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Occurrence of arrhenotoky and thelytoky in a parasitic wasp *Venturia canescens* (Hymenoptera: Ichneumonidae): Effect of endosymbionts or existence of two distinct reproductive modes?

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Abstract. Endosymbiotic organisms are known to manipulate the reproductive biology of their hosts. Incomplete prevalence of endosymbiont inducing thelytokous parthenogenesis results in the coexistence of sexual and asexual individuals, and could account for the maintenance of sexual reproduction in certain populations or species. In the parasitoid *Venturia canescens*, arrhenotokous (“sexual”) and thelytokous (“asexual”) individuals occur sympatrically. We aimed to determine whether endosymbionts are implicated in the thelytoky of *V. canescens*. After screening females of the two reproductive modes for several reproductive parasites: bacteria (*Wolbachia*, *Rickettsia*, *Bacteroidetes*, *Spiroplasma*, *Arsenophonus*) and Microsporidia, we concluded that thelytoky in *V. canescens* is not induced by any of these parasites and confirmed its suitability as a biological model for solving the evolutionary enigma of the maintenance of sexual reproduction.

INTRODUCTION

Often tackled but never resolved, the question of how sexual reproduction is maintained in eukaryotes remains one of the most important enigmas in evolutionary biology (Stearns, 1990; Otto, 2009). A key point of contention is in the imbalance between the obvious demographic and genetic costs of sex (Maynard-Smith, 1978) vs the lack of demonstrated short term advantages. Niche differentiation, highlighted by environmental dependent differences in fitness of sexual and asexual forms, seems to be a fruitful avenue of investigation to resolve this issue (Amat et al., 2006, 2009; Tobler & Schlupp, 2010). In order to evaluate this hypothesis, it is necessary to compare closely related sexual and asexual species or individuals of the same species that occur sympatrically.

However, the hallmarks of such ecological situations can be confused with altered genomic constitutions such as hybridization and polyploidization or be associated with parthenogenesis-inducing microbes. Particularly for arthropods, the presence of reproductive parasites, i.e. endosymbiotic organisms manipulating the reproduction of their hosts, was widely documented during the last decades, initially with *Wolbachia* (Stouthamer et al., 1990). Recently, the list of bacterial endosymbionts has grown with the addition of *Cardinium* (Zchori-Fein et al., 2001), *Rickettsia* (Hagimori et al., 2006), *Arsenophonus* (Gherna et al., 1991) and *Spiroplasma* (Hurst et al., 1999). Microsporidia are also capable of manipulating reproduction in species of amphipod and mosquito (Dunn & Smith, 2001). These maternally transmitted parasites manipulate their host’s reproduction in order to enhance their rate of transmission in host populations in different

ways: cytoplasmic incompatibility, feminisation of genetic males, male killing and induction of thelytokous parthenogenesis (Turelli & Hoffmann, 1995; Hogg et al., 2002; Bourtzis & Miller, 2003; Werren et al., 2008; Saridaki & Bourtzis, 2010).

Coexistence of sexual and asexual individuals within a population may be due to incomplete prevalence of organisms inducing parthenogenesis. Stouthamer et al. (2001) proposed three processes to explain an intermediate proportion of infected and non-infected individuals in a population: (i) inefficient transmission of the parasite, (ii) existence of suppressor genes in hosts and (iii) presence of male-biasing sex-ratio distorters, as in *Trichogramma kaykai* (Hymenoptera: Trichogrammatidae). In conjunction with these factors, costs of carrying endosymbiotic parasites may influence the level of prevalence. Such costs may be expressed in terms of higher mortality, lower fecundity and reduced locomotor activity of the hosts (Fleury et al., 2000).

In the hymenopteran parasitoid, *Venturia canescens* Gravenhorst (Hymenoptera: Ichneumonidae), the natural co-occurrence of wasps with two different modes of reproduction, arrhenotoky (called “sexual”) and thelytoky (“asexual”), has been interpreted as evidence for two coexisting modes of reproduction (Schneider et al., 2002; Amat et al., 2006; Thiel et al., 2006; Pelosse et al., 2007) rather than the induction of thelytokous parthenogenesis. However, the prevalences of reproductive parasites in *V. canescens* has to be clarified because Cook & Butcher (1999) highlighted the presence of *Wolbachia*, whereas Mateo-Leach et al. (2009), who used antibiotic treatment and PCR screening with prokaryotic primers, concluded

that prokaryotes are unlikely to be implicated in the thelytokous parthenogenesis in *V. canescens*. In addition to the lack of congruence between these results, we know that PCR screening with prokaryotic primers and antibiotic treatment are sensitive to the density and diversity of bacteria, potentially leading to false negative results (Suzuki & Giovannoni, 1996). Thus, complementary analyses are necessary before reaching a conclusion on the determinism of the thelytoky in *V. canescens*. Here, we aim to determine whether thelytoky in *V. canescens* is correlated with the presence of endosymbiotic bacteria, using specific and generic primers of the bacteria that manipulate reproduction. In addition, we test for the first time whether the presence of *Microsporidia* is implicated in thelytoky in *V. canescens*. Our study should indicate whether the co-occurrence of females producing only female offspring (thelytoky) with those producing males and females (arrhenotoky) is indicative of the co-occurrence of uninfected individuals with two different reproductive modes, or a result of the co-occurrence of parasitized and un-parasitized arrhenotokous individuals.

MATERIALS AND METHODS

Biological material

Venturia canescens is a solitary koinobiont endoparasitoid of mainly Pyralidae larvae. It possesses a haplodiploid sex determination system: haploid individuals from unfertilised eggs are males whereas diploid individuals from fertilised eggs are females. Arrhenotokous parthenogenesis contrasts with thelytokous parthenogenesis because under thelytoky diploid females develop from unfertilised eggs and no males are produced. Arrhenotokous strains of *V. canescens* were discovered a few years ago occurring naturally, with the two reproductive modes occurring sympatrically (Schneider et al., 2002). Gene flow occurs between arrhenotokous and thelytokous populations and it is hypothesized that thelytokous individuals originate from arrhenotokous ones (Schneider et al., 2002, 2003; Mateo-Leach et al., 2012), indicating that the two reproductive modes belong to the same species. In our experiment, we used arrhenotokous and thelytokous strains of *V. canescens* established from individuals collected near Valence (France, 44°58'34"N, 4°55'6"E) in 2007 and 2008. Individuals of each of these reproductive modes were reared separately in the laboratory with *Ephestia kuehniella* (Lepidoptera: Pyralidae) as the host and in a controlled environment (25 ± 1°C, 70 ± 5% RH and 12L : 12D).

DNA extraction and screening

If thelytokous parthenogenesis is induced by an endosymbiotic organism, then the expectation is that the analyses will reveal the presence of at least one species of reproductive parasite in all the thelytokous females and none in arrhenotokous ones. Consequently, the sample sizes for molecular analyses do not need to be large. Further, if a bacterium or a microsporidium induces thelytoky in *V. canescens*, we should find it in the ovaries of all thelytokous wasps. For each reproductive mode, analyses were performed on five nymphal females in both years sampled. They were carefully extracted from cocoons by dissection before the imaginal moult. Further, analyses were performed on three pools of gonads from wasps sampled in 2008. Each pool consists of ten ovaries and oviducts of adult wasps of the same reproductive mode. Ovaries and oviducts were dissected in 10 µl of ethanol (70%). Pools of gonads were used to

test for the presence of low numbers of reproductive parasites. Nymphs and gonads were then kept individually at -20°C.

DNA extraction and purification were performed using the Nucleospin Tissue Kit from Macherey-Nagel®, following the manufacturer instructions (volume of elution = 100 µl). The DNA quality was systematically tested using polymerase chain reaction (PCR) amplification of a region present in thelytokous and arrhenotokous *V. canescens*, which codes for a VLP (Virus-Like Particle) (Theopold et al., 1994), using primers listed in Table 1. We used specific primers (Table 1) to investigate the presence of six taxa of endosymbiotic bacteria: *Wolbachia*, *Rickettsia*, *Bacteroidetes*, *Cardinium*, *Spiroplasma* and *Arsenophonus*. We also performed PCR amplifications of a conserved region of the Prokaryote 16S rDNA and of a conserved region of the Microsporidia 16S rDNA, with respectively two sets of primers in order to limit potential bias of amplification (Suzuki & Giovannoni, 1996; Table 1). For all analyses, we included an appropriate positive control (Table 1). Positive amplifications were then directly sequenced in order to determine the nature of the organism present.

PCRs were performed in a final volume of 25 µl containing 200 µM dNTPs, 200 nM of each primer, 1.5 mM of MgCl₂, 1 X Taq buffer and 0.5 U of taq polymerase (EuroblueTaq, Eurobio, France) and 2 µl of the DNA template. PCRs were performed under the following conditions: initial denaturation at 95°C for 2 min, 35 cycles of denaturation (94°C, 30 s), annealing (50 to 63°C, depending on primers, 30 s), extension (72°C, 1 min) and a final extension at 72°C for 5 min. The PCR products were then visualized using agarose gel electrophoresis.

RESULTS AND DISCUSSION

Among the thelytokous and arrhenotokous females of *V. canescens* that were screened, all analyses on pools of gonads produced negative results and positive results were obtained with *Wolbachia* primers (FbpA and 16S) and prokaryotic primers (16S) for two and five thelytokous females, respectively. The sequencing of the two positive samples for *Wolbachia*, using forward (FbpA-F and Wolb-F) and reverse (FbpA-R and Wolb-R) primers (Biofidal, Villeurbanne, France), revealed an unequivocal single sequence in both senses, for each pair of primers, respectively (access numbers: JX399797 and JX399793). BLAST analysis of the sequences showed that the *Wolbachia* belong to clade A, which is one of the most common infecting Arthropods (Werren & Windsor, 2000). Two positive samples with prokaryotic primers were also sequenced, using both forward (E8F) and reverse (1541R and 1389R) primers (Genoscreen, Lille, France), and revealed an unequivocal single sequence in both senses and for both individuals tested, given the quality of each base. BLAST analysis showed 99% similarity with *Enterococcus mundtii* (GQ337033). Species of *Enterococci* are not recorded as reproductive parasites but are generally associated with the digestive organs of insects (Geiger et al., 2009). As *Wolbachia* and *E. mundtii* were not systematically detected in thelytokous females and never in pools of gonads, they are not implicated in the thelytoky of *V. canescens*. These positive results are not a consequence of contamination because reproductive parasites were never detected in the negative controls.

Thus, we conclude with confidence that thelytokous parthenogenesis in *V. canescens* is genetic and is not

TABLE 1. Primers used in polymerase chain reaction (PCR) assays to detect microbes manipulating reproduction and control quality DNA. Analyses with specific and generic primers were performed respectively on nymphal females and pools of ovaries of *V. canescens*. Tm = annealing temperature.

Organism (gene)	Primers	Sequence (5'–3')	Tm	Size	Positive control	Reference
<i>V. canescens</i> (VLP)	Vcan-F	-CTCAATATGTGGGGTGGTGG-	56°C	250bp	<i>V. canescens</i>	Theopold et al., 1994
	Vcan-R	-TCGCAGTGGCTTGTCAGAGT-				
<i>Wolbachia</i> (WSP/FbpA/16S)	81-F	-TGGTCCAATAAGTGATGAAGAAAC-	52°C	610bp	<i>D. melanogaster</i>	Braig et al., 1998 Baldo et al., 2006 O'Neill et al., 1992
	691-R	-AAAAATTAACGCTACTCCA-				
	FbpA-F	-CCRCCAGARAAAAYACTATTC-				
	FbpA-R	-TGGTCCAATAAGTGATGAAGAAAC-	55°C	509bp		
	Wolb-F	-TTGTAGCCTGCATGTTTACT-	52°C	936bp		
	Wolb-R	-GAATAGGTATGATTTTCATGT-				
<i>Bacteroidetes</i> (16S)	Ch-F	-TACTGTAAGAATAAGCACGGC-	57°C	900bp	<i>Bemisia tabaci</i>	Zchori-Fein & Perlman, 2004
	Ch-R	-GTGGATCACTTAACGCTTTCG-				
<i>Cardinium</i> (16S)	CLO-F	-GGAACCTTACCTGGGCTAGAATGTATT-	56°C	500bp	<i>Bemisia tabaci</i>	Gotoh et al., 2007
	CLO-R	-GCCACTGTCTTCAAGCTCTACCAAC-				
<i>Rickettsia</i> (16S)	Rb-F	-GCTCAGAACGAACGCTATC-	56°C	900bp	<i>Bemisia tabaci</i>	Gottlieb et al., 2006
	Rb-R	-GAAGGAAAGCATCTCTGC-				
<i>Spiroplasma</i> (16S)	Spixo-F	-TTAGGGGCTCAACCCCTAAC-	52°C	810bp	<i>Zygiella x-notata</i>	Duron et al., 2008
	Spixo-R	-TCTGGCATTGCCAACTCTC-				
<i>Arsenophonus</i> (23S)	Ars23s-F	-CGTTTGTATGAATTCATAGTCAAA-	52°C	650bp	<i>Omatissus lybicus</i>	Thao & Baumann, 2004
	Ars23s-R	-GGTCCTCCAGTTAGTGTACCAAC-				
Microsporidia (16S)	V1-F	-CACCAGGTTGATCT-	57°C	1300bp	<i>C. elegans</i>	Weiss et al., 1994 Weiss & Vossbrinck, 1998 Baker et al., 1995
	1492-R	-GGTTACCTTGTACGACTT-				
	V1-F	-CACCAGGTTGATCT-	63°C	450bp		
530-R	-CCGCGGCTGCTGGCAC-					
Procaryotes (16S)	E8-F	-AGAGTTTGTATCATGGCTCAG-	55°C	1500bp	<i>D. melanogaster</i>	Reysenbach et al., 1994 Suzuki & Giovannoni, 1996
	1541-R	-AAGGAGGTGATCCANCCRCA-				
	E8-F	-AGAGTTTGTATCATGGCTCAG-	55°C	1400bp		
1389-R	-GACGGCGGTGTGTACAA-					

manipulated by reproductive parasites. However, using several specific and generic primers, we highlight that *V. canescens* is not free of bacteria. We found a low incidence of *Wolbachia* in *V. canescens*, as noted previously by Cook & Butcher (1999). Additional analyses are required to locate *Wolbachia* and *E. mundtii* in wasp tissues and determine their origin and whether they affect the phenotype of *V. canescens*.

The absence of endosymbiont induced thelytoky in *V. canescens* could be explained by its sex determination mechanism, which is based on allelic composition at a single locus (sl-CSD: single locus Complementary Sex Determination) (Beukeboom, 2001). This sex determination diverges from the classical haplodiploid scheme in that diploid individuals homozygous for this locus are generally sterile or not viable males. In *V. canescens*, thelytokous parthenogenesis is automictic, leading to the maintenance of heterozygosity for loci located close to the centromere (Beukeboom & Pijnacker, 2000). Single locus-CSD appears to constrain the infection by endosymbiont-induced thelytoky because, for instance in the case of infection with *Wolbachia*, thelytokous induction involves gamete duplication in order to restore the diploidy and results in completely homozygous diploid offspring (Stouthamer & Kazmer, 1994). However, the recent discovery of *Rickettsia* inducing thelytoky in *Neochrysocharis formosa* (Hymenoptera: Eulophidae) through a cytogenetic mechanism that does not modify

heterozygosity (Adachi-Hagimori et al., 2008) justifies the investigation of reproductive parasites inducing parthenogenesis in species with sl-CSD, like *V. canescens*.

Finally, our results reveal that *V. canescens* presents a clear case of coexistence in sympatry of distinct thelytokous and arrhenotokous individuals. Such a biological system, though sporadic in its occurrence among animals, is highly relevant to the investigation of one of the major unsolved problems in evolutionary biology, namely the maintenance of sexual reproduction (Otto, 2009). Recent studies on *V. canescens* appear to demonstrate niche differentiation that could explain the coexistence of the two reproductive modes, i.e. thelytokous individuals seem to be more adapted to anthropogenic and arrhenotokous individuals to natural habitats (Amat et al., 2006, 2009; Thiel et al., 2006; Pelosse et al., 2007, 2010; Foray et al., 2011). Future studies should investigate the genetic basis of thelytoky in *V. canescens* since thelytoky in the parasitoid wasp *Lysiphlebus fabarum* (Hymenoptera: Braconidae) is determined by a single recessive gene (Engelstädter et al., 2011; Sandrock & Vorburger, 2011).

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