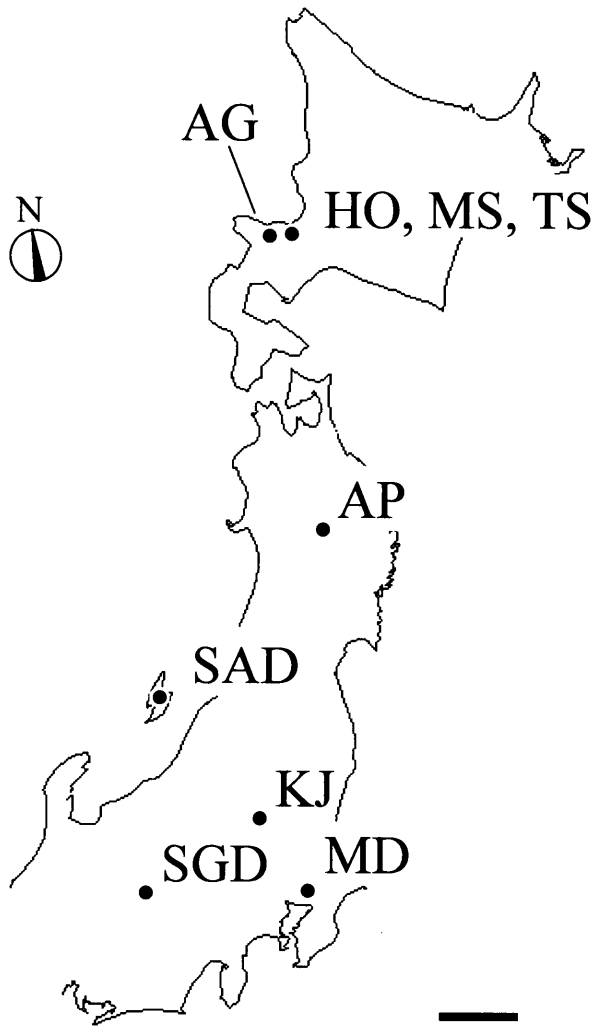

Figure 3.1 Collection sites of *Ostrinia orientalis*, *O. zaguliaevi* and *O. zealis* in north Japan. AG, Akaigawa; HO, Hoshioki; MS, Misumai; TS, Takinosawa; AP, Appi; SAD, Sado; KJ, Kawaji; MD, Matsudo; SGD, Sugadaira. The scale bar indicates 100 km.

3.2.2 Rearing and crosses

Insect rearing and crosses were conducted as described in Chapter 1.

3.2.3 DNA extraction

DNAs were extracted from female ovaries as described in section 1.2.4 of Chapter 1.

3.2.4 *Wolbachia* PCR

To examine the *Wolbachia* infection, PCRs specific to *Wolbachia* 16S *rRNA* gene were conducted as described in section 1.2.4 of Chapter 1.

3.2.5 Sequencing

Two *Wolbachia* genes, *ftsZ* and *wsp*, were amplified by PCR and directly sequenced as described in section 1.2.5 of Chapter 1.

3.2.6 Bacterial PCR

To examine bacterial infection, PCRs using general primers for eubacterial 16S *rRNA* gene were conducted as described in section 2.2.5 of Chapter 2.

3.3 Results

In *O. orientalis*, three (MD607, SAD801, SGD105) of 24 wild females were infected with *Wolbachia* (Table 3.1). Progenies (G_0) were obtained from the two infected females (MD607, SAD801), and both families consisted of females only (family size of SAD801 was 26 and data for MD607 were lost) (Table 3.2). All the 21 females free from *Wolbachia* infection produced progenies with sex ratio not significantly deviated from 1:1 ($P > 0.01$ by chi-squared test). In a *Wolbachia*-infected matriline (SAD801), maternal inheritance of the sex ratio trait was confirmed (Table 3.2). In the other *Wolbachia*-infected matriline (MD607), one G_1 family obtained consisted of females only (n=34). Of two G_2 families obtained, one consisted of females only (n=16) and the other consisted of males only (n=10). One G_3 family was obtained from the all-female G_2 family, and it consisted of females only (n=108).

In *O. zaguliaevi*, one (AG901) of nineteen females was infected with *Wolbachia* (Table 3.3). The AG901 female produced all-female progeny (n=10). The 18 wild females free from *Wolbachia* infection produced progenies with sex ratio not significantly deviated from 1:1 ($P > 0.01$ by chi-squared test). I could not obtain G_1 generation from the AG901 female.

In *O. zealis*, none of the ten wild females collected were infected with *Wolbachia* (Table 3.4). One wild female (AP902) produced all-female progenies. The other nine females produced progenies with nearly 1:1 sex ratio ($P > 0.01$ by

chi-squared test). Sex ratio was examined for G_1 families produced by two females in the all-female G_0 family (derived from AP902) (Figure 3.2). The G_1 families showed nearly 1:1 sex ratio. Each G_1 family was separated into two groups, and reared on a diet with and without tetracycline. Only one G_2 family was produced from one of G_1 females not treated with tetracycline, and showed nearly 1:1 sex ratio (17 females and 11 males). Each of the two G_1 females treated with tetracycline produced single family: one showed sex ratio not significantly biased from 1:1 (13 females and 6 males), and the other consisted of females only (n=47). The wild AP902 female was checked by PCR for infection with bacteria other than *Wolbachia*. The sequence of 16S *rRNA* gene was not amplified by PCR.

None of the 82 individuals of *O. nubilalis* were infected with *Wolbachia*. Sex ratios were examined for fifty-five G_0 families derived from Kuban Station population (Table 3.5). None of them were significantly deviated from 1:1 ($P >$ 0.01 by chi-squared test).

Wolbachia in the four infected wild females, i.e. three *O. orientalis* and one *O. zaguliaevi*, had the identical *wsp* and *ftsZ* gene sequences, being also identical to those of *Wolbachia* in *O. furnacalis* and *O. scapulalis* (Chapter 1, Chapter 2). The phylogenetic trees of *wsp* and *ftsZ* gene sequences are shown in Chapter 1.

Table 3.1 Sex ratio of the broods produced from wild females of *Ostrinia orientalis* and its association with *Wolbachia* infection

Location	Female collected	*Wolbachia* infection	Proportion of males (n)
Sado	**SAD801***	+	**0.00 (26)**
	SAD802*	–	0.54 (13)
	SAD803*	–	0.86 (7)
Sugadaira	SGD801	–	0.55 (107)
	SGD802	–	0.49 (53)
	SGD803	–	0.41 (58)
	SGD101	–	0.57 (106)
	SGD102	–	0.48 (130)
	SGD103	–	0.50 (8)
	SGD104	–	0.50 (109)
	SGD105	+	**n.e.** †
	SGD106	–	0.52 (87)
	SGD107	–	0.57 (70)
	SGD108	–	0.53 (19)
	SGD109	–	0.56 (97)
	SGD110	–	0.51 (65)
	SGD111	–	0.40 (35)
	SGD112	–	n.e.
	SGD113	–	0.48 (69)
	SGD114	–	n.e.
	SGD115	–	0.68 (40)
	SGD116	–	0.41 (51)
Matsudo	**MD607**	+	**0.00 (n.e.)**
	MD8101	–	0.45 (51)

*Sex ratio data are from Ohno (2000a).

† Not examined.

Table 3.2 Maternal inheritance of SR trait in two *Wolbachia*-infected matrilines of *O. orientalis*

Matriline	G_0		G_1		G_2		G_3	
	Female	Male	Female	Male	Female	Male	Female	Male
SAD801	26	0	10	0	7	0		
			7	0	26	0		
			5	0				
MD607	*	0	34	0	16	0	108	0
					0	10		

* Data lost.

Table 3.3 Sex ratios of the broods produced from wild females of *Ostrinia zaguliaevi* and its association with *Wolbachia* infection

Location	Female collected	*Wolbachia* infection	Proportion of males (n)
Hoshioki	HO901	–	0.35 (23)
	HO902	–	0.52 (23)
	HO903	–	0.59 (37)
	HO904	–	0.78 (9)
	HO905	–	0.44 (16)
Misumai	MS901	–	0.57 (42)
	MS902	–	0.53 (38)
	MS904	–	0.42 (19)
	MS905	–	0.44 (9)
	MS906	–	0.13 (8)
	MS907	–	0.48 (31)
	MS908	–	0.58 (19)
	MS910	–	0.46 (13)
	MS913	–	0.57 (21)
Takinosawa	TS901	–	0.50 (14)
Akaigawa	**AG901**	+	**0.00 (10)**
Appi	AP901	–	0.50 (6)
	AP905	–	0.18 (11)
	AP906	–	0.43 (21)

Table 3.4 Sex ratios of the broods produced from wild females of *Ostrinia zealis* and its association with *Wolbachia* infection

Location	Female collected	*Wolbachia* infection	Proportion of males (n)	
Appi	**AP902**	–	**0.00**	**(94)**
	AP903	–	0.49	(76)
	AP904	–	0.33	(46)
	AP907	–	0.45	(85)
Kawaji	KJ901	–	0.54	(68)
	KJ902	–	0.49	(142)
	KJ903	–	0.49	(110)
	KJ904	–	0.40	(42)
	KJ905	–	0.42	(57)
	KJ906	–	0.41	(54)

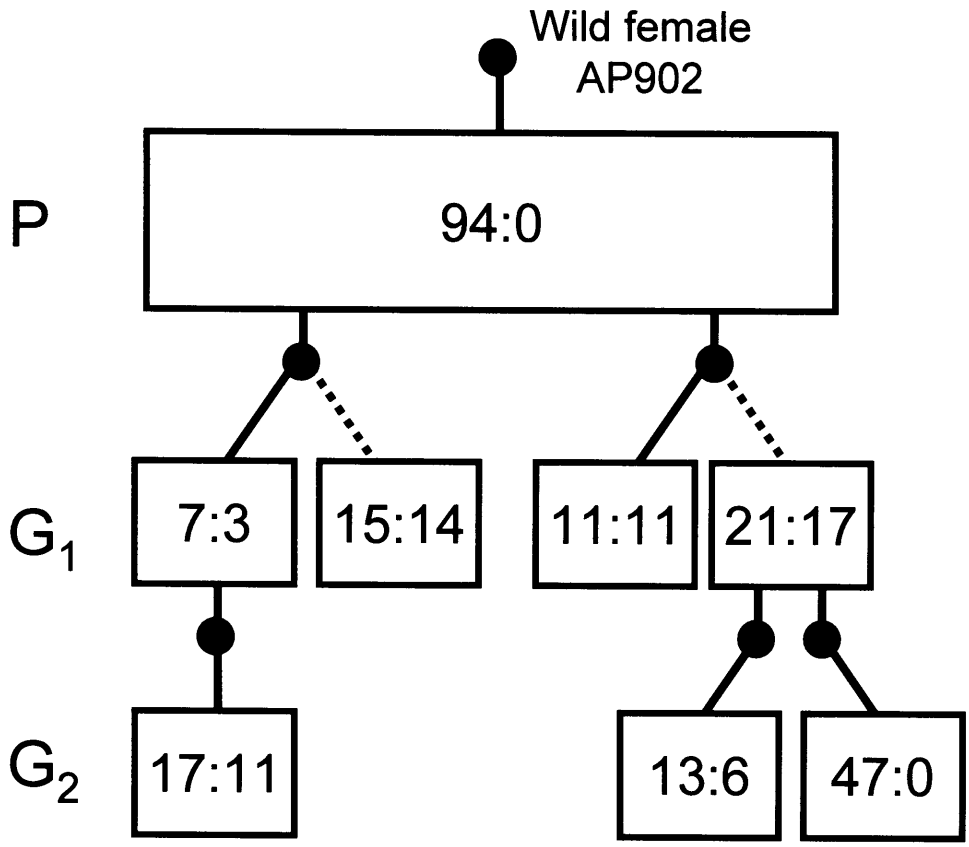

Figure 3.2 Inheritance of sex ratio trait in *O. zealis*. The pedigree shows the matriline derived from one wild *O. zealis* female (AP902). Black circle (●) depicts one female producing offspring. Dotted line depicts tetracycline treatment.

Table 3.5 Sex ratios of the broods produced from wild females of *Ostrinia nubilalis* and its association with *Wolbachia* infection

Female collected	*Wolbachia* infection	Proportion of males (n)
KS101	—	0.60 (115)
KS102	—	0.50 (52)
KS103	—	0.52 (91)
KS104	—	0.53 (68)
KS105	—	0.51 (80)
KS106	—	0.60 (100)
KS107	—	0.55 (113)
KS108	—	0.47 (75)
KS109	—	0.53 (73)
KS110	—	0.44 (70)
KS111	—	0.43 (120)
KS112	—	0.76 (17)
KS113	—	0.58 (81)
KS114	—	0.49 (68)
KS115	—	0.30 (27)
KS116	—	0.49 (83)
KS117	—	0.37 (78)
KS118	—	0.51 (85)
KS119	—	0.49 (109)
KS120	—	0.42 (53)
KS121	—	0.57 (30)
KS122	—	0.46 (63)
KS123	—	0.48 (21)
KS124	—	0.65 (26)
KS125	—	0.40 (5)
KS126	—	0.68 (25)
KS128	—	0.59 (39)
KS129	—	0.33 (58)
KS130	—	0.51 (55)
KS131	—	0.59 (66)
KS132	—	0.51 (106)
KS133	—	0.49 (61)
KS134	—	0.55 (99)
KS135	—	0.51 (75)
KS136	—	0.62 (60)
KS137	—	0.49 (109)
KS138	—	0.50 (84)
KS139	—	0.56 (97)
KS140	—	0.60 (30)
KS141	—	0.50 (26)
KS142	—	0.37 (30)
KS143	—	0.67 (57)
KS144	—	0.46 (91)

continued from left.

Female collected	*Wolbachia* infection	Proportion of males (n)
KS145	—	0.56 (107)
KS146	—	0.58 (31)
KS147	—	0.68 (34)
KS148	—	0.59 (70)
KS150	—	0.49 (53)
KS151	—	0.42 (111)
KS152	—	0.49 (55)
KS153	—	0.49 (39)
KS154	—	0.47 (57)
KS155	—	0.49 (97)
KS156	—	0.53 (88)
KS157	—	0.38 (16)

3.4 Discussion

The study in this chapter, together with the findings in Chapter 1 and Chapter 2, revealed that *Wolbachia* infection are found in at least four species of *Ostrinia*: *O. furnacalis*, *O. scapulalis*, *O. orientalis* and *O. zaguliaevi*. *Wolbachia* infection was not found from *O. zealis* female (n=10) and *O. nubilalis* (n=82). In *O. zealis*, since sample size is very small, it is possible that *Wolbachia* infection will be found in further examination. As to Kuban station population of *O. nubilalis* in which 62 females were checked for *Wolbachia*, frequency of *Wolbachia* infection, if existed, must be very low.

The *wsp* gene sequence, as well as the *ftsZ* gene sequence of *Wolbachia* in the four *Ostrinia* species were identical. The evolutionary rates of the *wsp* and *ftsZ* gene are the fastest and the second fastest among the *Wolbachia* genes being known to date. The identity of the *wsp* and the *ftsZ* gene sequences indicates that *Wolbachia* strain(s) in these host species are identical or at least very closely related. Therefore, the all-female trait found in *O. orientalis* and *O. zaguliaevi* is probably due to feminization of genetic males induced by *Wolbachia* infection, as in the case of *O. furnacalis* and *O. scapulalis* (Chapter 1 and Chapter 2). The all-male family observed in the *O. orientalis* matriline derived from the all-female family can be explained by spontaneous cure from *Wolbachia* infection.

The evolutionary origin of the *Wolbachia* infection shared among the four *Ostrinia* species can be explained in three ways (cf. Jiggins *et al.*, 2000a). First, *Wolbachia* may have resided in its ancestral hosts before the species differentiation of the hosts. Differentiation of these species would have occurred rather recently on an evolutionary time scale since the these species have a small divergence in mtDNA (Kim *et al.*, 1999). Second, *Wolbachia* may have moved horizontally by way of cannibalism or via a third species such as a parasitoid. The habitats of these host species are often overlapping in Japan, and so these species may have common some parasitoids. Third, the *Wolbachia* strain may have transferred to different host species through hybridisation. Even if these species can hybridise, it would not occur frequently, because components, blend ratios or the amount of their sex pheromone are different from each other (Ishikawa *et al.*, 1999). Occurrence of natural hybridisation between *O. scapulalis* and *O. orientalis* has been evidenced (Ohno, 2000). Examining the mitochondrial DNA (mtDNA) gene sequences of infected and uninfected individuals of the four species will be helpful in inferring which of these three explanations are most likely. This is because mitochondria, often used as genetic marker in examining genetic polymorphism in animals, are generally inherited maternally as well as *Wolbachia*. The occurrence of the same or closely related *Wolbachia* strains in closely related host species have been known in genera such as *Trichogramma*, *Gryllus*, *Acraea* and *Tribolium* (National centre for biochemistry information home page).

In the SR matriline of *O. zealis*, one G_2 family inherited the all-female trait (Figure 3.2). This may suggest the maternal inheritance of the sex ratio trait. This all-female family in G_2 generation was produced from a tetracycline-treated female. The bacterial PCR was negative. Therefore, it was indicated that the SR trait found in *O. zealis* was not associated with bacterial infection.

In *O. scapulalis*, an unidentified maternally inherited element (SR-w^-) causes all-female production, probably due to sex chromosome meiotic drive (Chapter 2). Production of 1:1 sex ratio families from SR-w^- matrilines in *O. scapulalis* is probably due to the presence of suppressor (Chapter 2). Sex ratio trait found in *O. zealis* is very similar to that found in *O. scapulalis*, and hence might be the same phenomenon.

Chapter 4

Effect of tetracycline concentration on feminization in *Wolbachia*-infected *Ostrinia scapulalis*

4.1 Introduction

Wolbachia-infected females of *O. scapulalis* and *O. furnacalis* are feminized genetic males (Chapter 1; Chapter 2). *Wolbachia*-infected larvae developed as females even if they were fed with diet containing tetracycline from the hatchling stage. These tetracycline-treated female adults were *Wolbachia*-negative by the PCR assay, and produced only male progenies. These findings suggest that, in *O. furnacalis* and *O. scapulalis*, sexual development will not be affected by the presence/absence of *Wolbachia*, once sex is determined during the egg and embryonic stages. This suggestion is consistent with the fact that a series of sex determining process starts at the embryonic stage in *Drosophila melanogaster* (Hoy, 1994). It is highly probable that *Wolbachia* affects sex determination processes during the embryonic stage of *Ostrinia*.

Then, how will the sexual development of *Wolbachia*-infected *Ostrinia* be affected by tetracycline treatment at the embryonic stage? Among the developmental stages capable of oral administration of tetracycline, the post-mating female adult is the closest to the embryonic stage. Mated female adults of *O. scapulalis* were treated with tetracycline, and sexuality of their progenies was examined. In addition, *Wolbachia*-infected *O. scapulalis* were treated with tetracycline at different concentrations during the larval stage, and sexuality of their progenies was examined.

4.2 Materials and methods

4.2.1 Insect collection and establishment of *Wolbachia*-infected matrilines

Two *Wolbachia*-infected matrilines (MD014 and KI730) were established from single wild females of *O. scapulalis* (Chapter 2).

4.2.2 Tetracycline treatment of *Wolbachia*-infected adults

Nine females of the matriline MD014 were crossed with normal males. Two days later, after removing the males, 3% sugar solution containing 0.24% tetracycline hydrochloride was given to the females. After the treatment with tetracycline for one day, eggs were collected daily from individual females until the sixth day. Eggs laid in the seventh, eighth and ninth day were pooled. Larvae were reared on a normal artificial diet (Nippon Nosan, Yokohama). Sexuality of the adults obtained was examined.

4.2.3 Tetracycline treatment of *Wolbachia*-infected larvae

Artificial diet containing tetracycline hydrochloride at nine concentrations was fed to

larvae of the matriline KI730. Developed females were crossed with normal males. Progenies were reared on a normal artificial diet. Sexuality of the adults obtained was examined.

4.2.4 Genitalia observation

Genitalia were macerated in 5% KOH overnight, and stained with Acid Fuchsin for two or three days. Stained genitalia were observed under a binocular microscope (Olympus SZX12).

4.3 Results

4.3.1 Tetracycline treatment of *Wolbachia*-infected female adults

During the first four days after the treatment with tetracycline, all the nine treated females laid eggs that developed to female adults (Figure 4.2). In contrast, eggs laid from the fifth to ninth days developed to females, sexual mosaics or males. The sexual mosaic individual had both male and female morphological characters on the body and wings, which are clearly distinguishable in *O. scapulalis* (Figure 4.1). Regarding the mosaic individuals, eggs laid later tended to develop to adults with larger male portion in an insect body.

Morphology of progenies of a tetracycline-treated female was examined in detail (Table 4.1). More than half (24 of 42) of mosaic individuals had morphological abnormalities, such as curled wings and abnormal external genitalia. Some of them had both male and female structures in genitalia, which could be easily distinguished in *uncus* (un)/ *papillae anales* (pap.a). A mosaic individual having two sets of *aedeagus* (ae) has been found. Examples of abnormalities in external genitalia are shown in Figure 4.3. Within the complete females and males in the same family, few individuals had curled wings (three out of 159 females and one out of 43 males) and no individuals had abnormal external genitalia (Table 4.1).

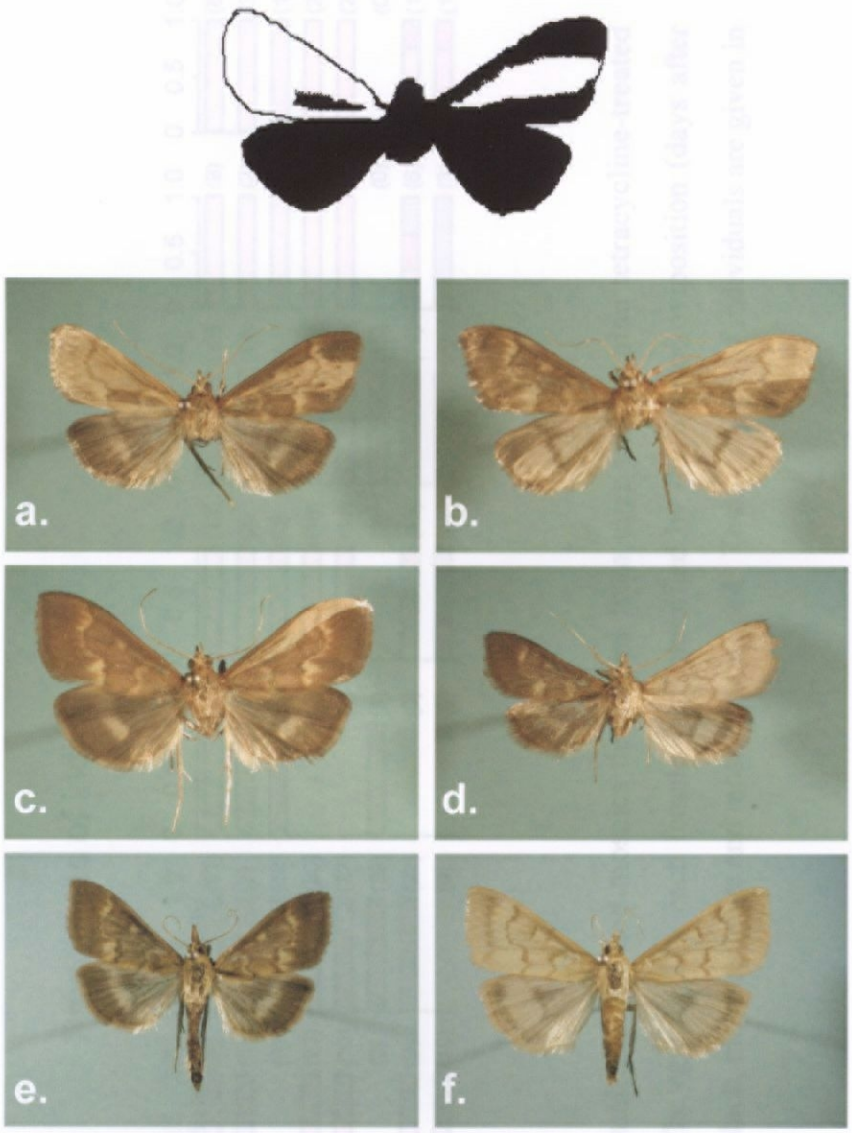

Figure 4.1 Mosaic individuals produced from *O. scapulalis* females treated with tetracycline at the adult stage. **a.-d.** mosaics, **e.** normal male, **f.** normal female. An illustration of **a.** is shown on the top. Black and white regions on wings represent male and female tissues, respectively.

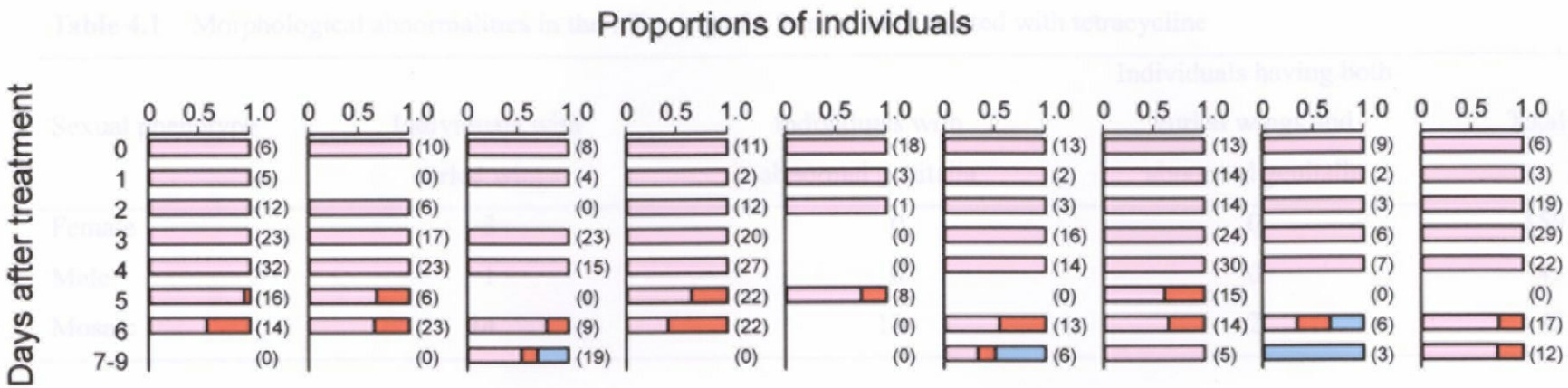

Figure 4.2 Proportions of females, males and mosaics developed from eggs laid by nine tetracycline-treated *Wolbachia*-infected females. The eggs laid by each female were classified by the date of oviposition (days after treatment). Females shown as black, males as white and mosaics as striped. Total numbers of individuals are given in parentheses.

Table 4.1 Morphological abnormalities in the offspring of a female adult treated with tetracycline

Sexual phenotype	Individuals with curled wings	Individuals with abnormal genitalia	Individuals having both curled wings and abnormal genitalia	Total
Female	3	0	0	159
Male	1	0	0	43
Mosaic	14	12	2	42

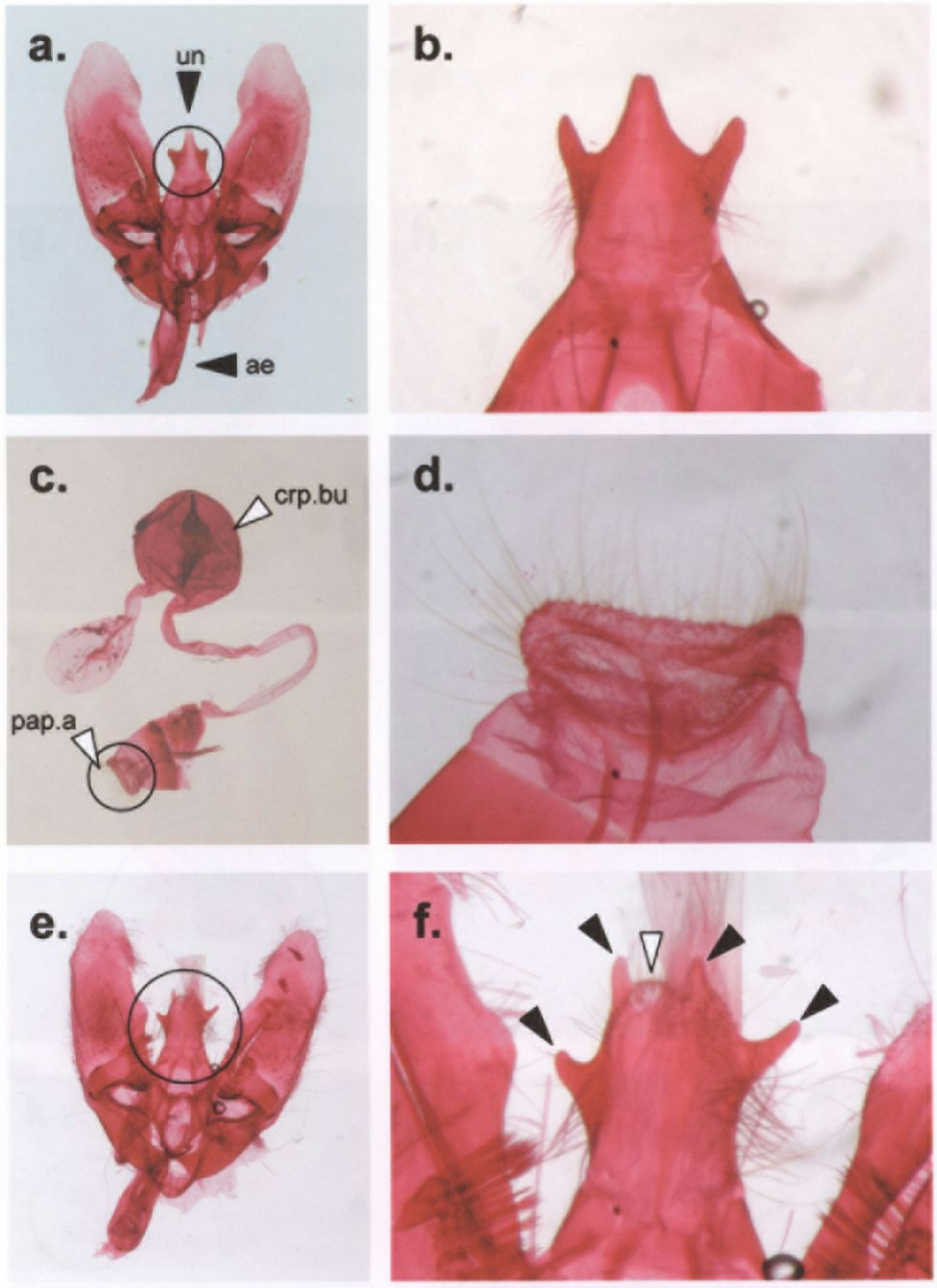

Figure 4.3 Genitalia (8th-10th abdominal segments) of a normal male (a, b), a normal female (c, d) and four sexual mosaic individuals (e-l) of *Ostrinia scapulalis*. b, d, f, h, j and l are magnified images of a, c, e, g, i and k, respectively. *uncus* (un), *aedeagus* (ae), *papillae anales* (pap.a), *corpus bursae* (crp.bu) are indicated. Many mosaic individuals had both male (shown with black triangles) and female (shown with white triangles) structures (f, h, l). A mosaic individual having two sets of *aedeagus* (ae) has been found (j).

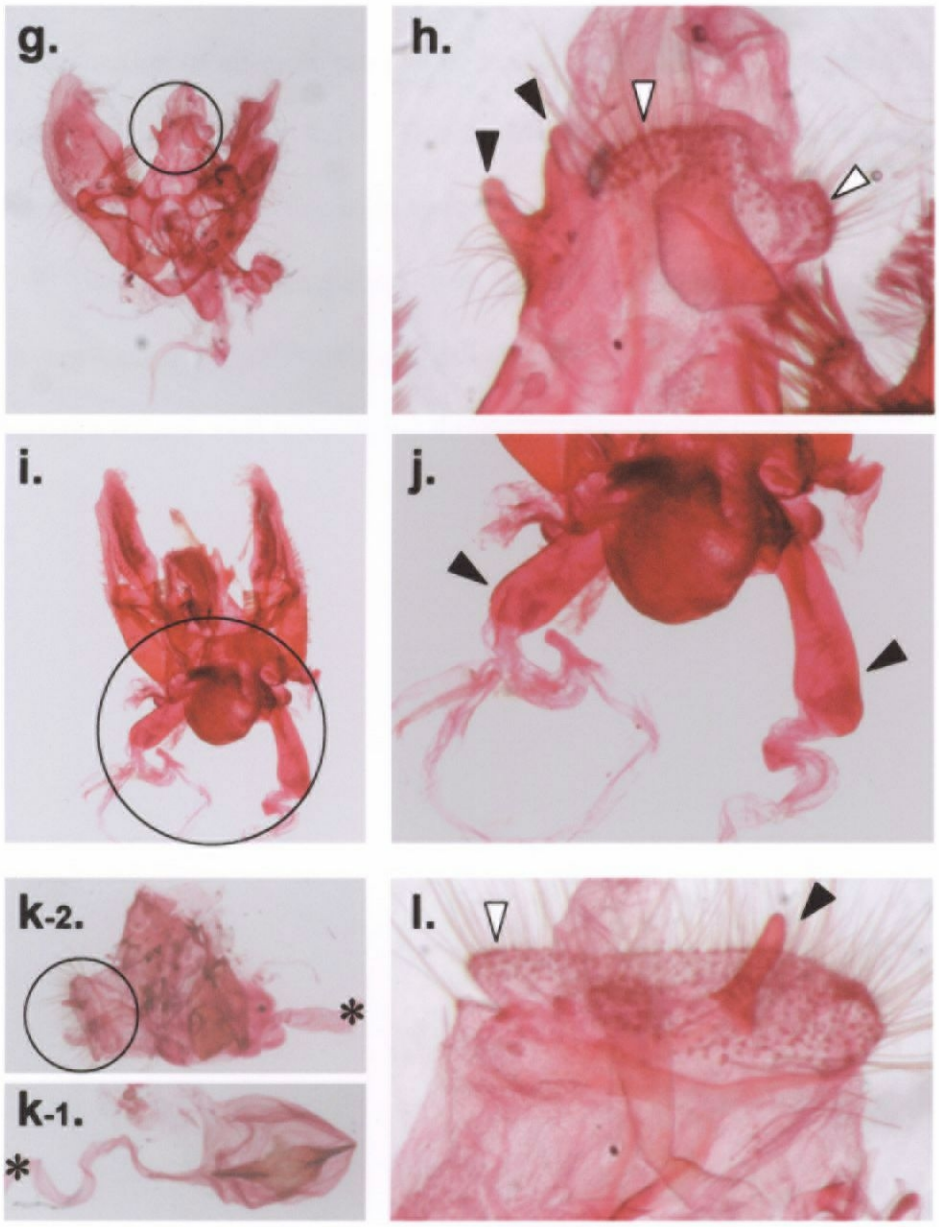

Figure 4.3 (Continued.)

4.3.2 Tetracycline treatment of *Wolbachia*-infected larvae

Treatment with tetracycline at higher six concentrations to *Wolbachia*-infected females resulted in production of all-male progenies, except for one family consisting of seven females and four males (Table 4.2). This family containing both males and females was produced from the female treated with tetracycline at an intermediate concentration, 1,200 x 10^{-8} (w/w). The tetracycline treatment at the three lowest concentrations resulted in production of all-female progenies. Sexual mosaics were not produced from the females treated with tetracycline during the larval stage.

Table 4.2 Number of families with all females, families with both males and females and families with all males, produced from females treated with tetracycline at the larval stage

Concentration of tetracycline ($\times 10^{-8}$ w/w)	Number of families		
	All male	Both male and female	All female
60,000	4 (240)	0	0
30,000	2 (69)	0	0
12,000	1 (21)	0	0
6,000	4 (267)	0	0
1,200	2 (22)	1 (11)*	0
600	4 (345)	0	0
120	0	0	7 (534)
60	0	0	3 (130)
6	0	0	4 (249)

Total numbers of progenies are given in parentheses.

*This family consisted of seven females and four males.

4.4 Discussion

A number of sexual mosaics were produced from *O. scapulalis* females of *Wolbachia*-infected matriline when they were treated with tetracycline at the adult stage. This finding suggests autonomous sex differentiation of the cells, and the lack of sex hormone in *O. scapulalis*, which are widely accepted postulates for sex differentiation in insects. It is suggested that *Wolbachia*-induced feminization in *O. scapulalis* has an association with sex determination in host cells.

4.4.1 Difference from *Wolbachia*-induced feminization in *A. vulgare*

Wolbachia-induced feminization of genetic males had been reported only in terrestrial isopod crustaceans such as *Armadillidium vulgare* (Rigaud, 1997). In general, diffusive sex hormone induces sex differentiation in crustaceans (Legrand *et al.*, 1987). In a male *A. vulgare*, sex hormone, produced in the androgenic gland, induces male differentiation after the fourth moult (Martin *et al.*, 1999; Okuno *et al.*, 1999). In a normal female and a *Wolbachia*-infected genetic male of *A. vulgare*, androgenic gland does not differentiate. When the *Wolbachia* was eliminated from infected young individuals by high temperature, they will gradually acquire male phenotype. On the other hand, in *O. scapulalis*, phenotype of infected individuals was not affected by elimination of *Wolbachia* although their progenies are affected. Moreover, in contrast to *O. scapulalis*, *Wolbachia*-infected lines of *A. vulgare* do not produce sexual mosaics

but occasionally produce intersex which has characters intermediate between male and female.

4.4.2 Possible mechanism of the sexual mosaic formation

Sexual mosaics produced in the present experiments could be recognized at the adult stage. Since no sexual dimorphism can be detected in the external morphology of *Ostrinia* at the egg and larval stages, it is virtually impossible to recognize sexual mosaics at these stages. The sexual mosaics of *O. scapulalis* should also have mosaic sexual characters in the egg and larval stages as well. In a genetic strain (*mo*) of the silk worm, *Bombyx mori* which produce sexual mosaics with high frequency, mosaic characters were found in eggs, larvae and adults (Ebinuma and Kobayashi, 1986).

It is possible that decrease of *Wolbachia* density within the egg due to tetracycline treatment of *O. scapulalis* adults leads to non-uniform distribution of *Wolbachia* in an egg and among embryonic cells; some parts/cells with high and others with low *Wolbachia* densities. The cells with high density and those with low density may develop as female and male cells, respectively.

With regard to sexual mosaics, eggs laid at later times after the tetracycline treatment tended to develop to mosaics having more male cells. This finding suggests that it takes a few days for the effect of tetracycline to appear. It is possible that a germ cell with *Wolbachia* density substantially decreased by tetracycline treatment at the embryonic stage is destined to develop as a male, while a germ cell with high

Wolbachia density is destined to develop as a female. A germ cell with an intermediate *Wolbachia* density may become a sexual mosaic. An illustration of this hypothetical scenario is shown in Figure 4.4.

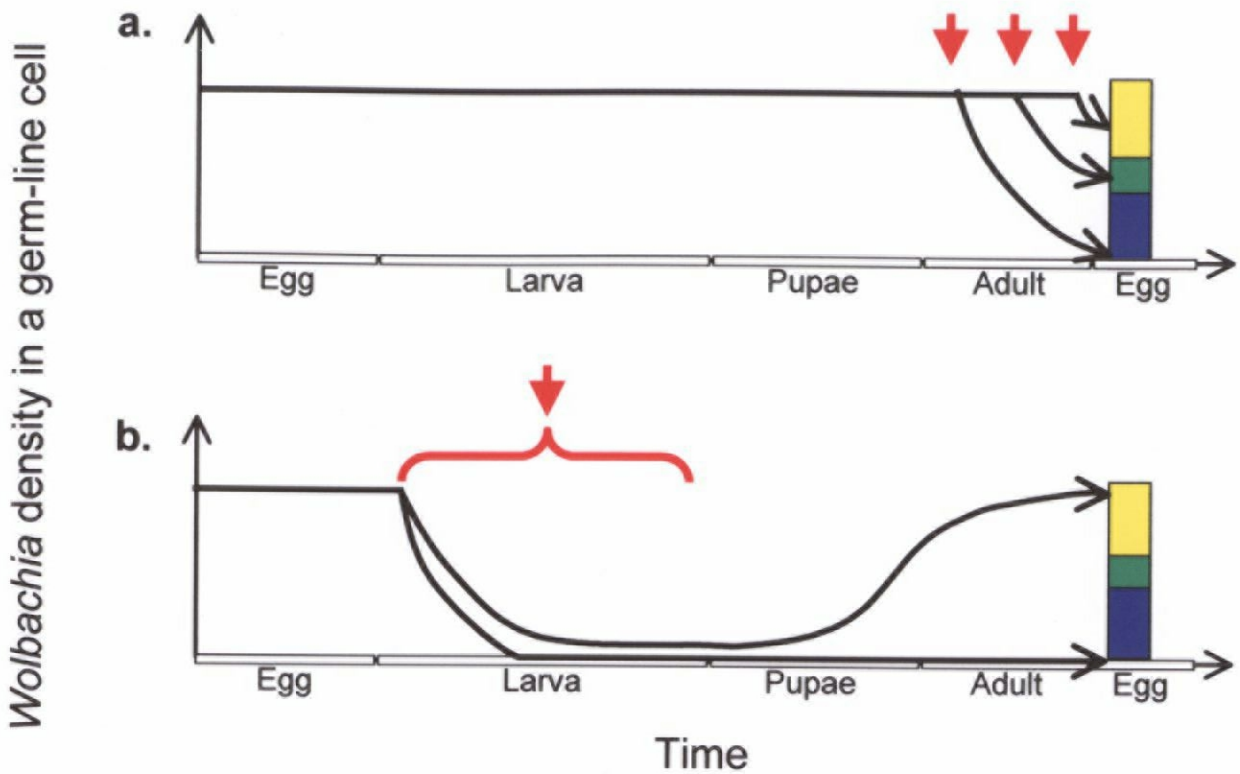

Figure 4.4 Illustration showing hypothetical dynamics of *Wolbachia* densities in a germ-line cell along with the developmental time course. Changes of *Wolbachia* densities in relation to tetracycline treatments are presented. Tetracycline treatment at adult stage (a) and larval stage (b), are depicted with red. The ranges of *Wolbachia* densities at the embryonic stage destined to develop as females, mosaics and males are depicted with yellow, green and blue, respectively.

4.4.3 Potential use of *Wolbachia*-mediated sexual mosaic formation for studies on sex determination in Lepidoptera

As mentioned above, in *Bombyx mori*, there are genetic strains that produce sexual mosaics with high frequencies. Genetical and cytological research has revealed that sexual mosaic formation is due to double fertilization (e.g. Goldschmidt and Katsuki, 1927). It is considered that the double fertilization represents the mechanism of sexual mosaic formation in Lepidoptera (Laugé, 1985). However, sexual mosaic formation from *Wolbachia*-infected females of *O. scapulalis* is not due to double fertilization, but due to partial feminization caused by *Wolbachia*. In *Wolbachia*-infected *O. scapulalis*, the genotype of all cells are ZZ, irrespective of their sexual phenotype.

Since the mosaic formation due to infection with feminizing microorganisms has not been known in lepidopteran species, the present finding will be a helpful tool for the elucidation of the mechanism of sex determination in Lepidoptera. A very recent finding of *Wolbachia*-induced feminization in the sulfur butterfly, *Eurema hecabe* (Hiroki *et al.*, personal communication), a species distantly related to *Ostrinia*, implies that feminization will be found in more lepidopteran species. Furthermore, it is possible in such cases that feminizing *Wolbachia* as found in *Ostrinia* and *Eurema* transfect to other lepidopteran species to cause feminization. Therefore, induction of sexual mosaic formation by administrating tetracycline to feminized adults may be applicable to lepidopteran species other than *Ostrinia*.

Although sexual mosaics are very rare in nature, they have been reported in many species of Lepidoptera (e.g. Nishiyama, 2001). Taking into account that a lot of insect

species are infected with *Wolbachia* (approximately 20%, see general introduction), it is possible that some of the natural sexual mosaics of lepidopteran species are due to partial elimination of feminizing *Wolbachia* by natural antibiotics or high temperature.

4.4.4 Effect of tetracycline treatment to *Wolbachia*-infected larvae on sexuality of their progenies

Wolbachia-infected *O. scapulalis* larvae were treated with tetracycline at nine concentrations. Progenies produced from females treated with higher concentrations of tetracycline were all males, and progenies from females treated with lower concentrations of tetracycline were all females. One family that contained both males and females was produced from a female treated with an intermediate concentration of tetracycline. Sexual mosaics were not found in these families. Here, I assume that these results accurately reflect the dose-dependent response of *Wolbachia*-infected larvae of *O. scapulalis* to tetracycline treatment, and discuss the significance of the results. However, since only one family was found to contain both males and females, more replications of tetracycline treatment at intermediate concentrations are necessary to verify this assumption.

The administration of tetracycline to larvae of *O. scapulalis* will decrease the *Wolbachia* density in germ cells. If tetracycline concentration is sufficiently high, *Wolbachia* will be eliminated from all the oogonia, which develop to *Wolbachia*-free oocytes, and then differentiate as males. If tetracycline concentration is not sufficiently high, *Wolbachia* may not be completely eliminated from some of the germ

cells. In such germ cells that still harbour *Wolbachia*, density of *Wolbachia* can increase during the tetracycline-free period of prepupal, pupal and adult stages, and her progenies can be feminized at the embryonic stage. Other germ cells that have lost *Wolbachia* will develop as *Wolbachia*-free oocytes, and then differentiate as males. Thus, when tetracycline is administrated to a *Wolbachia*-infected individual at the larval stage, each egg produced from the treated individual has two alternatives: having *Wolbachia* with the density sufficiently high to feminize all the embryonic cells, or having no *Wolbachia* infection at all. It follows that sexual mosaics are rarely produced when tetracycline treatment is conducted at larval stage, which is concordant with the present observation

In *Wolbachia*-infected matrilines of *O. furnacalis* and *O. scapulalis*, families with nearly 1:1 sex ratio were rarely found (Chapter 1; Chapter 2). Appearance of small proportion of males in a brood may be due to naturally occurred decrease of *Wolbachia* densities in oocytes.

In this chapter, although I discussed the results in relation to the *Wolbachia* densities, the *Wolbachia* densities have remained to be examined. It is necessary to examine the association between *Wolbachia* densities at the stages of embryo/larvae and the sexuality at the adult stage.

As is shown in Figure 4.2, the condition for sexual mosaic formation appears to be severe. Many sexual mosaics of *O. scapulalis* had morphology that would be disadvantageous in survival and reproduction (Table 4.1). It is therefore possible that the *Wolbachia* strain in *O. scapulalis* has evolved to exist at a high density in embryonic cells of the embryo in order to increase the efficiency of feminizing host cells and to

reduce production of the sexual mosaics.

General Discussion

5.1 Feminization of genetic males due to *Wolbachia* infection

Occurrence of *Wolbachia*-induced feminization was demonstrated in *O. furnacalis* and *O. scapulalis* (Chapter 1, Chapter 2). *Wolbachia*-infected females were found also in *O. orientalis* and *O. zaguliaevi*, and they produced all-female progenies. *Wolbachia* strains in *O. furnacalis*, *O. scapulalis*, *O. orientalis* and *O. zaguliaevi* were indistinguishable in terms of DNA sequences of *ftsZ* and *wsp* genes. Therefore, it is very likely that *Wolbachia*-induced feminization of genetic males is also occurring in *O. orientalis* and *O. zaguliaevi*.

In insects, feminization of genetic males had not been found when the present study was initiated. Even in a well-studied model organism, *Drosophila melanogaster*, feminization by a single mutation that converts an XY male into a functional female is not known. On the other hand, feminization induced by microorganisms has been known in some crustaceans (reviewed by Rigaud, 1997). Futhermore, crustacean males can readily be changed to females by simple experimental manipulations (Legrand *et al.*, 1987). Rigaud (1997) argued that the absence of feminization in

insects is relevant to the nature of insect sex determination, i.e. lack of circulating sex hormone and cell autonomous sex differentiation. In addition, it has been argued that strong constraint of sex chromosome dosage compensation makes the feminization difficult to occur in insects (Rigaud, 1997).

Partial elimination of *Wolbachia* from female adults resulted in production of sexual mosaic individuals in *O. scapulalis* (Chapter 4). This is concordant with the two widely accepted postulates: the lack of circulating sex hormone and the cell autonomous sex differentiation. The initial key stage of sex determination in *O. scapulalis* is probably the embryo, because an individual treated with tetracycline during the entire larval stage develops as a female (Chapter 2). Under the lack of sex hormone and the cell autonomous sex differentiation, feminization of genetic male can be explained only by assuming that *Wolbachia* affects sex differentiation of the cells at the embryonic stage.

In contrast to *Drosophila*, it is suggested that in lepidopteran insects, sex chromosome dosage compensation does not occur (Suzuki *et al.*, 1998). Therefore, the sex chromosome dosage compensation, one of the factors that are supposed to constrain the feminization in insects (Rigaud, 1997), may not work as a constraint against feminization in Lepidoptera. If the lack of dosage compensation is the general feature in Lepidoptera, *Wolbachia*-induced feminization would be found in other lepidopteran species. Actually, *Wolbachia*-induced feminization has recently been found in the sulfur butterfly, *Eurema hecabe* (Hiroki *et al.*, personal communication).

5.2 Why is the *Wolbachia* prevalence low in *Ostrinia* species?

In *O. furnacalis*, *O. scapulalis*, *O. orientalis* and *O. zaguliaevi*, *Wolbachia* infection appears to be held at low frequencies in the field (Chapter 1; Chapter 2; Chapter 3). Sex ratio distorters in arthropods are mostly maintained at low frequency in the natural populations of their hosts (see Hatcher, 2000 for review, but see Jiggins *et al.*, 2000c for notable exception).

It is not clarified what forces restrict *Wolbachia* prevalence in *Ostrinia* species. However, one can raise several factors that may underlie the low prevalence of feminizing *Wolbachia* in *Ostrinia*.

(1) Fitness effect on host.

It has been shown that, in the *Drosophila* parasitoid wasp, *Leptopilina heterotoma*, *Wolbachia* infection has a negative impact on several host fitness traits of both sexes (Fleury *et al.*, 2000). It is possible that fitness of infected individuals of *Ostrinia* species might be lower than uninfected individuals in the field condition. However, in the laboratory condition at least, fitness of infected individuals of *O. furnacalis* and *O. scapulalis* does not appear to be very lower than uninfected individuals of respective species.

(2) Vertical transmission rate.

It is possible that some factors such as high temperature, natural antibiotics and diapause decrease the vertical transmission rate of *Wolbachia* in the field (vertical transmission rate of *Wolbachia* is > 98% under non-diapause conditions in the laboratory). The decrease in the vertical transmission rate diminishes the proportion of

feminized progenies (i.e. production of male progenies), and thus hinders the increase of *Wolbachia* prevalence.

(3) Suppressors or insensitive genes.

It is possible to consider the presence of nuclear genes in *Ostrinia* that resist to the vertical transmission or feminizing activity of *Wolbachia*, as supposed in the case of *Wolbachia*-mediated feminization in isopods (Rigaud and Juchault, 1992; Juchault *et al.*, 1994). In the laboratory, however, *Wolbachia*-infected females of *O. furnacalis* and *O. scapulalis* rarely produced nearly 1:1 sex ratio progenies (Table 1.1; Figure 1.1; Table 2.2). Such resistance factor, if present, appears to be not functioning under the laboratory condition.

(4) Sexual selection against feminized males.

It is also possible that sexual selection against the feminized genetic males is preventing the spread of *Wolbachia* infection in *O. furnacalis* population, as argued in the case of *A. vulgare*, in which males have been shown to prefer real females (Moreau *et al.*, 2001).

5.3 Non-random segregation of sex chromosome during female meiosis

Female-biased sex ratio not associated with bacterial infection (SR-w$^-$) was found in *O. scapulalis* (Chapter 2). Non-random segregation during female meiosis probably underlies its mechanism. The causal agent should be present either in W chromosome or in a cytoplasmic element, because the sex ratio trait was maternally inherited. The sex ratio trait found in *O. zealis* may also be caused by the same mechanism (Chapter

3).

The SR-w$^-$ trait in *O. scapulalis* is very similar to the SR trait in a moth *Abraxus grossulariata* (Geometridae). In *A. grossulariata*, maternal inheritance of SR condition was imperfect; all-female production occasionally changed to and from nearly 1:1 sex ratio (Doncaster, 1913; 1914). Genetic analysis and observation of egg hatchability indicated that the SR condition in *A. grossulariata* was probably neither due to parthenogenesis nor due to male-killing (Doncaster, 1914). Hurst (1993) suggested the mechanism of the SR trait in *A. grossulariata* to be the sex-chromosome meiotic drive system involving suppressors. Considering that *O. scapulalis* and *A. grossulariata* are distantly related, it is also possible that SR traits due to non-random segregation in female meioses are occurring in other taxonomic groups of Lepidoptera.

Meiosis is one type of cell division that results in the formation of haploid gametes from diploid cells through two steps of divisions (MI and MII). Although basically the same process occurs in both males and females, there are important differences in meiosis between the sexes. Generally, MI and MII are symmetric in males, and the meiosis generates four equivalent gametes (Figure 5.1). In contrast, each of the two divisions in females is asymmetric and meiosis generates an oocyte and polar bodies, and only one gamete is generated per oocyte (Figure 5.1).

This asymmetry in female meiosis makes it possible for non-random segregation during meiosis to occur. Therefore, non-random segregation of sex chromosome during meiosis must be restricted to female-heterogametic organisms. This restriction does not apply to meiotic drive for autosomes, and non-random segregation of an autosome during female meiosis has been well studied cytologically and genetically in

mouse (e.g. Pardo-Manuel de Villena *et al.*, 2000). At present, in contrast, no cytological evidence has been obtained for the sex chromosome meiotic drive in lepidopteran insects, although it has been invoked in explaining female-biased sex ratio in several lepidopteran species (reviewed by Jiggins *et al.*, 1999).

Meiotic drive that produces SR condition has been frequently found in Diptera (Jaenike, 1996; Jiggins *et al.*, 1999). Dipteran species possessing the sex ratio meiotic drive are male heterogametic, and thus in those species, X-chromosomal factors drive against Y chromosomes through dysfunction of Y-bearing sperm. Therefore, 'meiotic drive' in Diptera and that in Lepidoptera cannot be compared directly.

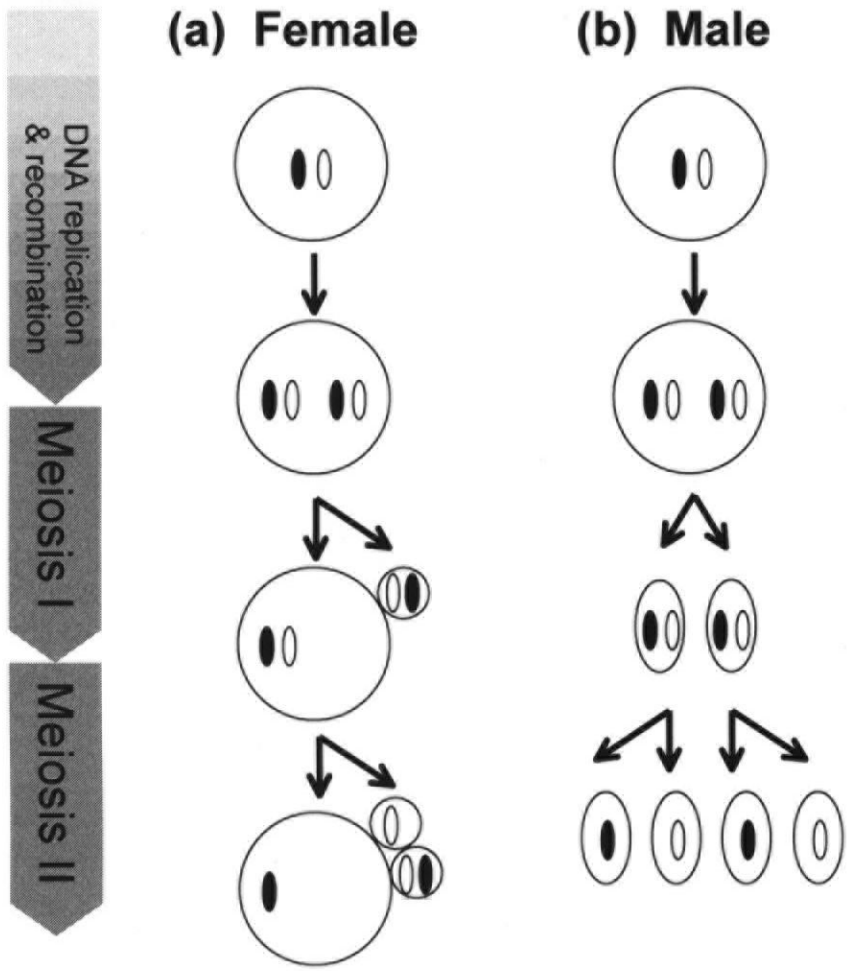

Figure 5.1 A schematic presentation of meiosis in females (a) and males (b). Meiotic divisions are asymmetric in females but symmetric in males. The process of DNA replication, recombination and chromosome/chromatid segregation is shown on the left (modified from Pardo-Manuel de Villena and Sapienza, 2001).

5.4 *Ostrinia* species may have genetic constraint against male-killing

Based on two lines of evidence described below, it is believed that male-killing, compared to other types of SR mechanisms, is easy to be evolved in insects (Hurst *et al.*, 1999a). First, male-killing bacteria have been found in five insect orders (Diptera, Hemiptera, Coleoptera, Hymenoptera and Lepidoptera) that include diverse sex determining systems such as male heterogametic, female heterogametic and haplo-diploid etc. Therefore, it is suggested that male-killing is not restricted to particular host genetic systems (Hurst *et al.*, 1997a). Second, male-killing bacteria belong to diverse groups in the eubacteria (see Table 0.1). In Lepidoptera, male-killing has been found in more than ten species from various taxonomic groups (Table 0.2; reviewed in Hurst, 1993; Hurst and Majerus, 1993). Among these, causal agents have been identified in three species. Two *Acraea* species have been shown to harbour male-killing *Wolbachia*, and in *Danaus chrissipus*, *Spiroplasma* bacterium induces male-killing (Hurst *et al.*, 1999a; Jiggins *et al.*, 2000a; 2000b).

When I initiated the present study (Chapter 1; Chapter 2), I expected that male-killing would be found at least in either *O. furnacalis* or *O. scapulalis*. In fact, however, male-killing was not found in *O. furnacalis* and *O. scapulalis* through intensive surveys. Moreover, to my surprise, the *Wolbachia* strain causing feminization in *O. scapulalis* induced male-killing in the Mediterranean flour moth, *Ephestia kuehniella*, after experimental transfection (Fujii *et al.*, 2001). These findings imply that *Ostrinia* species have some genetic constraint against evolution of male-killing.

To test the above idea, more transfection experiments between species harbouring naturally occurring feminizing bacteria and species harbouring male-killing bacteria are necessary. Besides, when a new insect species that harbour feminizing bacteria is found, survey of male-killing in that species should have particular significance.

5.5 Potential utilization of *Ostrinia* species in investigation of sex determination system in Lepidoptera

Wolbachia-infected *Ostrinia* species may be used as a unique and useful material/model for studies on expression of sex determining genes during the embryonic stage in Lepidoptera.

First, *Wolbachia*-infected *Ostrinia* species produce only females. Conversely, when they are treated with tetracycline at the larval stage, they produce only male progenies (Chapter 1; Chapter 2). Therefore, one can obtain female eggs only or male eggs only from each female. Second, sexual mosaic individuals are easily produced from *Wolbachia*-infected females by treatment with tetracycline at the adult stage (Chapter 4). I believe sexual mosaics will provide new facets to the studies on sex differentiation, as well as tissue differentiation, in insects.

Most of the sex ratio distortions known in other lepidopteran insects are caused by male-killing. *Ostrinia* species, in which both feminization and sex-chromosome drive occur, may have sex-determination system somewhat different from other lepidopteran insects. In this sense, investigating sex determination system in *Ostrinia* species must

be of great interest.

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Summary (in Japanese)

アワノメイガ属(*Ostrinia*)には20種が記載されている. そのうちアワノメイガ種群に属す10種は互いに外部形態が酷似しており, 分子系統解析からも非常に近縁であると分かっている. 私は, アワノメイガ*O. furnacalis*を室内飼育する間に, 野外採集したあるメス成虫の子孫の性比が著しくメスに偏るという現象を偶然発見した. 本研究はまず, この性比異常の出現頻度, 遺伝様式, メカニズム, 原因因子を解明するために, 飼育と交配を中心に遺伝学的な調査と実験を行った. また, 同種群の他種においても同様の性比異常現象が存在する可能性を考え, アワノメイガ種群の5種(アズキノメイガ*O. scapulalis*, オナモミノメイガ*O. orientalis*, フキノメイガ*O. zaguliaevi*, ゴボウノメイガ*O. zealis*, ヨーロッパアワノメイガ*O. nubilalis*)についても調査と実験を行った. これらの結果に基づき, アワノメイガ種群における性比異常の実態と発現メカニズムを考察した.

アワノメイガにおける*Wolbachia*が引き起こすメス化

アワノメイガにおいて, メスに偏った性比が生じるメカニズムと原因因子を明らかにすることを試みた. 千葉県松戸で採集した91頭のメス成虫のうち12頭がほぼメスのみを産んだ. メスに偏った性比は母系遺伝したことから, 原因は細胞質因子かW染色体であると示唆された. さらに, 細菌感染の関与を調べるため, 性比異常系統の幼虫にテトラサイクリンを投与した結果, 次世代は当初の予想に反しオスのみになった. このようにテトラサイクリン投与によりオスのみが産まれたことは細菌感染による遺伝的オスのメス化により説明できる. すなわち, 一般に鱗翅目昆虫の性染色体構成はZW(♀)/ZZ(♂)またはZO(♀)/ZZ(♂)であるので, 性比異常が遺伝的なオスがメス化されていることによって生じている場合, 母親は遺伝的にはオス(ZZ)であり正常なオス(ZZ)と交尾すれば遺伝的オス(ZZ)だけが産まれるはずである. ここでテトラサイクリンにより細菌が除去されると, 遺伝的オスのメス化が解除され, 全ての子がオスになったと考えることができる.

最近, αプロテオバクテリア群に属す細菌*Wolbachia*が節足動物の生殖を様々な方法で操作することで注目されている. そこで, アワノメイガに性比異常を引き起こす細菌も*Wolbachia*である可能性を調べるため, PCRアッセイにより*Wolbachia*感染をチェックした. 性比異常を示した12頭に感染が認められた. それ以外の79頭のうち67頭に関しては, 1頭を除く66頭は感染していなかった. *Wolbachia*感染がメスに偏った性比と強くリンクしていることから, *Wolbachia*の感染がメス化の原因

因子であることが示された.

*Wolbachia*感染によるメス化は, これまで甲殻類(等脚目)のみで知られていた. そこで, *ftsZ*遺伝子と*wsp*遺伝子の塩基配列に基づいて*Wolbachia*の系統関係を調べた. どちらの遺伝子についてもアワノメイガに感染している*Wolbachia*の系統は等脚目に感染しているそれとは姉妹関係は認められなかった. また, 昆虫の性決定にホルモンは関与していないとされているのに対し甲殻類の性決定にはホルモンが関与しているなど, 性決定システムは両者で大きく異なっているため, 等脚目におけるメス化現象とアワノメイガにおけるそれは全く異なると考えられた.

アズキノメイガにおける異なる要因による2種類の性比異常

アワノメイガの近縁種アズキノメイガでも同様の性比異常現象が生じているのかを調べるために, 関東から東北にかけて6地点でアズキノメイガのメス成虫372頭を採集し産卵させた. 31頭がほぼメスのみを産んだ. そのうち17頭は*Wolbachia*に感染しており(SR-w^+), 14頭は感染していなかった(SR-w^-).

SR-w^+形質は母系遺伝し, 抗生物質処理により次世代はオスのみになった. この結果は, アワノメイガの場合と同じようにSR-w^+形質が*Wolbachia*感染による遺伝的オスのメス化であることを示している. また, アズキノメイガに感染していた*Wolbachia*の*wsp*遺伝子と*ftsZ*遺伝子の塩基配列は, アワノメイガに感染していた*Wolbachia*と同一であったことから, これらは同じ系統の*Wolbachia*であることが推察された.

一方, SR-w^-形質においてメスに偏った性比を生じるメカニズムはオス殺し, 産雌性単為生殖, メス化のいずれでもないことが示された. 残る可能性として, 卵の減数分裂時における性染色体の不平等分離(マイオティックドライブ)が考えられる. 性染色体構成がZW(♀)／ZZ(♂)またはZO(♀)／ZZ(♂)である鱗翅目昆虫では, メスにおける減数分裂の際にZ染色体が選択的に極体に移動させられることにより性比の偏りが達成される. 今までに, 動物のメスにおける減数分裂の際に染色体が不平等分離することにより性比の偏りが起こる例は知られていなかった. SR-w^-形質は不完全ながら母性遺伝したことから, 原因は細胞質因子かまたはW染色体であると示唆された. 性比の偏りの垂直伝播はときおり失敗したが, 世代を重ねる間に, メスに偏った性比に復帰することがあった. この現象はマイオティックドライブを抑制する核の因子によって説明できる. 今までに昆虫で知られている細胞質因子による性比の偏りのほとんどは細菌が原因で生じていた. しかし, アズキノメイガにおけるSR-w^-形質の原因因子は, PCRと抗生物質処理の結果から細菌ではないことが示

された.

アワノメイガ種群のその他の種における*Wolbachia*感染とメスに偏った性比

東日本から北海道にかけて採集したオナモミノメイガ, フキノメイガ, ゴボウノメイガ, およびロシア南西部で採集したヨーロッパアワノメイガ(いずれもメス個体)の*Wolbachia*感染および次世代の性比を家族ごとに調べた.

オナモミノメイガ24頭中3頭が*Wolbachia*に感染しており, これらはメスのみを産んだ. そのうち2頭に由来する系統で, メスに偏った性比の母系遺伝が確かめられた. フキノメイガ19頭中1頭が*Wolbachia*に感染しており, この個体はメスのみを産んだ. アワノメイガ, アズキノメイガ, オナモミノメイガ, フキノメイガの4種に感染している*Wolbachia*の*wsp*遺伝子と*ftsZ*遺伝子の塩基配列が同一であったことから, これら4種は同じ系統の*Wolbachia*に感染している可能性が高い. 感染したオナモミノメイガ, フキノメイガでのメスに偏った性比のメカニズムは追求できなかったが, おそらくメス化によるものと推測される.

ゴボウノメイガ10頭およびヨーロッパアワノメイガ55頭には, *Wolbachia*感染は認められなかった. しかし, ゴボウノメイガについては1頭がメスのみを産んだ. ヨーロッパアワノメイガについては, さらに27頭のメス成虫を用いてPCRを行ったが, やはり*Wolbachia*の感染は検出されなかった. ゴボウノメイガのメスに偏った性比は, 母系遺伝したこと, テトラサイクリンの効果が見られなかったこと, および細菌特異的PCRの結果がネガティブであったことから, アズキノメイガにおけるSR-w$^-$と同様のメカニズムに基づくものであることが示唆された.

*Wolbachia*感染アズキノメイガに対するテトラサイクリン投与によって起こる性モザイク形成

アズキノメイガの*Wolbachia*感染メス成虫にテトラサイクリンを投与したところ, 次世代にメスとオスのほか多くの性モザイク個体が出現した. このことから*Wolbachia*がアズキノメイガの個々の細胞の性決定に関与していることがうかがえる. テトラサイクリン投与後産卵までの日数が長いものほどオスの出現する割合が多く, またモザイク個体もオス形質をより多く持っていた. 性モザイクが生じた原因は, テトラサイクリン投与により卵母細胞内の*Wolbachia*の量が減り, 性決定カスケードの開始点である胚期に, *Wolbachia*の細胞内密度にばらつきが生じ, メスに分化するように決定された細

胞とオスに分化するように決定された細胞ができたと推察された.

つぎに, アズキノメイガの*Wolbachia*感染幼虫に様々な濃度のテトラサイクリンを投与し, 次世代の性比への影響を調査した. 高濃度を投与した場合, オスばかりが生じたが, 低濃度を投与した場合はメスばかりを生じた. 中間の濃度ではオス個体とメス個体が現れたが, 性モザイクは現れなかった. 幼虫にテトラサイクリンを投与した場合には, 投与後テトラサイクリンにさらされない期間が長いため, その間に*Wolbachia*が完全にメス化できる密度にまで十分に増殖できた卵と, ほとんど*Wolbachia*が増殖できない卵とが, テトラサイクリン濃度依存的に二極化していることが示唆された.

本研究で見つかった2種類のメスに偏る性比異常現象はいずれも母性遺伝するものである. 性比をメスに偏らせることは, 母性遺伝する因子にとって有利に作用すると考えられる. メスに偏った性比が, 実際に母性遺伝する因子(特に細胞質因子)によって引き起こされる例は多くの節足動物で見つかっている. メカニズムとして, (1)オスのみが死亡する, いわゆる「オス殺し」, (2)処女メスがメスを産む「産雌性単為生殖」, (3)遺伝的なオスがメス化する, の3種類が知られていた. 鱗翅目昆虫でも, メスに偏った性比は数多く報告されているが, メカニズムが分かっているケースのほとんどはオス殺しであり, 原因因子としては*Wolbachia*とスピロプラズマが報告されている. 本研究により, アワノメイガ種群の4種においておそらく同一系統の*Wolbachia*感染による遺伝的なオスのメス化が起きていることが示された. また, 本研究では, *Wolbachia*感染系統のアズキノメイガにおいては, *Wolbachia*の不完全除去により, 性モザイク個体が形成されることを示した. さらにアズキノメイガでは, *Wolbachia*感染によるメス化以外に, 原因因子は不明であるが, 性染色体のマイオティックドライブにより性比異常が生じていることが示唆された. 以上, 本研究で得られた知見は, 農業害虫を数多く含む鱗翅目昆虫の性決定機構の解明に向け新たな切り口を与えるものと考えられる.