

Epidemiology of asexuality induced by the endosymbiotic *Wolbachia* across phytophagous wasp species: host plant specialization matters

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Abstract

Among eukaryotes, sexual reproduction is by far the most predominant mode of reproduction. However, some systems maintaining sexuality appear particularly labile and raise intriguing questions on the evolutionary routes to asexuality. Thelytokous parthenogenesis is a form of spontaneous loss of sexuality leading to strong distortion of sex ratio towards females and resulting from mutation, hybridization or infection by bacterial endosymbionts. We investigated whether ecological specialization is a likely mechanism of spread of thelytoky within insect communities. Focusing on the highly specialized genus *Megastigmus* (Hymenoptera: Torymidae), we first performed a large literature survey to examine the distribution of thelytoky in these wasps across their respective obligate host plant families. Second, we tested for thelytoky caused by endosymbionts by screening in 15 arrhenotokous and 10 thelytokous species for *Wolbachia*, *Cardinium*, *Arsenophonus* and *Rickettsia* endosymbionts and by performing antibiotic treatments. Finally, we performed phylogenetic reconstructions using multilocus sequence typing (MLST) to examine the evolution of endosymbiont-mediated thelytoky in *Megastigmus* and its possible connections to host plant specialization. We demonstrate that thelytoky evolved from ancestral arrhenotoky through the horizontal transmission and the fixation of the parthenogenesis-inducing *Wolbachia*. We find that ecological specialization in *Wolbachia*'s hosts was probably a critical driving force for *Wolbachia* infection and spread of thelytoky, but also a constraint. Our work further reinforces the hypothesis that community structure of insects is a major driver of the epidemiology of endosymbionts and that competitive interactions among closely related species may facilitate their horizontal transmission.

Keywords: ecological specialization, *Megastigmus*, multilocus sequence typing, parthenogenesis, thelytoky

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Introduction

Sex is by far the most predominant mode of reproduction among eukaryotes, such that only one of a thousand animal species shows some type of asexual

reproduction (Suomalainen *et al.* 1987). Among the diverse mechanisms involved in asexual reproduction, parthenogenesis is defined *sensu stricto* as the development of an egg without fertilization. The origin of parthenogenesis is polyphyletic in both invertebrates and vertebrates, suggesting that systems maintaining sexuality are labile and stimulating elucidation of the evolutionary routes to parthenogenesis (reviewed in Simon

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et al. 2003). Spontaneous loss of sexuality may occur through mutations or intra- and interspecific hybridization (Dedryver *et al.* 2001; Simon *et al.* 2003), but it can also result from infection by bacterial endosymbionts that are predominantly transmitted vertically through the female egg cytoplasm and distort the host sex ratio towards females to their own advantage (Engelstädter & Hurst 2009).

In the insect order Hymenoptera, several forms of parthenogenesis have been defined according to the sex of offspring produced by a virgin female (Cook 1993; Cook & Crozier 1995). The dominant and ancestral form of parthenogenesis is arrhenotoky, in which fertilized eggs develop as diploid females and unfertilized eggs develop as haploid males. However, numerous species display thelytoky, which is a form of complete parthenogenesis where unfertilized eggs develop into diploid females. Thelytoky can be under the control of the insect itself or its endosymbionts (Stouthamer *et al.* 1990; Vavre *et al.* 2004). Parthenogenesis inducers include bacteria of the genera *Wolbachia* (Werren 1997), *Cardinium* (Zchori-Fein & Perlman 2004) and *Rickettsia* (Hagimori *et al.* 2006). While induction of male production is impossible in genetically based thelytoky, endosymbiotic thelytoky can be characterized by its reversibility, as antibiotic or heat treatment of females leads to bacteria elimination and production of males in their progeny (Stouthamer *et al.* 1990). Generally, infection by parthenogenesis-inducing (PI) *Wolbachia* is fixed within species or populations and results in an obligate parthenogenesis associated with a loss of sexual function in females (Jeong & Stouthamer 2005; Kremer *et al.* 2009), or in both sexes (Gottlieb & Zchori-Fein 2001), making *Wolbachia* indispensable. Only in very rare cases, polymorphism of infected and uninfected females is maintained (Werren *et al.* 2008).

Despite vertical transmission via the egg cytoplasm being the predominant transmission mode of PI endosymbionts (Werren 1997), phylogenetic incongruence between host and endosymbiont histories strongly indicates occasional horizontal transmission events (O'Neill *et al.* 1992; Stouthamer *et al.* 1993; Baldo *et al.* 2006; Perlman *et al.* 2010). Horizontal transmissions of *Wolbachia* have been successfully demonstrated experimentally by injection (Braig *et al.* 1994; Grenier *et al.* 1998), introgression (Jaenike 2007), maintenance of close contacts between conspecifics (Rigaud & Juchault 1995) or in host-parasitoid associations (Vavre *et al.* 1999). Effective horizontal transmission of endosymbionts depends (i) on intimate ecological associations which provide within-community horizontal transmission opportunities (Vavre *et al.* 1999; Sintupachee *et al.* 2006; Stahlhut *et al.* 2010) and (ii) on the phylogenetic similarity of donor and recipient host species because internal

defence mechanisms against infections are likely to be more similar between closely related hosts (Stahlhut *et al.* 2010). Studies addressing how insect phylogeny and ecology affect patterns of similarity between strains of PI endosymbionts would help gain critical insights on how these two forces affect shifts in reproduction modes. In particular, study systems involving several groups of closely related thelytokous species but displaying different levels of ecological proximity due to habitat specialization can be of critical interest to define the possible ecological boundaries to the evolutionary trajectory of thelytoky in insects.

In this study, we address the potential for phylogeny, ecology and infection by PI endosymbionts to drive the spread of asexuality within insects. For this purpose, we focused on the genus *Megastigmus* (Hymenoptera: Torymidae), which counts more than 125 arrhenotokous and thelytokous wasp species worldwide (Grissell 1999). Half of these species are specialist seed feeders, and the other half are presumed to be parasitoids, gall-makers or to have unknown hosts. Within the seed-specialized group, two-thirds of species exploit gymnosperm hosts (Pinaceae and Cupressaceae families), whereas one-third exploits preferentially angiosperms, especially the Rosaceae and Anacardiaceae families (Roques & Skrzypczyńska 2003). Being highly specialized in the seed resource, several wasp species exploiting the same host strictly require the same ecological niche, which is then particularly propitious to direct intra- or interspecific interactions within this insect group (Boivin *et al.* 2008; Auger-Rozenberg & Roques 2012). In the genus *Megastigmus*, the dominant and ancestral form of parthenogenesis is by far arrhenotoky as thelytoky characterizes only a minority of species (Grissell 1999), but both arrhenotokous and thelytokous species can occur in sympatry on gymnosperm or on angiosperm hosts (Boivin *et al.* 2008; Auger-Rozenberg & Roques 2012). Thelytoky has only been established from records of highly female-biased sex ratios in field populations, but neither its determinism nor its evolutionary trajectory has been elucidated yet. We first performed a large literature survey to examine the distribution of thelytoky in *Megastigmus* across their known obligate host plant families. Second, we tested for thelytoky caused by endosymbionts by screening in a large sample of arrhenotokous and thelytokous species for endosymbionts and by performing antibiotic treatments. Finally, we performed phylogenetic reconstructions to examine the evolution of endosymbiont-mediated thelytoky in *Megastigmus* and its possible connections to host plant specialization. This study strongly suggests that thelytoky is caused by *Wolbachia* endosymbionts, and interestingly, we show that host plant specialization is a key determinant of *Wolbachia* infection and thelytoky.

Material and methods

Distribution of thelytoky among phytophagous Megastigmus species

Parthenogenesis, that is, arrhenotoky or thelytoky, is the only mode of reproduction in the *Megastigmus* genus. It has never been formally studied and it was generally deduced from sex ratio estimates in field-sampled wasp populations. Here, we used extensive catalogs of seed-feeding *Megastigmus* species to gather the current knowledge on the relative prevalence of arrhenotoky and thelytoky in the genus *Megastigmus* (Grissell 1999; Roques & Skrzypczyńska 2003; Roques *et al.* 2003; Auger-Rozenberg *et al.* 2006). A bibliographic search of nonredundant articles in the Web of Science (2003–2012) and CAB abstracts (2003–2012) was also performed on key terms '*Megastigmus* and seed*' to check for potential new species descriptions or increase knowledge in the biology of the species described in the above catalogs. We considered only wasp species for which arrhenotoky or thelytoky was ascertained by authors, or for which male frequencies estimated at emergence from sampled seed lots provided unambiguous support for arrhenotoky (high male abundance) or thelytoky (absence or <1%). Species for which the parthenogenesis mode was not mentioned or for which sample sizes were insufficient (<10) to estimate sex ratios were not included. The prevalence of thelytoky was assessed as the percentage of thelytokous species in all of the selected wasp species. The distributions of both arrhenotoky and thelytoky relative to host plant specialization were assessed by assorting both arrhenotokous and thelytokous wasp species according to their host plant group (gymnosperm or angiosperm), family and genus.

Wasp sampling

Megastigmus species were collected across the Northern Hemisphere (Nearctic and Palaearctic) by seed samplings on diverse host plants. Insect-infested seeds were separated from noninfested ones by X-ray radiography (using Faxitron-43855[®], 15 kV, 3 mA, 3'30" to 4'30" depending on seed species with X-ray-sensitive films Kodak 'Industrex M', and Faxitron-MX20[®], 20 kV, 0.3 mA, 1'45" with an EZ20 digital scanner). The insect-infested seeds were placed in individual rearing boxes stored in outdoor insectaries located at INRA, Orléans, France, and at INRA, Avignon, France. Adult emergence was recorded over the 3 years following seed maturation because of a possible prolonged diapause (Roques & Skrzypczyńska 2003). After emergence, insect species determination was ascertained using the morphological keys of Grissell (1999) and Roques &

Skrzypczyńska (2003). Emerged insects were then sorted by species and collection site and preserved in 100% alcohol at -20 °C. A total of 25 *Megastigmus* species were used in this study: 10 were thelytokous and 15 were arrhenotokous. These species, their reproduction mode, their host plants, collecting localities and distributions are summarized in Table 1. Voucher material of specimen remnants and associated complete specimens from the same original series are kept in ethanol in the collection of the National Institute of Agromonomical Research, Orléans, France.

DNA extraction

Total genomic DNA was isolated by crushing individually whole adult *Megastigmus* females using two different procedures, depending on sample size and further DNA use. Screening for PI endosymbiont infection, which involves large sample sizes, was performed using the Chelex method. This fast and cheap method consisted of digesting tissues kept for 2 h at 56 °C in a 200 µL 10% Chelex solution (Bio-Rad). After 30 min at 100 °C, samples were centrifuged and supernatants were used as DNA sources. For sequencing and further phylogenetic analyses, which requires smaller sample sizes and long-term conservation of DNA, we used the DNeasy extraction Tissue Kit (Qiagen). Total genomic DNA was eluted in 200 µL of AE buffer in this case. In addition, a polymerase chain reaction (PCR) was systematically carried out on Qiagen extractions with *Wolbachia* primers in order to confirm the lack of bias in the detection of the symbiont due to different methods of DNA extraction.

Wasp sequences

We used Sigma RedTaq for PCR amplification. The forward and reverse primers used were 1775-COI-F (Clyde, 5'-CGAATAAATAATATAAGATTTTG-3'), and 2773-COI-R (Bonnie, 5'-GGATAATCTCTATATCGACGAGGTAT-3'; Scheffer & Grissell 2003) for the segment of the cytochrome oxidase I (COI) gene. Cycling programmes were as follows: a denaturation step at 94 °C for 1 min, annealing for 1 min at 48 °C and extension at 72 °C for 1 min with 30 cycles being performed. All PCR products were then purified with a QIAquick PCR purification kit (Qiagen) and were directly sequenced with the amplification primers. Sequencing was performed using the big-dye terminator sequencing kit (PE Applied Biosystem) and carried out with an ABI 3100 automatic sequencer. The gene fragment was sequenced on two to four individuals for each species, except for cryptic and rare species because of the small number of specimens available. When individuals were identified as originating from a different biogeographical region

Table 1 Collection data for the specimens used in the study of 15 arrhenotokous and all the currently known thelytokous species (N = 10) in the *Megastigmus* genus. A: arrhenotokous parthenogenesis. T: thelytokous parthenogenesis. Note that *Torymus azureus* was used as an outgroup for building the cytochrome oxidase I (COI) phylogenetic tree of the *Megastigmus* genus presented in Fig. 1. Specimens collected outside their native range belonged to introduced populations

Species name	Reproduction	Host plant group	Host plant family	Host plant species	Collection site	Native range
<i>M. aculeatus</i>	T	Angiosperm	Rosaceae	<i>Rosa majalis</i>	Krasnoyarsk, Russia	Palaeartic
<i>M. aculeatus nigroflavus</i>	T	Angiosperm	Rosaceae	<i>Rosa multiflora</i>	Dulles, USA	Nearctic
<i>M. borriesi</i>	T	Gymnosperm	Pinaceae	<i>Abies koreana</i>	Rold skov, Denmark	East Asia
<i>M. brevicaudis</i>	T	Angiosperm	Rosaceae	<i>Sorbus sp.</i>	Ojcow, Poland	Palaeartic
<i>M. pictus</i>	T	Gymnosperm	Pinaceae	<i>Larix decidua</i>	Krynica, Poland	Palaeartic
<i>M. pinsapinis</i>	T	Gymnosperm	Pinaceae	<i>Cedrus atlantica</i>	Tala-Guilef, Algeria	Palaeartic
<i>M. pistaciae</i>	T	Angiosperm	Anacardiaceae	<i>Pistacia terebenthus</i>	Exocori, Greece	Palaeartic
<i>M. rosae</i>	T	Angiosperm	Rosaceae	<i>Rosa tomentosa</i>	Briançon, France	Palaeartic
<i>M. rosae alba</i>	T	Angiosperm	Rosaceae	<i>Rosa sp.</i>	Mytilini, Greece	Palaeartic
<i>M. suspectus</i>	T	Gymnosperm	Pinaceae	<i>Abies alba</i>	Villers-Cotterêts, France	Palaeartic
<i>M. amicorum</i>	A	Gymnosperm	Cupressaceae	<i>Juniperus phoenicea</i>	Baux-de-Provence, France	Palaeartic
<i>M. atedius</i>	A	Gymnosperm	Pinaceae	<i>Picea sp.</i>	Vernon, Canada	Nearctic
<i>M. atlanticus</i>	A	Gymnosperm	Cupressaceae	<i>Cupressus atlantica</i>	Idni, Morocco	Palaeartic
<i>M. bipunctatus</i>	A	Gymnosperm	Cupressaceae	<i>Juniperus communis</i>	Briançon, France	Palaeartic
<i>M. cryptomeriae</i>	A	Gymnosperm	Cupressaceae	<i>Cryptomeria fortunei</i>	Hangzhou, China	East Asia
<i>M. hoffmeyerii</i>	A	Gymnosperm	Pinaceae	<i>Tsuga canadensis</i>	North Bay, Canada	Nearctic
<i>M. lasiocarpae</i>	A	Gymnosperm	Pinaceae	<i>Abies amabilis</i>	Rutherford Creek, Canada	Nearctic
<i>M. nigrovariegatus</i>	A	Angiosperm	Rosaceae	<i>Rosa sp.</i>	Victoria, Canada	Nearctic
<i>M. pinus</i>	A	Gymnosperm	Pinaceae	<i>Abies grandis</i>	Vernon, Canada	Nearctic
<i>M. rafni</i>	A	Gymnosperm	Pinaceae	<i>Abies grandis</i>	Mt Bachelor, USA	Nearctic
<i>M. schimitscheki</i>	A	Gymnosperm	Pinaceae	<i>Cedrus libani</i>	Kapidag, Turkey	Palaeartic
<i>M. spermatrophus</i>	A	Gymnosperm	Pinaceae	<i>Pseudotsuga mensiezii</i>	Chico, USA	Nearctic
<i>M. thyoides</i>	A	Gymnosperm	Cupressaceae	<i>Chamaecyparis sp.</i>	Wilmington, USA	Nearctic
<i>M. transvaalensis</i>	A	Angiosperm	Anacardiaceae	<i>Schinus molle</i>	Marrakech, Morocco	Afro-tropical
<i>M. tsugae</i>	A	Gymnosperm	Pinaceae	<i>Tsuga heterophylla</i>	Saanichton, Canada	Nearctic
<i>Torymus azureus</i>	A	Gymnosperm	Pinaceae	<i>Picea abies</i>	Suchora, Poland	Palaeartic

(i.e. when invasive specimens were found), additional specimens from native areas were sequenced in order to ensure genetic similarity between native and introduced individuals. Sequences were aligned using Clustal W (Thompson *et al.* 1994) as implemented in BioEdit 7.05 (Hall 1999).

In addition to the COI gene, we studied a nuclear fragment, the D2 region of the 28S ribosomal subunit (rDNA), to build a phylogenetic tree of the studied *Megastigmus* species. Nuclear primers previously used for reconstructing a molecular phylogeny of *Megastigmus* spp. on conifers (Auger-Rozenberg *et al.* 2006) were chosen due to their utility for molecular identification at intrageneric taxonomic level. However, the 28S data were not used further because their resolution was insufficient for the goal of this study (results not shown).

Wasp population screenings for endosymbionts

In a wide range of insect species, sex ratio distortion has been associated with infection by diverse bacterial

symbionts such as *Wolbachia* (Werren 1997), *Cardinium* (Zchori-Fein & Perlman 2004), *Arsenophonus* (Thao & Baumann 2004) and *Rickettsia* (Hagimori *et al.* 2006). As a first test for an association between such endosymbionts and thelytoky in the *Megastigmus* genus, we screened for their presence in both arrhenotokous and thelytokous species (listed in Table 1). Separate PCRs were performed on each *Megastigmus* female using specific primers for *Wolbachia* surface protein (wsp; Braig *et al.* 1998), *Cardinium* 16S rRNA (Zchori-Fein & Perlman 2004), *Arsenophonus* 23S rDNA (Thao & Baumann 2004) and *Rickettsia* 16S rRNA (Hagimori *et al.* 2006) gene fragments.

Polymerase chain reactions were carried out in a 25-µL final volume reaction mixture containing 200 µM dNTP, 10 pM primers, 0.5 IU HotStarTaq® DNA polymerase and 1.5 µL DNA solution. All reactions were run in a ABI thermal cycler (PE Applied Biosystems PCR System 9700) with an initial denaturing step at 94 °C for 4 min, an annealing step for 1 min, an elongation step at 72 °C for 1 min 30 s and a final

extension step of 72 °C for 1 min 30 s. Annealing temperatures and primer pairs used for *Wolbachia*, *Cardinium*, *Arsenophonus* and *Rickettsia* PCR screening are detailed in Table S2 (Supporting Information). All PCRs included a positive control of *Asobara tabida* (*Wolbachia*-infected) and *Bemisia tabaci* biotype Q (*Cardinium*-, *Arsenophonus*- or *Rickettsia*-infected) samples. For each bacterial gene, 5 µL of amplified reaction product was run on a 1% agarose gel stained with ethidium bromide after the PCRs in order to determine the presence of an amplified DNA fragment. If a sample of a *Megastigmus* species was negative for any bacterial-specific amplification, but COI amplification succeeded, the insect was considered uninfected. When amplification with a bacterial-specific primer yielded visible bands, the insect was considered infected, and samples were kept for sequencing and strain characterization.

Wolbachia strain characterization

Only *Wolbachia* was detected in our samples (see Results section). We used multilocus sequence typing (MLST; Baldo *et al.* 2006) to characterize *Wolbachia* strain similarity among *Megastigmus* host species. A *wsp* sequence was also obtained from each infected insect. MLST was based on the methods of Baldo *et al.* (2006) using the standard primers that amplify the five conserved *Wolbachia* genes *hcpA*, *ftsZ*, *gatB*, *coxA* and *fbpA*. These are degenerate primers designed to amplify sequences from diverse *Wolbachia* strains. Despite repeated attempts, we could not amplify the *fbpA* gene. Thus, four MLST loci (*hcpA*, *ftsZ*, *gatB* and *coxA*), plus the *wsp* gene, were sequenced in both directions to provide double coverage of the region of interest, with the exception of both *Megastigmus borriesi* and *Megastigmus rosae* var. *alba*, which could be sequenced for the *wsp* gene only.

Phylogenetic analyses

Wolbachia. Two samples of each species were sequenced and revealed no polymorphism between individuals. A single sequence was therefore chosen for alignment to a set of database sequences for phylogenetic analysis. Our multilocus gene phylogeny was based on the four successfully amplified loci (*coxA*, *gatB*, *hcpA* and *ftsZ*) used by Baldo *et al.* (2006) and includes many *Wolbachia* strains identified as from A and B supergroups. Sequences are accessible from the GenBank database under Accession nos KF531859 to KF531890. *Wolbachia* from *Brugia malayi* was used as an outgroup. As more sequences are available for *wsp* gene (in our sampling as well as in GenBank database), the analysis was

conducted separately for *wsp* sequences for fine-scale genotyping of *Wolbachia* harboured by *Megastigmus* species because this gene often evolves rapidly (GenBank Accession nos KF531891 to KF531900).

For all data sets, alignments were initially generated using the MUSCLE software (Edgar 2004) implemented in CLC Main Workbench version 6.7.1 (CLC Bio). Phylogenetic analyses were performed using maximum-likelihood (ML) inference with PhyML version 3.0 (Guindon *et al.* 2010). The appropriate model of evolution was evaluated with jModeltest version 0.1.1 (Posada 2008). The models selected were TIM1+G for *coxA* and *gatB*, TIM3+G for *ftsZ* and *hcpA* and GTR+I+G for the concatenated MLST data set (*coxA* + *gatB* + *ftsZ* + *hcpA*) and for the *wsp* gene. The robustness of the nodes was assessed with 100 bootstrap replicates. Additionally, Bayesian inferences were also used to reconstruct phylogenies with MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003) using appropriate settings leading to convergence between two independent runs. Finally, trees were edited with Figtree version 1.4.0 (A. Rambaut, <http://tree.bio.ed.ac.uk/software/figtree>).

Megastigmus. The same procedure as for *Wolbachia* data was conducted on COI gene sequences. Sequences are accessible from the GenBank database under Accession nos KF531833 to KF531858. Models of substitution selected were GTR+I+G for the COI gene.

Analysis of congruence. The Shimodaira–Hasegawa (SH) and approximately unbiased (AU) tests were used to evaluate the significance of topological incongruence among trees. First, to check for some methodological artefacts, topologies obtained with the two methods (ML and Bayesian inferences) were compared and showed no significant difference. Second, for *Wolbachia* phylogenies, we compared topologies obtained with the different loci and also with the concatenated data set. These comparisons were made on the entire data set (i.e. the *Wolbachia* from *Megastigmus* species and other sequences retrieved from GenBank) and for the *Wolbachia* infecting *Megastigmus* only. Differences in log likelihood between the best ML topology and an alternative topology were used as values of the AU test and the SH test, and then, *P*-values were calculated with Consel program (version 0.1i; Shimodaira & Hasegawa 2001).

Analysis of host–symbiont associations

Parafit statistic was used to test whether hosts and symbionts evolution was independent or whether there was a pattern of co-cladogenesis between them (Legendre *et al.* 2002). The AxiParafit program implemented in the

Copypat software version 2.00.02 (Meier-Kolthoff *et al.* 2007) was used with patristic distances as input matrix and 999 permutations both for COI sequences data and for 28S sequences compared with the *Wolbachia* sequences concatenated data set.

Antibiotic treatments

When thelytoky is induced by symbionts, antibiotic curing of infected thelytokous females is expected to show reversion to male production with elimination of the bacteria (Stouthamer *et al.* 1990; Zchori-Fein *et al.* 1992). Because it was not possible to rear and treat all of the *Wolbachia*-positive *Megastigmus* species due to the variety of their ecological requirements, we chose to focus antibiotic treatments on one model species, *Megastigmus pinsapinis*, to ascertain the association between thelytoky and *Wolbachia* infection. Antibiotic treatments were also performed on a closely related arrhenotokous species, *Megastigmus schimitscheki*, to exclude any toxic effects of antibiotics on *M. pinsapinis* reproduction.

Two hundred *M. pinsapinis* females within 24 h of emergence from seeds of *Cedrus atlantica* (Pinaceae) were split into two groups of 100, one dedicated to antibiotic treatment and the other used as a control. The 100 females to be treated with antibiotics were placed in ventilated plastic tubes and were provided with cotton soaked in a sugared solution containing tetracycline hydrochloride (Sigma) at a final 0.2% concentration in the diet. These females were referred to as being cured. The 100 control females were provided with sugared water only. Both cured and control females were left for 5 days at 19 °C and natural daylight. Fifty females from each of the cured and control groups were then removed and stored at –20 °C in 100% ethanol for subsequent DNA tests for the presence of *Wolbachia* with PCR tests using the *wsp* gene. The remaining females of each group were allowed to lay eggs on young conelets of *C. atlantica* after their introduction in insect-proof bags. Because emergence of a cohort of this species may require up to 5 years due to prolonged diapause and because sex is indistinguishable at the larval stage using morphological characters, proportions of males and females in the progeny of both cured and control females were estimated directly on larvae using flow cytometry (Boivin & Candau 2007). Antibiotic treatments, *Wolbachia* detection and male proportion estimations in the *M. schimitscheki* progeny strictly followed the same procedure as that in *M. pinsapinis*, using 160 newly emerged females to constitute two groups of 80 for the establishment of both cured and control groups, and 30 and 50 females of each group for PCR and progeny production, respectively.

Results

Distribution of thelytoky among phytophagous *Megastigmus* species

A review of the current knowledge of parthenogenesis in the seed-feeding *Megastigmus* group showed a low prevalence of thelytoky (15%) as only 10 of 69 species for which the reproduction mode could be unambiguously established are thelytokous (Table S1, Supporting Information). Thelytoky tends to be more frequently associated with species developing on angiosperms (26%) than on gymnosperms (9%), but the difference was not statistically significant (Fisher's exact test, $P = 0.0729$). Seed wasps of gymnosperms count 4 thelytokous species, all specialized on Pinaceae, specifically in the genus *Abies*, *Larix* and *Cedrus* (Table S1, Supporting Information). Arrhenotokous species of *Megastigmus* are also found on the same host plant genus. Among seed wasp of angiosperms, thelytoky occurs almost exclusively in species specialized on the *Rosa* genus (Rosaceae) except for one on Anacardiaceae (Table S1, Supporting Information).

In order to study the evolutionary history of thelytoky in *Megastigmus*, thelytokous species must be placed on the phylogenetic tree of the *Megastigmus* genus. COI reconstruction was performed on sequences obtained from the 25 species sampled in this study (Fig. 1). There was an ancestral split between the species related to Anacardiaceae and the others. Wasp species related to Rosaceae form two clades, supported by high bootstrap values, with an initial split between the *aculeatus* group on the one hand and all others species associated with Rosaceae on the other hand. This latter clade is sister to a monophyletic clade comprising all species exploiting gymnosperms (posterior probability 0.73). In accordance with previous studies, *Megastigmus* developing on gymnosperms also show specialization, with, for example, species exploiting the Pinaceae forming a monophyletic group (except for *Megastigmus thyoides*, the only species related to Cupressaceae in the Nearctic region, which could rather reflect a secondary change of host family than an ancestral association).

Replacing species reproducing through thelytoky on that tree does not show any strong phylogenetic signal: thelytokous species occur sporadically on the tree. While the ancestral mode of reproduction is difficult to assess, one clear result is that transitions from one mode of reproduction to the other have regularly occurred during the evolutionary history of seed-specialized *Megastigmus*.

Wasp population screenings for endosymbionts

A total of 25 arrhenotokous and thelytokous *Megastigmus* species (15 and 10 species, respectively) were

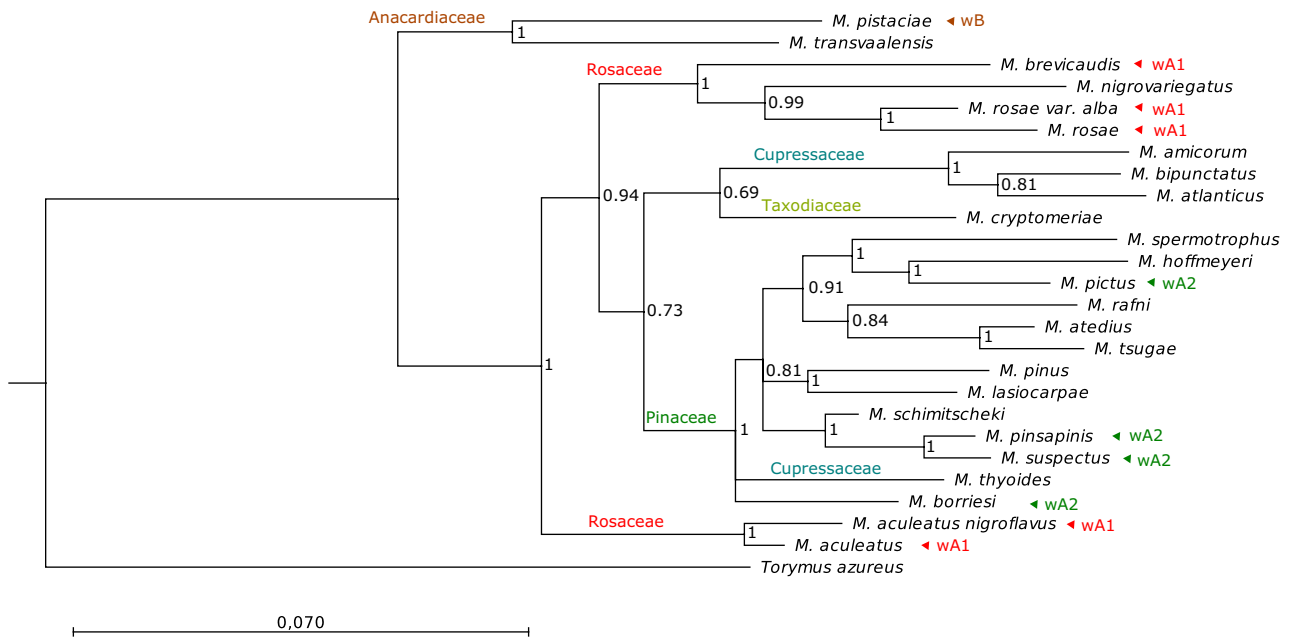


Fig. 1 Bayesian-likelihood inference phylogeny based on cytochrome oxidase I (COI) sequences in seed-specialized wasps of the *Megastigmus* genus (26 taxa, 962 bp). *Tormus azureus* was used as an outgroup. Posterior probability values are indicated at each node. Host plant families are indicated at each branch forming a monophyletic group (except for the Cupressaceae). Anacardiaceae and Rosaceae are angiosperms, and Pinaceae, Cupressaceae and Taxodiaceae are gymnosperms. *Megastigmus* species infected by *Wolbachia* are indicated by arrowheads followed by the name of the *Wolbachia* lineage.

sampled and screened for the presence of *Wolbachia*, *Cardinium*, *Arsenophonus* and *Rickettsia* using specific primers. None of the arrhenotokous species was found infected by any of the targeted endosymbionts (Table 2). On the contrary, *Wolbachia* was found fixed in all of the thelytokous samples tested, while *Cardinium*, *Arsenophonus* and *Rickettsia* were not detected in any of these species (Table 2). The correct amplification of both positive controls and arthropod COI indicated true lack of infection and not a failure in the set-up of the PCRs. These results demonstrate the strong association of *Wolbachia* infection with thelytokous parthenogenesis in *Megastigmus* (Fisher's exact test's P -value < 0.001).

Antibiotic treatments

Tetracycline treatments led to an almost complete loss of *Wolbachia* infection in *Megastigmus pinsapinis* females and a significant recovery of male production, whereas control ones remained infected and produced only females (Table 3). As expected for arrhenotokous species, both control and treated *Megastigmus schimitscheki* females were not infected by *Wolbachia* and produced exclusively males. In addition, for each species, the mean brood size was not significantly altered by tetracycline treatment (Table 3). These data suggest that

arrhenotoky restoration in treated *M. pinsapinis* was due to symbiont curing rather than direct toxicity of antibiotics to insects and that *Wolbachia* is probably the causative agent of thelytoky.

Diversity and distribution of *Wolbachia* strains in *Megastigmus*

Topologies obtained with the four MLST genes were not congruent among each other on the entire data set (34 taxa; all P -values < 0.01 for SH test, < 3.10^{-5} with AU test), as already shown (Baldo *et al.* 2006). In contrast, for the eight *Wolbachia* infecting *Megastigmus* for which sequences have been obtained on the four MLST genes, topologies were globally congruent as all SH tests and most AU were not significant. The only consistent exception was for the *coxA* gene, for which topology was significantly different from the other genes based on AU tests. In relation to that, a closer look at the *Wolbachia* strain infecting *Megastigmus pistaciae* suggests that this strain has undergone a recombination event between A and B *Wolbachia*. Indeed, this strain belongs to the B supergroup based on all sequenced genes, except for *coxA* for which it falls within the A supergroup (Fig. S1, Supporting Information). In summary, despite the complex history of *Wolbachia* strains at a global scale, the tree based on the

Table 2 *Megastigmus* species screened for bacterial infection (+) or noninfection (–) using PCR with *Wolbachia*-, *Cardinium*-, *Arsenophonus*- and *Rickettsia*-specific primers. Each female was screened for all target endosymbionts. The mitochondrial cytochrome oxidase I (COI) gene of the *Megastigmus* host was amplified when no bacterial-specific primers yielded PCR products to test for correct DNA extraction in the procedure

Parthenogenesis and species (N)	<i>Wolbachia</i> (infection %)	<i>Cardinium</i>	<i>Arsenophonus</i>	<i>Rickettsia</i>	Arthropod COI
Thelytoky					
<i>M. aculeatus</i> (43)	+ (100)	–	–	–	
<i>M. aculeatus nigroflavus</i> (21)	+ (100)	–	–	–	
<i>M. brevicaudis</i> (1)	+ (100)	NA	NA	NA	
<i>M. borriesi</i> (1)	+ (100)	NA	NA	NA	
<i>M. pictus</i> (30)	+ (100)	–	–	–	
<i>M. pinsapinis</i> (60)	+ (100)	–	–	–	
<i>M. pistaciae</i> (30)	+ (100)	–	–	–	
<i>M. rosae</i> (30)	+ (100)	–	–	–	
<i>M. rosae alba</i> (5)	+ (100)	–	–	–	
<i>M. suspectus</i> (65)	+ (100)	–	–	–	
Arrhenotoky					
<i>M. amicornum</i> (20)	–	–	–	–	+
<i>M. atlanticus</i> (20)	–	–	–	–	+
<i>M. cryptomeriae</i> (20)	–	–	–	–	+
<i>M. pinus</i> (20)	–	–	–	–	+
<i>M. rafni</i> (30)	–	–	–	–	+
<i>M. spermotrophus</i> (30)	–	–	–	–	+
<i>M. schimitscheki</i> (30)	–	–	–	–	+
<i>M. transvaalensis</i> (20)	–	–	–	–	+

N, number of females tested for infection; +, amplification of the primer; –, no amplification of the primer tested; NA, nonavailable data.

Table 3 *Wolbachia* infection rates in control and tetracycline-treated females of *Megastigmus pinsapinis* (thelytokous) and *Megastigmus schimitscheki* (arrhenotokous), and brood size and proportions of males produced by control and tetracycline-treated females of these species

Species	<i>Wolbachia</i> infection		Progeny produced by treated females		
	Treatment (N*)	% of <i>wsp</i> positives**	Number of mothers	Mean brood size***	Mean % of males
<i>M. pinsapinis</i>	Control (44)	100	44	21.4 ± 5.3 ^a	0
	Tetracycline (42)	4.7	42	18.7 ± 7.8 ^a	92 ± 1.1
<i>M. schimitscheki</i>	Control (30)	0	30	30.3 ± 4.5 ^a	100
	Tetracycline (28)	0	28	32.1 ± 7.5 ^a	100

*Number of treated females.

**Infection rates were determined by PCR using *Wolbachia*-specific primers of the *wsp* gene.

***For each species, similar letters indicate that the mean number of larvae (±SE) produced does not differ significantly using a Kruskal–Wallis test ($P < 0.05$).

concatenated data set is a good mean representation of the history of *Wolbachia* infection in *Megastigmus* (Fig. 2) and is also consistent with the tree obtained on the *wsp* gene (Fig. S2, Supporting Information).

Three highly homogeneous lineages of *Wolbachia* infecting *Megastigmus* species were identified (namely WA1, WA2 and WB). We refer to these as lineages as opposed to strains, because although they are closely related, some substitutions were observed. A strict association between *Wolbachia* lineage and host plant is

observed. The WB lineage is restricted to *M. pistaciae*, the only thelytokous species developing on Anacardiaceae. A second lineage, named WA1, was only found in thelytokous species developing on Rosaceae, that is, *Megastigmus brevicaudis*, *M. rosae* (both subtypes) and *Megastigmus aculeatus* (both subtypes). The third lineage, named WA2, is closely related to WA1 and grouped the *Wolbachia* infecting *M. borriesi*, *Megastigmus pictus*, *M. pinsapinis* and *Megastigmus suspectus*, which all develop on Pinaceae.

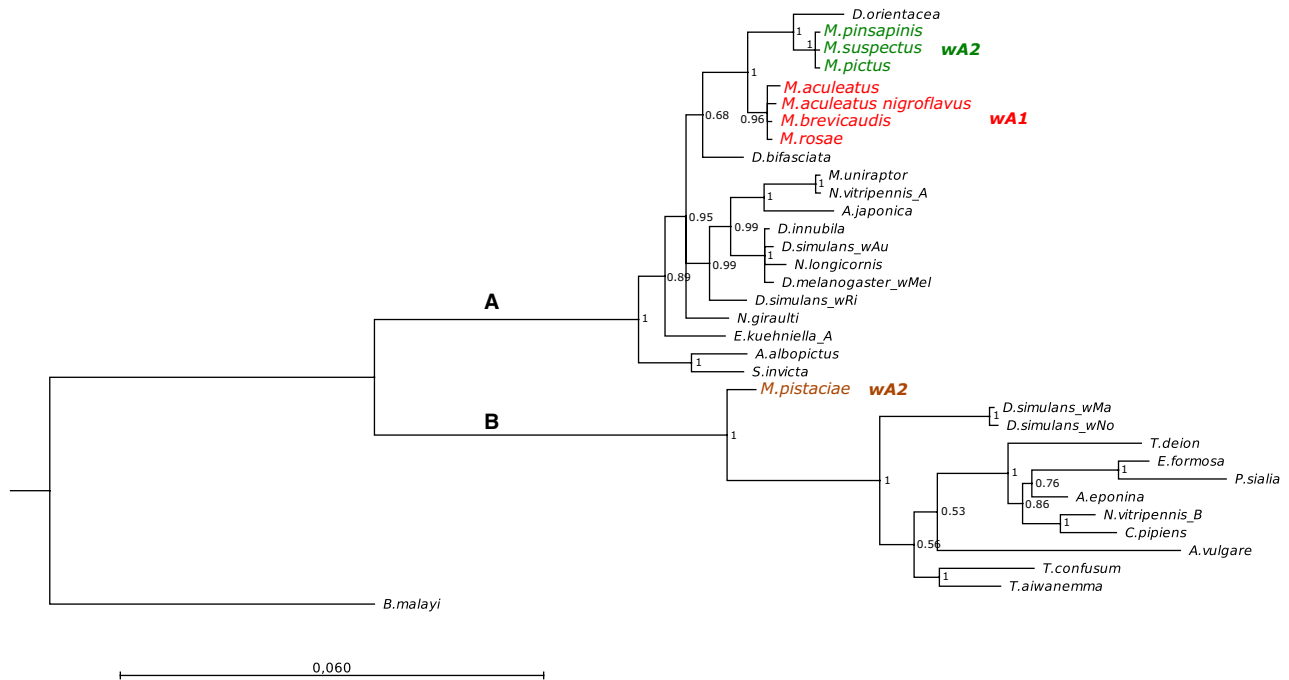


Fig. 2 Phylogenetic placement of the *Wolbachia* strains infecting the seed-specialized wasps *Megastigmus* spp. among other *Wolbachia* belonging to A and B supergroups. Bayesian-likelihood inference phylogenies are shown, while maximum-likelihood analyses gave substantially the same results. *Wolbachia* sequences are labelled with the name of their host. *Wolbachia* of *Brugia malayi* was used as an outgroup. Posterior probability values are indicated at each node. This phylogeny is based on concatenated sequences data set for the four multilocus sequence typing (MLST) loci *coxA*, *gatB*, *ftsZ* and *hcpA* (34 strains, 1650 bp).

Importantly, and despite this strict association between host plant and *Wolbachia* lineage, no significant association was detected between *Wolbachia* and host lineages using Parafit test. In addition, *Wolbachia* (concatenated data set) and *Wolbachia*-infected *Megastigmus* (COI) topologies were not congruent (SH test, $P < 0.014$ and AU test, $P < 0.003$). Such analyses on these two maternal markers exclude both co-cladogenesis and transfer following hybridization, suggesting that horizontal transfers have played a major role in the distribution of the *Wolbachia* strains.

The distribution of *Wolbachia* strains among *Megastigmus* species suggests that horizontal transmission routes are constrained by host plant family, with only one family per strain. Our tree in Fig. 1 shows no exception to this rule, but this could nevertheless be obtained by chance, at least in principle. To evaluate how likely this is, we performed a simple test, stating as our null hypothesis that a strain can appear in an insect irrespective of the plant it parasitizes. Because infection with PI-*Wolbachia* rapidly leads to the irreversible loss of sexual reproduction, rendering *Wolbachia* indispensable for the insect, infection loss is highly improbable (Stouthamer *et al.* 2010). Under this hypothesis, from the 10 infected species, at least seven independent *Wolbachia* acquisitions have occurred during *Megastigmus*

evolution. Indeed, in three cases, it is not possible to distinguish between independent acquisition and ancestral infection (e.g. in *M. rosae* and *M. rosae* var. *alba*). In all other cases, the presence of an uninfected species within the clade excludes ancestral acquisition. Using the constraint solver clingo (Gebser *et al.* 2011), we first enumerated all possible scenarios with seven independent *Wolbachia* acquisitions (at least one by strain) possibly occurring at each node of the actual *Megastigmus* phylogeny and leading to 10 infected extant species. Among all these scenarios, we reported the proportion p of cases where a strain is strictly specific of a plant family and obtained a value for p of 0.0051. This result indicates that the probability of getting such a plant-specific distribution by chance is exceptional, which constitutes reasonable evidence that the events of horizontal transmission are not occurring randomly, but are rather influenced by the host plant.

Discussion

Distribution and endosymbiotic origin of thelytoky in phytophagous Megastigmus species

A few hundred species in all of the major animal groups are characterized by thelytokous parthenogenesis;

its patchy taxonomic distribution in Hymenoptera is consistent with several independent evolutionary origins from ancestral arrhenotokous species (Cook 1993). The relatively high prevalence of thelytoky in Hymenoptera might reflect that some mechanisms of their haplodiploid sex determination are rather easily redirected to thelytoky by mutations (Lattorff *et al.* 2005) or bacteria (Cordaux *et al.* 2011). Our literature review of parthenogenesis in the *Megastigmus* genus estimated the prevalence of thelytoky to 15%, which supported the assumption that arrhenotoky is the dominant and ancestral form of parthenogenesis in these phytophagous wasps. Thelytoky was unambiguously described in species exploiting diverse habitats, although it appears to occur in only three of the ten host plant families currently known to host *Megastigmus* species in both gymnosperms and angiosperms (Pinaceae, and Rosaceae and Anacardiaceae, respectively). Interestingly, several thelytokous species share congeneric host plants (e.g. on the *Rosa* genus), but each group of thelytokous species associated with Pinaceae, Rosaceae or Anacardiaceae remains confined at the host family taxonomic level. This observation supports the potential for intimate ecological connections within a given host plant family, but not between different host plant families.

Thelytoky may be under the control of the insect itself or its endosymbionts (Stouthamer *et al.* 1990; Vavre *et al.* 2004). Strict association between thelytoky and *Wolbachia* infection and reversibility of thelytoky through antibiotic treatments provide a particularly strong support for an endosymbiotic origin of thelytoky in *Megastigmus* wasps. Within the Hymenoptera, *Wolbachia*-induced thelytoky is found in three superfamilies: the Cynipoidea (Rokas *et al.* 2002), the Braconidae (Kremer *et al.* 2009) and the Chalcidoidea (Stouthamer 1997), within which only entomophagous families were concerned. To our knowledge, we document here the first case of *Wolbachia*-induced thelytoky in phytophagous chalcids.

Host plant specialization matters in the spread of thelytoky

The phylogenetic tree of the *Megastigmus* genus including all main ecological groups (angiosperm- and gymnosperm-specialized seed wasps) indicated that thelytokous *Megastigmus* species do not form a monophyletic group, but occur sporadically on the COI phylogenetic tree (Fig. 1). This pattern is consistent with the hypothesis of independent evolutionary origins of thelytokous species from ancestral arrhenotokous species (Cook 1993), and thus, that transitions from arrhenotoky to thelytoky have repeatedly occurred. While transition from arrhenotoky to thelytoky is possible, the reverse is much more constrained and even impossible after

sexual traits have decayed (Jeong & Stouthamer 2005; Kremer *et al.* 2009).

The phylogenetic tree obtained on the concatenated MLST data set provided critical insights on the history of *Wolbachia* infection in *Megastigmus*. Three homogeneous lineages of *Wolbachia* (WA1, WA2 and WB) were identified and shown to be associated with the host plant families of their hosts (Fig. 1). Although both lineages were found closely related, WA1 infected exclusively thelytokous wasp species exploiting the Rosaceae, while WA2 was detected only in those exploiting the Pinaceae. The third lineage WB was restricted to the only currently known thelytokous wasp species exploiting Anacardiaceae (*M. pistaciae*). More interestingly, there was no statistical support for an association between *Wolbachia* and host lineages. For example, WA1 is found in five species developing on Rosaceae, but belonging to highly divergent clades (10% COI). Similarly, WA2 is only found in four species exploiting Pinaceae, despite a divergence of as high as 5–6% on COI among these species. On the opposite, we found a nonrandom association between *Wolbachia* strains and the host plant used by the insects. Altogether, this led us to postulate on the one hand that thelytoky may have spread across the *Megastigmus* genus through horizontal transmission of *Wolbachia* among wasp species and on the other hand that these transfers occurred preferentially between species exploiting similar host plants.

Within-community horizontal transmission of *Wolbachia* is likely to result from ecologically mediated pathways such as host–parasitoid associations (Vavre *et al.* 1999; Huigens *et al.* 2004). Shared feeding and breeding sites may also act as a route through which *Wolbachia* can be transmitted from one host species to another (Sintupachee *et al.* 2006). Primary infection by *Wolbachia* has to face two main filters (Vavre *et al.* 2003). First, *Wolbachia* must come into contact with the recipient species (i.e. has to pass the encounter filter). Second, *Wolbachia* has to evade the host immune system and to develop in the new host (i.e. has to pass the compatibility filter), which is facilitated by the relatedness of the donor and recipient species. Community structure may affect both filters and thus impact the epidemiology of *Wolbachia* across species, as recently showed theoretically using small-world networks (Zug *et al.* 2012). Whether communities are composed of generalist or specialist species, and of distantly or closely related species, will clearly impact these two filters. On the one hand, interspecific horizontal transmission of *Wolbachia* may be favoured in particular habitats that are attractive for a wide set of generalist host species, which will simultaneously feed and develop on it and/or which can be connected by shared parasitoids (Stahlhut *et al.*

2010). The situation encountered in *Trichogramma* may be representative of such case with the existence of a specialized PI-*Wolbachia* clade infecting these generalist wasps, but within which horizontal transmission is rampant (Schilthuizen & Stouthamer 1997). On the other hand, intimate interspecific connections between host species may also arise from host plant specialization, which may favour *Wolbachia* transmission due to narrow shared ecological niches. This situation opens widely the encounter filter, but also restricts the host spectrum to the few species exploiting this particular resource. When species within the community are moreover closely related species exploiting the same resource, the compatibility filter will also be open and facilitates symbiont infection. This latter situation is probably the one encountered in the seed-feeding *Megastigmus* analysed here, revealing two essential features of the spread of thelytoky in the context of insect ecological specialization.

First, the COI reconstruction of the present 25 *Megastigmus* species strongly confirmed host plant specialization in this genus (Fig. 1). Within the species on angiosperms, the clade of species attacking Anacardiaceae first diverged. Within one of the sister groups, wasp species exploiting gymnosperms form a monophyletic group and even show specialization, as depicted by the monophyletic group developing on the Pinaceae (except for *Megastigmus thyoidea*). The existence of a common *Wolbachia* lineage in all wasps exploiting the Rosaceae (WA1) and another one in all those developing on the Pinaceae (WA2) suggests that host ecological specialization can promote the spread of thelytoky through seed use. All *Megastigmus* females oviposit during a rather narrow period of the development of the host reproductive structures and progeny develop exclusively within seeds at a final density of one larva per seed, while several eggs can be found within the same seed (Turgeon *et al.* 1994; Rouault *et al.* 2004; T. Boivin, personal observation). On both wild roses and Pinaceous trees, many *Megastigmus* species show some restricted behavioural plasticity allowing shifts onto different congeneric host species or even onto a new host genus (Grissell 1999; Auger-Rozenberg & Roques 2012). The possibility of short-range host shifts may have favoured novel wasp assemblages and novel opportunities for them to interact and potentially exchange symbionts. If *Wolbachia* infection occurs mainly via ingestion during the early larval stages, we suggest that both seed tissues and cannibalism by larvae competing for the seed tissues may facilitate interspecific horizontal transfers of the endosymbiont. A similar pattern of endosymbionts sharing among sibling species competing for a common resource has recently been shown in weevils (Merville *et al.* 2013). Additionally, we cannot

rule out a *Wolbachia* transfer involving an unsampled community member, which would harbour the same strain and transmit it into a wasp clade serially or as a single event followed by horizontal transfer among wasps. Although shared parasitoids between phytophagous *Megastigmus* spp. have been sporadically observed (Mailleux *et al.* 2008; M.-A. Auger-Rozenberg, personal observation), host-parasitoid associations in this genus remain too poorly documented to formally invoke this *Wolbachia* horizontal route at this point. Expanding our investigations to other co-occurring species within wasp habitats may be a further key step to understand how a wasp clade can be originally infected.

Another key result of this study is that *Megastigmus* ecological specialization at the host plant family level probably constrained the invasion of *Wolbachia* lineages throughout the whole host genus. Indeed, diverging strategies in the use of angiosperms and gymnosperms may be an essential feature of the strict host radiation depicted here in the evolutionary history of *Megastigmus*. For this reason, intimate ecological connections promoting horizontal transfers of *Wolbachia* (see above) are thus unlikely to arise at this community scale. More interestingly, thelytoky appears to have still not invaded wasps developing on the Cupressaceae (Fig. 1, Table S1, Supporting Information). According to Auger-Rozenberg *et al.* (2006), wasps developing on the Cupressaceae exhibit a higher level of host specificity, because they seem host species specific, while wasps on Pinaceae or Rosaceae are specialized to particular host genera, but frequently attack several congeneric species if they occur in sympatry. This is consistent with a taxonomic radiation following initial host adaptation, which might have constituted an efficient barrier to the spread of thelytoky in this group, due to unlikely opportunities for interspecific horizontal transfers of *Wolbachia*.

Altogether, our results show that ecological specialization can be a driving force of the spread of endosymbiotic thelytoky, but also a constraint. This further reinforces the hypothesis that community structure of insects is a major driver of the epidemiology of endosymbionts (Ferrari & Vavre 2011) and that competitive interactions among closely related species may facilitate horizontal transmission (Merville *et al.* 2013).

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M.-A.A.R., F.V. and T.B. conceived and designed the study. M.-A.A.R., A.R. and T.B. acquired the samples and provided funding support. E.M., C.G., J.-N.C. M.-A.A.R. and T.B. performed the experiments and produced the data. H.H., M.-A.A.R., F.V., P.V. and T.B. analysed the data and wrote the paper. All authors have checked and approved the final version of the manuscript.

Data accessibility

Wolbachia and wasp DNA sequences: GenBank Accession Nos KF531859–KF531890 and KF531891–KF531900 and KF531833–KF531858, respectively.

Sequence alignments, tree files and museum accession number have been deposited in TreeBASE: <http://purl.org/phylo/treebase/phyloids/study/TB2:S15268?x-access-code=5077b288616e8249eae95b31f48742e8&format=html>.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Phylogenetic placement of *Wolbachia* infecting the seed-specialized wasps *Megastigmus* spp., among other *Wolbachia* strains belonging to A and B supergroups, based on the sequences of each of the four multilocus sequence typing (MLST) genes used in this study (*coxA*, *gatB*, *ftsZ* and *hcpA*).

Fig. S2 Phylogenetic placement of the *Wolbachia* strains infecting the seed-specialized wasps *Megastigmus* spp., among

other *Wolbachia* strains belonging to A and B supergroups, based on *wsp* sequences of 49 strains (530 bp).

Table S1 Literature review of the distribution of arrhenotoky, thelytoky and host plant specialization among the seed-feeding wasps of the *Megastigmus* genus.

Table S2 The primer pairs used for endosymbiont PCR screening in *Megastigmus* spp.