A new cytogenetic mechanism for bacterial endosymbiont-induced parthenogenesis in Hymenoptera

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Vertically transmitted endosymbiotic bacteria, such as \textit{Wolbachia}, \textit{Cardinium} and \textit{Rickettsia}, modify host reproduction in several ways to facilitate their own spread. One such modification results in parthenogenesis induction, where males, which are unable to transmit the bacteria, are not produced. In Hymenoptera, the mechanism of diploidization due to \textit{Wolbachia} infection, known as gamete duplication, is a post-meiotic modification. During gamete duplication, the meiotic mechanism is normal, but in the first mitosis the anaphase is aborted. The two haploid sets of chromosomes do not separate and thus result in a single nucleus containing two identical sets of haploid chromosomes. Here, we outline an alternative cytogenetic mechanism for bacterial endosymbiont-induced parthenogenesis in Hymenoptera. During female gamete formation in \textit{Rickettsia}-infected \textit{Neochrysocharis formosa} (Westwood) parasitoids, meiotic cells undergo only a single equational division followed by the expulsion of a single polar body. This absence of meiotic recombination and reduction corresponds well with a non-segregation pattern in the offspring of heterozygous females. We conclude that diploidy in \textit{N. formosa} is maintained through a functionally apomictic cloning mechanism that differs entirely from the mechanism induced by \textit{Wolbachia}.

Keywords: parthenogenesis; apomixis; cytogenetics; \textit{Neochrysocharis formosa}; \textit{Rickettsia}

1. INTRODUCTION

Some vertically transmitted bacteria increase in frequency by manipulating host reproduction in ways that enhance their own transmission, but do not necessarily benefit their host (for review see Bourtis & Miller 2003). Such manipulations range from cytoplasmic incompatibility, male killing and feminization, to induction of thelytokous parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis. The latter appears to be most common in the order Hymenoptera, insects that normally reproduce by parthenogenesis.

Cytogenetic mechanisms involving thelytokous diploidization can be divided into meiotic, considered most common (Suomalainen et al. 1987), and post-meiotic modifications (Stouthamer 1997). A schematic of different mechanisms in thelytokous diploidization is given in figure 1: (a) apomictic parthenogenesis (e.g. Vavra et al. 2004), (b) automictic terminal fusion (e.g. Rössler & DeBach 1973), (b) automictic central fusion (e.g. Belshaw & Quicke 2003), (b) automictic random fusion (e.g. Lampert et al. 2007) and (b) automictic gamete duplication (e.g. Pannebakker et al. 2004). In most species, meiosis is suppressed and the division has a mitotic character. However, the mechanism of diploidization in Hymenoptera infected with endosymbiotic bacteria, especially \textit{Wolbachia}, is a post-meiotic modification. The restoration of the diploid number takes place in the first mitotic division when in the anaphase two haploid sets of chromosomes fail to separate and result in a single diploid nucleus containing two identical sets of chromosomes. This diploidization process is called gamete duplication and is shown in figure 1b(v).

The eulophid parasitoid wasp \textit{Neochrysocharis formosa} (Westwood) is one of the most important natural enemies of leafminers, \textit{Liriomyza trifolii} (Burgess) and \textit{Liriomyza sativae} Blanchard, in Japan (Saito et al. 1996; Tokumaru & Abe 2006). \textit{Neochrysocharis formosa} has thelytokous and arthropotokous strains in the field (Arakaki & Kinjo 2006). Thelytokous reproduction in \textit{N. formosa} is known to be induced by \textit{Rickettsia} (Hagimori et al. 2006). To date, the cytological mechanism of diploidization by \textit{Rickettsia} has not been reported.

Here, we compare \textit{Rickettsia}-induced thelytoky with that of uninfected arthropotokous individuals using genetic analysis of microsatellite markers and chromosome behaviour analysis in young eggs. We report a new cytogenetic mechanism for symbiont-induced thelytoky in Hymenoptera.

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2. MATERIAL AND METHODS

(a) Insect collection

Rickettsia-infected thelytokous lines were established from a stock culture originating from the western districts of Japan at Sumitomo Chemical Co. Ltd and from the collections at Fukuyama, Hiroshima, Japan, in autumn 2004. An uninfected arrhenotokous line was established from collections in Nagaokakyo, Kyoto, Japan, in autumn 2005. All cultures were maintained on mining third-instar larvae of *L. sativae*.

(b) Development of microsatellite primers

Methods used for the isolation of microsatellites were based on the 5' anchored polymerase chain reaction (PCR) technique (Fisher et al. 1996). DNA was extracted from a Sumitomo thelytokous line individual by crushing with a clean plastic rod in 30 μl of a Tris–EDTA buffer (5N NaCl, 0.5 mM EDTA (pH 8.0) and 1 M Tris–HCl (pH 8.0)) incubated with 2 μl of proteinase K (0.5 mg ml⁻¹) at 37°C for 0.5 h. The homogenate was boiled at 99.9°C for 3 min to inactivate the proteinase K and was used as a template for PCR.

Figure 1. Different cytological mechanisms in apomictic and automictic parthenogenesis and their impact on the transition to homozygosity of a heterozygous locus whether crossing over between the locus and the centromere occurs or not (modified from Pearcy et al. 2006). Large and medium circles represent nuclei. Horizontal lines represent chromatids, with small circles showing the location of the centromere and letters representing alleles at a given locus. The parent is heterozygous (Aa) and the parthenogenesis causes inbreeding when its progeny becomes homozygote (AA or aa). (a(i)) Typical apomixis, (a(ii)) apomixis in Vavre et al. (2004), (b(i)) automixis, (b(ii)) automictic terminal fusion, (b(iii)) automictic central fusion, (b(iv)) automictic random fusion and (b(v)) automictic gamete duplication.

Initial reaction was performed using 33 μl PCRs: 1 μl template DNA; 1.5 U of Taq polymerase (PE Applied Biosystems, Tokyo, Japan); 0.66 μl of 10 mM dNTPs; 2.6 μl of 10 pmol μl⁻¹ PCT4 primer (5'-KKVRVRV(CT)₆-3'; Fisher et al. 1996); 3.3 μl of 10×PCR buffer with MgCl₂ and 26.2 μl of sterile water. PCR amplification was carried out in an ABI thermocycler (PE Applied Biosystems PCR System 9700, PE Applied Biosystems) with the following programme: an initial denaturing step at 92°C for 1 min; 30 cycles of 92°C for 1 min; an annealing step at 55°C for 1 min, 72°C for 1 min 30 s; and a final extension step of 72°C for 1 min 30 s. The PCR included a negative control (sterile water instead of DNA) to detect contamination. PCR products were resolved on a 2% agarose gel, stained with ethidium bromide and visualized under an UV transilluminator.

The PCR products were cloned according to the p-GEMT Easy Vector system protocol (Promega, Tokyo, Japan). Based on colony PCR analysis, recombinant clones larger than 400 bp were isolated, purified and directly sequenced using M13M4 and M13RV universal primers. A dye-terminator-labelled cycle sequencing reaction was conducted with BigDYE DNA Sequencing Kit v. 3.1 (PE Applied Biosystems). The temperature profile was 1 min at 96°C followed by 25 cycles of 10 s at 96°C, 5 s at 50°C and 4 min at 60°C. Reaction products were analysed using an ABI PRISM 3130xl Genetic Analyzer (PE Applied Biosystems). Partial sequences were edited and assembled with the Contig Express program in VECTOR NTI ADVANCE v. 10.1 (Invitrogen InforMax, Frederick, MD, USA).

For the detection of microsatellites in sequences, we used a TROLL program (Martins et al. 2006). Each sequence contained at least two microsatellite repeats on each end of the insert. Some microsatellite clones contained an additional internal microsatellite, implying microsatellites clustered in some genomic regions. Primers were designed to amplify regions containing microsatellite repeats using primer 3 (Rozen & Skaltsky 2000). PIG-tail was attached at the 5’ end of the reverse primer to enhance the 3’ overhang and to avoid typing error due to variability in non-template addition of nucleotides at the 3’ end of the PCR products (Brownstein et al. 1996). The forward primer was dye-labelled with NED (PE Applied Biosystems). Resulting forward and reverse primers were named NFTH9-F (5’-AAC TTC TCG CCG CTC ATT TA-3’) and NFTH9-R (5’-GTT TAC GAT CTC CCG TGC TTA TAA-3’). The microsatellite sequence has been deposited in GenBank (accession number AB428375).

(c) Allele segregation and mode of parthenogenesis in N. formosa
We treated four adult female N. formosa, two from the Sumito thletytokous line and the other two from the Hiroshima thletytokous line, with antibiotic tetracycline hydrochloride; provided them with hosts and collected their progeny, as per Hagimori et al. (2006). The segregation of the microsatellite markers was tested in the male offspring of these antibiotic-treated females. In addition, eight adult females from the Hiroshima thletytokous line without antibiotic treatment were allowed to oviposit into hosts and their progeny were collected. DNA was extracted from all progeny and genotyped for the microsatellite locus NFTH9.

To assess allele segregation under thletytokous parthenogenesis, we determined the proportion of homozygous female offspring produced by heterozygous mothers, R. We compared R-values with theoretical expectations (r) for five different modes of thletytokous parthenogenesis (as a convention, we use lower case letters to denote parameters and capital letters for the corresponding estimators): apomixis (r=0); automixis with gamete duplication (r=1); terminal fusion (r=1/3-1); fusion of two products of the first meiotic division, here referred to as random fusion (r=1/3); and central fusion (r=0-1/3). Fisher’s exact tests were used to determine which mode of parthenogenesis was consistent with the observed rate of transition to homozygosity for the locus. When r comprised a range of values, the value closest to the observed R was used for the test. The statistical software package R (R Development Core Team 2007, http://www.R-project.org) was used for all statistical analyses.

PCR amplifications were performed using an ABI thermocycler (PE Applied Biosystems PCR System 9700, PE Applied Biosystems) and each PCR consisted of 33 μl: 1 μl template DNA; 1.5 U of Taq polymerase (Applied Biosystems, Foster City, CA, USA); 0.66 μl of 10 mM dNTPs; each 1.3 μl of 10 pmol μl⁻¹ NFT99-F and NFT99-R primers; 3.3 μl of 10×PCR buffer with MgCl₂ and 23.6 μl of sterile water. Cycling parameters were as follows: 92°C for 1 min; 30 cycles of 1 min denaturation at 92°C; 1 min annealing at 55°C; and 1 min 30 s extension at 72°C. The final extension was conducted at 72°C for 1 min 30 s. For genotyping, the PCR products were electrophoresed along with GeneScan LIZ 500 internal size standard on an ABI PRISM 3130xl DNA sequencer (Applied Biosystems). Allele sizes were assigned against the internal size standard and individuals were genotyped using GENOTYPE v. 4.0 software (Applied Biosystems).

(d) Egg collection and cytological techniques
For investigation of meiotic stages in eggs prior to oviposition, we provided arrenotokous and thletytokous adult N. formosa females with L. sativae larvae to stimulate egg development in their ovaries. Eggs were dissected from N. formosa females on a microscope depression slide in a drop of Drosophila Ringer’s solution (18 mM KCl, 46 mM NaCl, 3 mM CaCl₂, 10 mM Tris/HCl (pH 7.2)). Most of the solution was removed by absorption and the eggs were transferred to a glass vial containing Carnoy’s fixative (99.5% ethanol : chloroform : acetic acid = 6 : 5 : 2) for at least 1 day. The eggs were then transferred to a slide and stained with DAPI (4’,6-diamidino-2-phenylindole; 1.5 μg ml⁻¹) in Vectashield (Vector Laboratories, Burlingame, CA, USA) and covered with a coverslip. They were stored at room temperature in the dark for at least 1 day to allow sufficient stain penetration into them. They were squashed prior to examination under an Olympus VANOX AH2-FL microscope (Olympus, Tokyo, Japan), equipped with epifluorescence at 200×, 400× or 1000× magnification. Images were collected using an HCC-3800, a 3-CCD colour camera from Flowl Co. Ltd (Tokyo, Japan).

To observe meiotic and mitotic stages in more mature eggs, three N. formosa females were allowed to oviposit into a third-instar L. sativae larvae for 30 min. Fly larvae containing parasitoid eggs were kept for each of 30 min time intervals from 0 to 120 min after oviposition. The eggs were dissected from hosts, fixed and stained, as described above. Over 70 eggs were examined for each N. formosa strain.
Table 1. Genotype of 14 *N. formosa* females and their parthenogenetically produced progeny for the microsatellite locus NFTH9. (Alleles are indicated by their length in base pairs (bp), with males being haploid and therefore carrying only one allele per locus. Females 1–6 were treated with tetracycline, while the others were not. Numbers in parentheses indicate the number of progeny with that genotype.)*

<table>
<thead>
<tr>
<th>Female</th>
<th>Tetracycline treatment</th>
<th>Parental genotype (bp)</th>
<th>Progeny genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sumitomo</td>
<td>yes</td>
<td>219/234</td>
<td>219 (22) 234 (14)</td>
</tr>
<tr>
<td>Hiroshima</td>
<td>yes</td>
<td>219/224</td>
<td>219 (10) 224 (9)</td>
</tr>
<tr>
<td>Hiroshima</td>
<td>no</td>
<td>219/224</td>
<td>219/224 (84)</td>
</tr>
</tbody>
</table>

*aThe segregation for the two alleles in the males does not differ significantly from the expected 1 : 1 (p > 0.05).*

Table 2. Observed rates of transition to homozygosity during parthenogenesis and consistency with different modes of parthenogenesis. Nt, number of offspring from a heterozygous mother; No, number of transitions to homozygosity; *R*, observed rate of transition to homozygosity; *r*, expected rate (or range of rates) of transition to homozygosity (see table 1 in Pearcy et al. 2006). Fisher’s exact tests of consistency of *R*-values with *r*; n.s., not significant; **highly significant (*p < 0.001.* When *r* is a range, the test was performed considering the *r* closed to *R* within the range.

<table>
<thead>
<tr>
<th>Nt</th>
<th>No</th>
<th><em>R</em></th>
<th><strong>Gameze duplication</strong></th>
<th><strong>Terminal fusion</strong></th>
<th><strong>Central fusion</strong></th>
<th><strong>Random fusion</strong></th>
<th><strong>Apomixy</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>98</td>
<td>0</td>
<td>0.00</td>
<td>***</td>
<td>***</td>
<td>n.s.</td>
<td>***</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

3. RESULTS

(a) Allele segregation

Infected females were all heterozygous for the NFTH9 locus. The male offspring produced by these females showed the expected segregation with each male carrying a single allele, and the segregation of the two alleles in the male population does not differ significantly from 0.5 (table 1). The proportion of homoygous female offspring produced by 10 heterozygous infected mothers (*R*) was 0.00 (*n* = 98) (tables 1 and 2). The value was significantly different for a locus from those expected under automixis with terminal fusion, gamete duplication or random fusion. By contrast, it was not different from the values expected under apomixis and autamixis of the egg. Subsequently, we did cytogenetic observations to distinguish between these two potential outcomes. Under central fusion, the expectation is the presence of two polar bodies at the end of meiosis, while in apomixes, at best a single polar body will be present.

(b) Cytogenetic observation of an arrhenotokous strain

Cytological analysis of arrhenotokous adult females revealed that mature eggs in their ovaries are in first meiotic metaphase (figure 2a). The chromosomes at this stage are packed together and it is impossible to count them with certainty (figure 2a). The first division is reductional, resulting in a first polar body with five chromosomes (dyads) on the periphery and a second set of about five chromosomes (dyads) of the second meiotic division pre-destined to form the pronucleus, and the second polar body, somewhat inward from the surface (figure 2b). This second set divides parallel to the periphery and results in two groups of five chromosomes, while the first polar body is delayed at interphase (figure 2c). The second division results in one set of five chromosomes forming the second polar body close to the two sets of the first polar body, and another set that forms the female pronucleus (figure 2d), which takes on an interphase appearance and migrates away from the polar bodies in no distinct direction, remaining in the anterior region (figure 2e). The pronucleus starts with synchronous mitotic divisions parallel to the surface, typically from 2 h onwards after oviposition (figure 2f). Chromosomes in the eggs could not be captured photographically in one plane due to their small size and the high density of the yolk. In 90 to 120 min old eggs, the number of chromosomes is easier to count when they have a haploid number (figure 2g(i)(ii)).

(c) Cytogenetic observation of a thelytokous strain

Cytological analysis of thelytokous adult females revealed that mature eggs in their ovaries are also in the first meiotic metaphase (figure 3a). In newly laid eggs, we observed condensed chromosomes at first metaphase (figure 3b). The clumped chromosome mass did not allow us to study chromosome pairing. In 0 to 30 min old eggs, the nuclei decondense and orient towards the equatorial plate (figure 3c). Although individual chromosomes were difficult to assess, we were able to count approximately 10 chromosomes in the complement, a diploid chromosome number also known from arrhenotokous strains.

In the metaphase of meiosis II, one set of chromosomes (figure 3d(i)(ii)) lies close to the periphery of the egg and forms the first polar body, while the other set, with approximately 10 discernable chromosomes, lies in the central part of the egg (figure 3d(iii)). After a short lasting interphase, the central nucleus went through a mitotic division with 10 doubled chromosomes at metaphase and a set of chromatids at anaphase (figure 3e(i)—(iii)), indicating that meiosis is replaced by a single equational division followed by the expulsion of a single polar body. We observed more than 70 eggs, and none of the two nuclei that entered interphase (figure 3e(i)—(iii)) resulted in a second polar body. Instead, they again divided mitotically giving rise to four nuclei (figure 3f(i)—(v)).
We never observed cell complements with the haploid chromosome number \((n=5)\), indicating that meiosis is exclusively non-reductional. This cytological mechanism is similar to that of a non-bacteria-induced thelytoky in the parasitoid wasp *Trichogramma cacoeciae* and is also known as apomictic cloning (figure 1a(ii); Vavre et al. 2004).

### 4. DISCUSSION

Our results suggest that meiosis in the *N. formosa* thelytokous strain is achiasmatic, with only a single equational division and the expulsion of a single polar body, and heterozygous females do not segregate for microsatellite markers in their offspring. This mechanism...
is typically apomictic and differs from parthenogenesis-induced *Wolbachia* systems in parasitoids where diploidy is restored through automixis resulting in homozygous females in a single generation (Stille & Dävring 1980; Stouthamer & Kazmer 1994; Gottlieb et al. 2002; Pannebakker et al. 2004). This is, therefore, a new cyto- genetic mechanism of parthenogenesis induced by endosymbiotic bacteria in Hymenoptera. In mites, Weeks & Breeuwer (2001) reported that the mechanism of parthenogenesis in *Bryobia practica* would be functionally apomictic and not gamete duplication, based on the data that all of the progeny produced by heterozygous mothers maintained heterozygosity in all the three microsatellite loci they examined. Unfortunately, their experiments used only molecular techniques and they never observed chromosomes, so we could not judge whether the mechanism is apomixis or automictic parthenogenesis with central fusion. We suggest a future comparison of their mechanism with that of thelytokous *N. formosa* in detail.

In all the cases established within the Hymenoptera to date, bacterial infection by *Wolbachia* results in gamete duplication for the restoration of diploidy (Stille & Dävring 1980; Stouthamer & Kazmer 1994; Gottlieb et al. 2002; Pannebakker et al. 2004). This mechanism has been thought to be functionally restricted to haplodiploid systems (Stouthamer 1997; Stouthamer et al. 1999), because many species with a haplodiploid sex determination routinely inbreed. Consequently, the transition from normal sexual reproduction to bacterium-induced parthenogenesis does not result in a strong inbreeding depression as would be expected for species that commonly outbreed. However, the mechanism of the *Rickettsia*-induced parthenogenesis described here maintains heterozygosity, and consequently takes away one of the barriers involved in the transition from sexual to thelytokous reproduction for species that commonly outbreed, such as most diplodiploid species. It is interesting to note that apomictic parthenogenesis is the most common form of parthenogenesis within diplodiploid arthropods (Suomalainen et al. 1987) and has been found in some Hymenoptera where parthenogenesis is not associated with microbial infection (Vavre et al. 2004). Our results suggest that in the future we may detect the presence of parthenogenesis-inducing symbionts in diplodiploid species.

Within the insect order Hymenoptera to which the parasitoids, wasps, bees, ants and sawflies belong, two different sex determination mechanisms are known, one the so-called complementary sex determination (CSD; Cook 1993) and another referred to as the 'chalcidoid' system (Luck et al. 1993). Wasps that have the CSD system generally become diploid males that are sterile or die in the larval stage. It is interesting to note that although there are many cases of thelytoky in Hymenoptera with CSD, no cases of *Wolbachia*-induced thelytoky have been found. A survey of thelytokous Hymenoptera belonging to the families with CSD may result in additional cases of *Rickettsia*-induced thelytoky.

This cyrogenetic mechanism may have important consequences for the infected populations. Under gamete duplication, there is a constant selection against recessive lethal mutations in genes expressed in females; however, the apomictic mechanism found here should allow the accumulation of recessive mutants in the genome of infected wasps. Such recessive lethals should have little effect on the fitness of the females since the mutations will not be expressed; however feeding antibiotics to such thelytokous females will result in the death of their recessive inherited haploid offspring. Consequently, we expect that a complete reproductive dependence of the host on its apomixis-inducing bacterial symbiont will evolve rapidly.

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