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Feminizing *Wolbachia* in *Zyginidia pullula* (Insecta, Hemiptera), a leafhopper with an XX/X0 sex-determination system

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Zyginidia pullula is a grass-dwelling leafhopper characterized by a bisexual reproduction mode. In this species, some females collected in Northern Italy, when mated with males, gave origin to an exclusively female brood. Here, we demonstrated that in these lineages an infection by a new strain of *Wolbachia pipientis*—designated as *w*Zygpul—was detected by amplifying and sequencing the *wsp* and 16S rRNA genes. About half of the female progeny were characterized by intersexual phenotypes, i.e. showing upper pygofer appendages, a typical male secondary sexual feature. The karyological analysis proved that while phenotypically normal females had a female genotype, those with upper pygofer appendages had a male genotype and were thus feminized males.

The complete removal of *W. pipientis* after tetracycline treatment of morphologically normal females, and the consequent re-appearance of males in the brood, permitted us to connect the feminizing effect with the presence of the bacterium. This is the first case of feminization by *W. pipientis* in an XX/X0 sex-determination system, and is the second case reported in insects.

Keywords: *Zyginidia pullula*; *Wolbachia pipientis*; 16S rRNA; *wsp*; feminization; XX/X0 sex-determination system

1. INTRODUCTION

Zyginidia pullula (Boheman; Hemiptera, Cicadellidae) is a widespread central European leafhopper about 3 mm long. It is a multivoltine species, with bisexual reproduction, able to produce up to four generations a year in Northern Italy, and feeds on grasses and maize crops as a mesophyll sucker (Verzé & Mazzoglio 1994).

In laboratory rearings, anomalous cases of isofemale broods were reported for the first time in 1984, using single virgin females collected in the field, and mated only once with a male (P. J. Mazzoglio 1989, unpublished data). In 1993, some other females collected in different localities produced only daughters (Verzé & Mazzoglio 1994). Later, an infection of *Wolbachia pipientis* was detected in *Z. pullula* by molecular methods (Negri *et al.* 2002). This Gram-negative α-proteobacterium belonging to Rickettsiales is probably one of the best examples of a 'reproductive parasite' infecting a wide range of arthropods, where it is maternally transmitted through the egg cytoplasm, and is therefore strictly associated with the gonads, but might also be contained in somatic tissues (Werren & O'Neill 1997; Cheng *et al.* 2000). In fact, *W. pipientis* is known to induce various reproductive alterations in its hosts, including cytoplasmic incompatibility, thelytokous parthenogenesis, male-killing and feminization of genetic males (reviewed in Stouthamer *et al.* 1999). Feminization should not be considered unique to *Wolbachia*: other symbionts, such as bacteria unrelated to *W. pipientis* in the mite *Brevipalpus phoenicis*, and microsporidians in crustaceans, are able to induce male individuals to develop as females (Terry *et al.* 1997; Weeks *et al.* 2001). The mechanism involving feminization in the mite—where haploid genetic males reproduce as parthenogenetic females—is not yet known; conversely, some details are available on the *Wolbachia*-infected *Armadillidium vulgare*, where it seems that the bacterium acts by suppressing the androgenic gland, thus ensuring female development (LeGrand *et al.* 1987).

Until now, feminization by *W. pipientis* in insects has been demonstrated only for the butterfly *Eurema hecabe* where the sex-chromosome system is WZ/ZZ, and the females are the heterogametic sex (WZ; Hiroki *et al.* 2002). The authors showed that the antibiotic removal of *Wolbachia* from the thelygenic females causes the production of only male progeny, after coupling with males. In these infected females, the absence from somatic nuclei of the sex chromatin body (which derives from the W chromosome) leads to the conclusion that they are actually feminized ZZ males, which generate feminized male progeny.

Other cases of feminization induced by *W. pipientis* in insects, reported for the two Lepidoptera species *Ostrinia furnacalis* and *Ostrinia scapulalis*, have proved instead to be male-killing occurrences (Kageyama *et al.* 2002; Kageyama & Traut 2003).

On the basis of morphological, cytological and molecular evidence, in the present study we prove that,

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in the all-female populations of *Z. pullula*, *W. pipientis* feminizes the genetic males of the leafhopper. These feminized males maintain some male features, leading to the formation of phenotypic intersexes. This is the first case of feminization by *W. pipientis* found in an XX/X0 sex-determination system, and is the second case reported in insects.

2. MATERIAL AND METHODS

(a) *Sample collections and rearings*

One hundred and twenty-nine females of *Z. pullula* were collected on grasses in different localities of the Po Valley (North Italy) along a transect of over 450 km. Some of them ($n=31$) were reared individually in the laboratory inside climatic chambers (23 ± 1 °C, 60 ± 10% relative humidity) on potted maize plantlets. After the oviposition and hatching of the first eggs, the mother was moved to another isolator on new maize where it carried on laying eggs. After the emergence of the first F_1 adults, they were sexed and the isofemale lines were identified. Some females belonging to these lines were reared individually so as to obtain F_2 isofemale broods. The F_1 broods with a normal 1 : 1 sex ratio were kept, so as to have males to be used as partners in breedings.

The other collected females ($n=98$) were preserved in absolute alcohol and stored at −20 °C.

(b) *Morphological and karyological analysis*

Before extracting DNA, specimens were examined under a stereomicroscope. The pygofer was dissected and prepared on a microscope slide in a lactic acid droplet, while the rest of the insect was preserved in absolute alcohol and stored at −20 °C.

For scanning electron microscopy (SEM), some individuals were dehydrated in progressive ethanol solutions up to absolute ethanol, then they were air-dried and gold-coated by sputtering. Observations were carried out with a Cambridge 200 scanning electron microscope.

Gonads of newly emerged females from laboratory rearings were dissected in Ringer solution and treated by the Cokendolpher method (Cokendolpher & Brown 1985).

(c) *DNA extraction, amplification, cloning and sequencing*

Total DNA was extracted from single individuals previously deprived of the pygofer using 80 µl of InstaGene Matrix (BioRad) following the manufacturer's instructions. Six microlitres of the solution containing total DNA were used as template for PCR reactions. Amplifications were performed using the primers V1–V6 (targeted for the *Wolbachia* 16S rRNA gene; O'Neill *et al.* 1992) and 81F–691R (targeted for the *Wolbachia* surface protein (*wsp*) gene; Braig *et al.* 1998), with the PCR conditions described by the authors. DNA extracted from a parthenogenetic and *Wolbachia*-infected population of the springtail *Folsomia candida* Willem was used in the PCR reactions as a positive control (Frati *et al.* 2004). To verify the presence of other bacteria co-infecting *Z. pullula*, universal primers for eubacterial 16S rRNA (O'Neill *et al.* 1992; Rousset *et al.* 1992) were used: the 'forward' primer 5′-GCTTAACACATGCAAG-3′ (position 45 in *Escherichia coli*) and the 'reverse' primer 5′-CCATTGTAG CACGTGT-3′ (position 1242 in *E. coli*).

Purified PCR products were sequenced directly on a CEQ 8000 Genetic Analysis System (Beckman Coulter). In addition, a number of PCR products of V1–V6 and 81F–691R primers were cloned using the TOPO TA cloning kit (Invitrogen) and fully sequenced on both strands using universal M13 forward and reverse primers.

(d) *Phylogenetic analysis*

Sequences were edited with Chromas v. 1.45 (Technelysium Pty Ltd, Australia) and aligned using the ClustalW algorithm (Thomson *et al.* 1994).

Phylogeny of 16S rRNA sequences was generated with a distance matrix method (neighbour-joining; Saitou & Nei 1987), using Paup* v. 4.0 (Swofford 2000). The Modeltest program (Posada & Crandall 1998) output was used to choose the distance method (general time reversible model), with consideration of the gamma shape parameter. Bootstrap analysis was carried out using 1000 replicates.

An analogous approach was adopted for generating phylogeny of *wsp* sequences. In this case, the Hasegawa–Kishono–Yano model was chosen, with the consideration of the proportion of invariable sites and of the gamma shape parameter.

The 16S rRNA and *wsp* sequences from this study have been deposited in GenBank under the accession numbers DQ203854 and DQ203853, respectively.

For generating the 16S rRNA dataset, sequences belonging to all the known *Wolbachia* supergroups were obtained from Lo *et al.* (2002) and Czarnetzki & Tebbe (2004). Moreover, sequences of *Wolbachia* strains found in Homoptera were downloaded from GenBank. A Blastn search (Altschul *et al.* 1990) was carried out with the new *Wolbachia* sequence from *Z. pullula*, and sequences with the highest scores (M84686; AF390864) were included in the phylogenetic analysis.

The *wsp* dataset was built considering all *Wolbachia* strains found in Hemiptera that have been already published (Zhou *et al.* 1998; Jeyaprakash & Hoy 2000; Mitsuhashi *et al.* 2002; Kikuchi & Fukatsu 2003; Kittayapong *et al.* 2003; Nirgianaki *et al.* 2003). Also, in this case, a Blastn search was performed and sequences with the highest scores were included in the phylogenetic analysis.

(e) *Tetracycline treatment*

Ten third–fourth instar nymphs of one of the all-female F_2 broods were fed an artificial diet: five of them with a mixture of distilled water and 2% sucrose containing tetracycline (20 mg per 100 g H_2O), the remaining five as controls with only distilled water and 2% sucrose. The treatment lasted for 6 hours and was repeated for 6 days. On the other days, the leafhoppers were placed individually on a maize plantlet leaf in the dark in a climatic chamber.

At the end of the treatment when the specimens emerged, the adults were reared in individual cages together with a partner from *Wolbachia*-free rearings for a reproductive test. Similar tests were also conducted on their F_1 brood.

After the emergence of the first F_1 adults, they were sexed, the treated mothers were killed and their pygofer was dissected for morphological analysis.

3. RESULTS

(a) *Molecular analyses*

Of 129 females collected in the field and tested for the presence of *W. pipientis*, 44 (about 34%) were positive to the amplification.

Of 31 reared females, 13 gave an exclusively female brood. Molecular analyses were performed on 20 F_1 adults per female line, revealing infection by *W. pipientis* in all individuals of the isofemale lines. On the contrary, females belonging to lines with a 1 : 1 sex-ratio trait were negative to the *Wolbachia* amplification.

A 16S rRNA gene fragment (900 base pairs) and a *wsp* fragment (610 base pairs) were both amplified by means of specific primers, V1–V6 and 81F–691R, respectively.

The screened individuals belonging to the all-female lines also gave positive amplifications with the universal primers for bacterial 16S rRNA gene. The bands obtained in 10 individuals were sequenced, and in all of them we found only one sequence which was identical in all the individuals tested: this was 1077 bases long, corresponding to a fragment of the gene encoding for the 16S rRNA subunit of *W. pipientis*. This sequence was also congruent with those amplified by the specific primers V1–V6.

In order to verify the presence of multiple bacterial strains coexisting in the same host, the *wsp* PCR products of five females per line were cloned into plasmid vectors, and 10 recombinant plasmids per female were sequenced. The amplicons of the remaining 15 females per line were sequenced directly.

All the examined specimens showed identical nucleotide sequences, suggesting that all tested *Z. pullula* were infected by a single *Wolbachia* strain. A BLASTn search suggested that the nucleotide sequences of *Wolbachia* found in *Z. pullula* might belong to a new strain.

To better understand the phylogenetic relationship of the 16S rRNA gene sequence of *Wolbachia* from *Z. pullula*, a 31 sequence dataset was built, and a neighbour-joining unrooted tree was generated by the PAUP program. The new *Wolbachia* sequence clusters into the B supergroup (see electronic supplementary material, figure S1). Within this group, the *Nasonia giraulti* strain is basal and shows the most differentiated sequence, while the *Z. pullula* strain is grouped together with other *Wolbachia* strains found in Hemiptera. In this cluster, only small differences are recognizable among diverse strains, which thus lacks a well-structured phylogeny.

The *wsp* sequence was analysed to identify the phylogenetic position of the bacteria hosted by *Z. pullula* within the *Wolbachia* strains harboured by Hemiptera.

A dataset comprising 87 sequences, of 522 nucleotides each, was generated. In order to eliminate the saturated and divergent regions from the alignment, GBLOCKS software was used. This computer program is able to select blocks of conserved positions which must fulfil certain requirements with respect to the lack of large segments of contiguous non-conserved positions, lack or low density of gap positions and high conservation of flanking positions, making the final alignment more reproducible and more suitable for phylogenetic analysis (Castresana 2000).

After removing positions 80–104, the resulting alignment consisted of sequences of 496 base pairs, and the corresponding neighbour-joining unrooted tree is shown in figure 1.

As in previous studies, the subdivision of the *W. pipientis* *wsp* sequences into two supergroups (A and B) is clear; however, the excision of the variable region from the alignment seems to be responsible for a small but marked shift in the phyletic relationships of different strains inside the two supergroups. The *Z. pullula* strain belongs to the B supergroup, and it lies in the clade identified by the leafhopper *Cofana spectra*. In this cluster, *Wolbachia* causes cytoplasmatic incompatibility and sex-ratio distortion (Kondo *et al.* 2002; Vala *et al.* 2003), or unknown effects on the hosts (Mitsuhashi *et al.* 2002).

(b) *Morphological analysis*

The all-female progeny of the infected lines also consists of some individuals with typical male secondary sexual characteristics localized in the last abdominal segments, which is the region showing sexual dimorphism. Such peculiar females possess upper pygofer appendages, i.e. forked chitinous structures, homologous with what can be seen in males (figure 2*a*,*b*). Upper pygofer appendages do not exist in normal females (figure 2*c*).

A screening of the specimens bearing these characteristics (intersexes) was conducted on only one of the infected lines that was randomly selected for all further inspections: among 48 females screened, upper pygofer appendage were observed in 20 specimens.

These particular phenotypes were also found in the field, among the females collected from different sampling sites.

Upper pygofer appendages appeared well developed in some specimens (figure 2*a*), or were not completely developed; in some cases, one of the forked branches was entirely lacking or shorter than the other one (figure 2*d*), or the whole appendage was reduced to a stump.

The other morphological characteristics of intersexes are completely identical to those of normal females; even SEM did not reveal appreciable differences.

(c) *Karyological analyses*

Zyginidia pullula has an XX/X0 sex-determination system. This species showed the following karyotype: $2n=8AA+XX$ for the female and $2n=8AA+X0$ for the male (data not shown).

As expected, cytological observations on newly emerged adults belonging to the infected lines showed that nuclei of females bearing upper pygofer appendages, i.e. the phenotypic intersexes, have male genotypes: diploid nuclei were characterized by the presence of 17 chromosomes (figure 3*a*) and haploid nuclei had 8 and 9 chromosomes (figure not shown). Females without upper pygofer appendages have 18 chromosomes (figure 3*b*).

Figure 1. (*Overleaf*.) Condensed phylogenetic tree of *Wolbachia* hosted by hemipteran species, based on the *wsp* gene. Sequences from *Tetranychus urticae* (Acari: Tetranychidae) and *Callosobruchus chinensis* (Coleoptera: Bruchidae) were those with the highest scores after a BLASTn search. The name of the host species is followed by GenBank accession numbers. The tree shown is midpoint rooted. Bootstrap values (1000 replicates) are labelled above branches; bootstrap values less than 50 are not shown. On the right-hand side, the *Wolbachia* supergroups (A and B) are indicated.

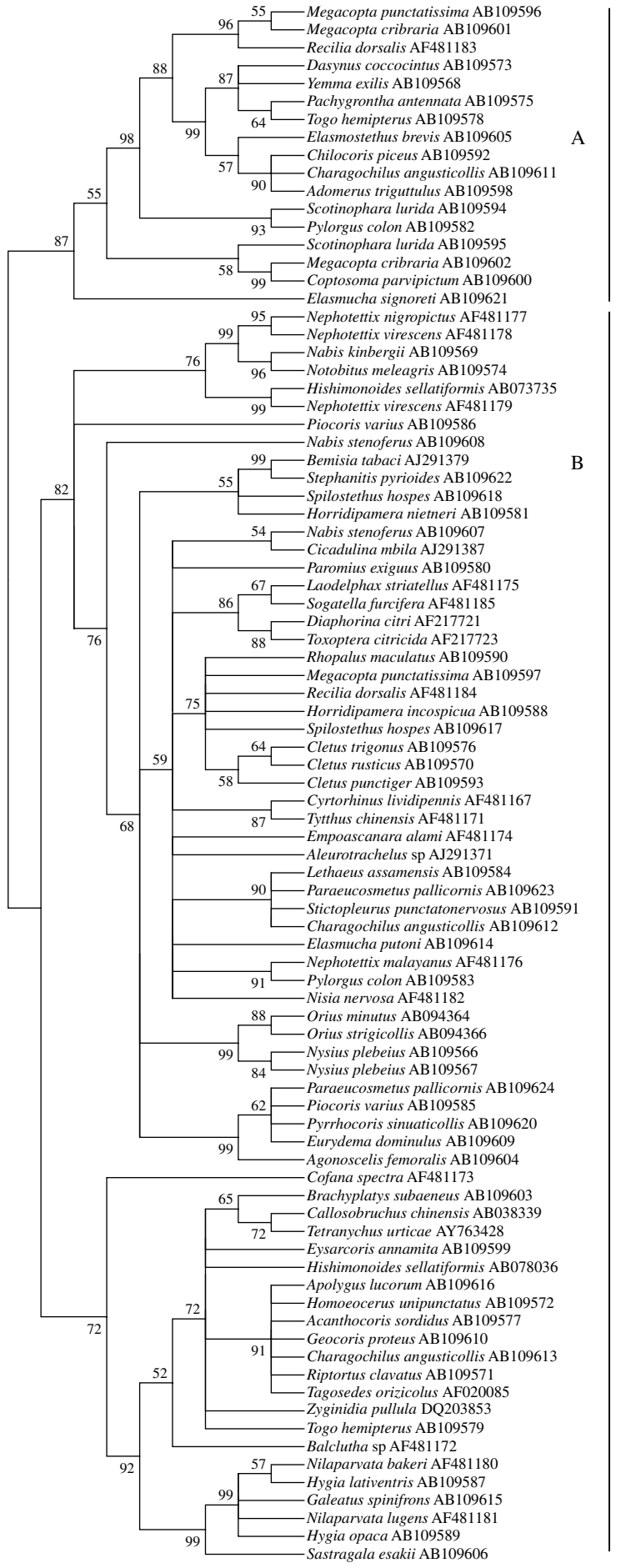

Figure 1. (*Caption overleaf.*)

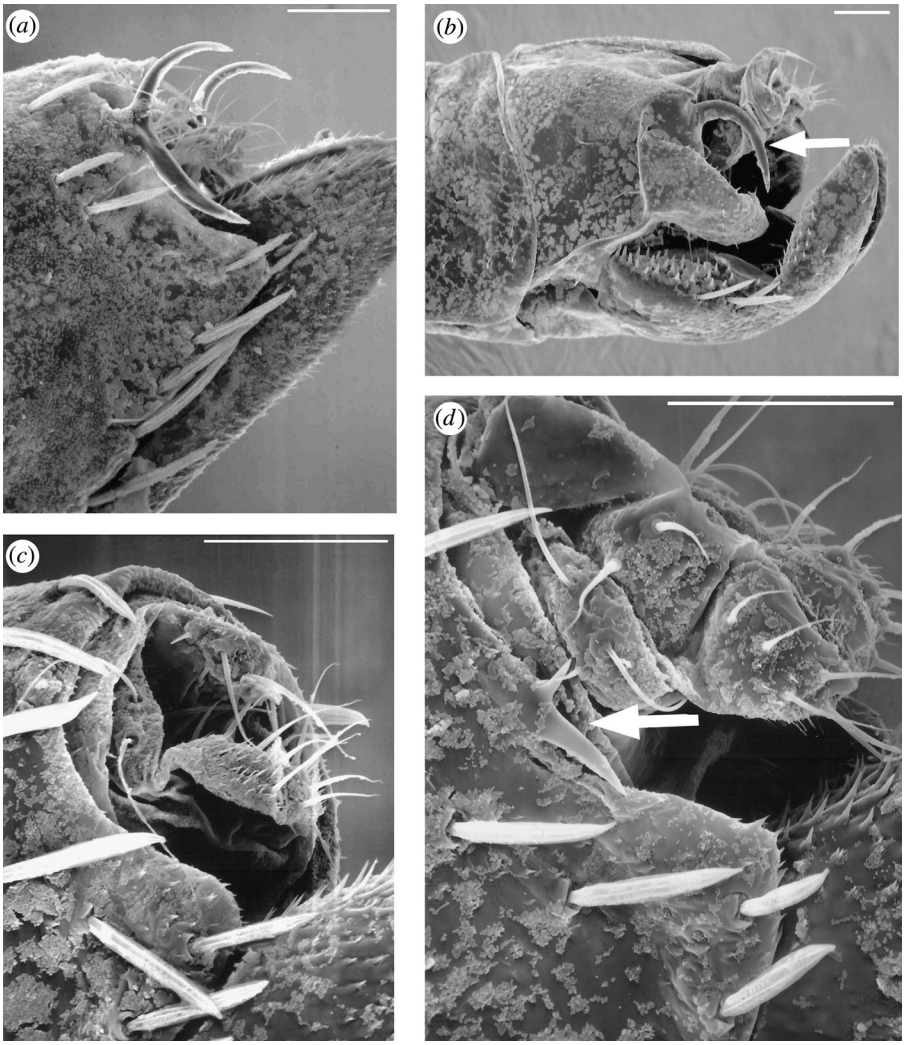

Figure 2. (*a*) Abdominal distal end of feminized male with well-developed upper pygofer appendages; (*b*) abdominal distal end of a male; the arrow shows the left upper pygofer appendage, the lower branch of which is covered by the lateral process of the pygofer; (*c*) perianal edge of the pygofer in a female; (*d*) poorly developed upper pygofer appendage on the perianal edge of the pygofer in a feminized male (scale bar, 50 μm).

(d) *Tetracycline treatment*

Both tetracycline-treated specimens and controls gave progeny. While control females produced an all-female brood, four of the treated individuals (T1, T2, T3, T4) produced male and female broods (T1 = 1 : 1,3; T2 = 1 : 1; T3 = 1 : 1; T4 = 1 : 1,2). The fifth treated female (T5) died 2 days after the administration of the antibiotic without laying eggs.

Morphological analysis of the treated females showed that they were all phenotypically 'normal', thus lacking upper pygofer appendages.

From 12 randomly chosen individuals of their broods, DNA was extracted but the *wsp* and 16S rRNA genes of *W. pipientis* could not be amplified by performing a further PCR using the PCR product as template.

Moreover, both F_1 females and males of the treated lines were fertile; they all produced progeny when mated with uninfected partners. The molecular screening performed on some specimens of these F_2 generations confirmed the absence of the bacterium.

4. DISCUSSION

Our data support the evidence that *Z. pullula* is infected by a strain of *W. pipientis* which causes feminization of genetic males. The phylogenetic analyses, based on both 16S rRNA and *wsp* genes, clearly set this new *Wolbachia* strain—designated as *w*Zygpul—in the B supergroup, where almost all the known strains found in Hemiptera are clustered. Within this order of insects, the B supergroup *Wolbachia* are known to induce cytoplasmatic incompatibility (*Laodelphax striatellus* and *Sogatella furcifera*; Noda *et al.* 2001) or to have an unknown effect (Kittayapong *et al.* 2003).

The feminizing effect induced by *W. pipientis* in *Z. pullula* is manifest by the production of females having a male genotype and some typical phenotypic male features, i.e. the upper pygofer appendages. *Zyginidia pullula* has an XX/X0 sex-determination system, as observed in other genera of the family Cicadellidae (Whitten 1965), with the uninfected populations showing the karyotype $2n = 8AA + XX$ in females and $2n = 8AA + X0$ in males.

The infected females bearing upper pygofer appendages have a male karyotype, both in somatic cells (17 chromosomes) and in gametic cells (gametes with 8 and 9 chromosomes), and are thus considered feminized males. On the contrary, infected females without the secondary male features (normal females) possess the typical female karyotype.

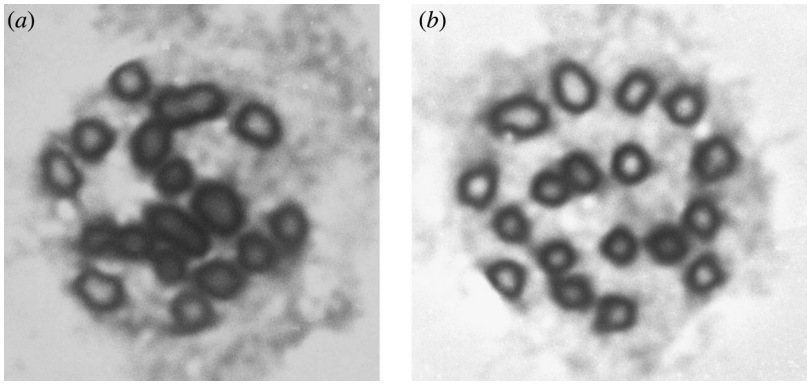

Figure 3. (*a*) Karyotype of a feminized male: 17 chromosomes; (*b*) karyotype of a *Wolbachia*-infected female: 18 chromosomes.

The removal of *W. pipientis* by tetracycline treatment of normal females, and the consequent re-appearance of males in their F_1 broods, permits correlation of the feminizing effect with the presence of the bacterium.

In insects, the simultaneous presence in the same specimen of male and female features is quite common, but only few occurrences of such aberrations have been correlated with a *Wolbachia* infection. In particular, some gynandromorphs have been described in parasitic wasps infected with a parthenogenesis-inducing *Wolbachia* strain (Stouthamer 1997) and in isopods infected with feminizing *Wolbachia* (Rigaud & Juchault 1998).

Furthermore, sexual mosaics of the lepidopteran *O. scapulalis* are generated after incomplete curing of *Wolbachia* or bacterial transfection into uninfected eggs (Kageyama & Traut 2003; Kageyama *et al.* 2003). These individuals are considered partly feminized males by the authors, whereas complete feminization seems to be absolutely incompatible with the survival of the male genotype (Kageyama & Traut 2003).

In *Z. pullula*, instead, *Wolbachia* confers a feminizing effect on genetic males, which are not only vital, but also active reproductively. Couplings were often observed, meaning that these individuals have a feminine 'sex appeal', but progeny were only occasionally obtained. Specific experimental trials concerning the fitness of feminized males, including fertility and prolificity, are in progress, and will be the subject of a future publication.

Wolbachia pipientis is often referred to as a 'selfish genetic element' which aims to produce a strongly female-biased sex ratio for increasing its own transmission and spread through a population. On the other hand, selection on host autosomes acts to prevent the *Wolbachia* action, such that the bacterium does not attain a high frequency in the population, and the host sex ratio tends to return to unity (Taylor 1990; Charlat *et al.* 2003). The presence of such modifiers (resistance genes) has been demonstrated in the isopod *A. vulgare*, and recently hypothesized in the lepidopteran *E. hecabe* (Rigaud & Juchault 1993; Hiroki *et al.* 2004).

Only few exceptions are reported to this model, e.g. in the Lepidoptera *Hypolimnas bolina*, *Acraea encedon* and *Acraea encedana*. In these species, a son-killer *Wolbachia* produces high infection frequencies (from 70 to 99%) in wild populations; it causes an extremely biased sex ratio towards females, still persisting after many generations (Dyson & Hurst 2004; Charlat *et al.* 2005).

In the sampled populations of *Z. pullula*, the sex ratio was only moderately female-biased (1 : 1.8 male : female), with the *Wolbachia* infection rate not exceeding 34% of the collected females. The moderate prevalence of the infection in the field suggests that, in the leafhopper, resistance genes could operate against bacterial action; otherwise, the theoretical model predicts a very high prevalence of the parasite in host populations.

Finally, how can *W. pipientis* determine the feminization in *Z. pullula*? A hypothesis could be that the bacterium interacts with the genetic control system involved in the leafhopper's sex determination. This is in view of the recent demonstration of the interaction between *W. pipientis* and Sex-lethal (*Sxl*), which is master regulator gene of sex determination in *Drosophila melanogaster* (Starr & Cline 2002). Moreover, in fruitfly males, feminization may be induced, modifying the doublesex (*dsx*) gene expression. This gene is essential for a proper differentiation of the sexually dimorphic somatic features, and is subjected to an alternative splicing in both sexes (Saccone *et al.* 2002; Pomiankowski *et al.* 2004). In *D. melanogaster* males, the ectopical expression of *dsx*F (specific female protein) with the complete removal of endogenous *dsx*M (specific male protein) caused external complete feminization. These individuals are sterile, but they are actively courted by other males and even copulate with them (Waterbury *et al.* 1999).

Although the similarity with these occurrences is suggestive, at the moment the lack of information about the genetic and molecular bases of the sex-determination system in *Z. pullula*, and the mechanism of interaction of the feminizing *Wolbachia*, do not yet allow us to validate this intriguing scenario.

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