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

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

3

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23 **Running title:** Spread of thelytoky in phytophagous wasps

24 **Abstract**

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

46

47 **Introduction**

48 Sex is by far the most predominant mode of reproduction among eukaryotes, such that only  
49 one out of a thousand animal species shows some type of asexual reproduction  
50 (Suomalainen *et al.*, 1987). Among the diverse mechanisms involved in asexual reproduction,  
51 parthenogenesis is defined *sensus stricto* as the development of an egg without fertilization.  
52 The origin of parthenogenesis is polyphyletic in both invertebrates and vertebrates,  
53 suggesting that systems maintaining sexuality are labile and stimulating elucidation of the  
54 evolutionary routes to parthenogenesis (reviewed in Simon *et al.*, 2003). Spontaneous loss of  
55 sexuality may occur through mutations or intra- and interspecific hybridization (Simon *et al.*,  
56 2003; Dedryver *et al.*, 2001), but it can also result from infection by bacterial endosymbionts  
57 that are predominantly transmitted vertically through the female egg cytoplasm and distort  
58 the host sex ratio towards females to their own advantage (Engelstädter and Hurst, 2009).

59 In the insect order Hymenoptera, several forms of parthenogenesis have been  
60 defined according to the sex of offspring produced by a virgin female (Cook, 1993; Cook and  
61 Crozier, 1995). The dominant and ancestral form of parthenogenesis is arrhenotoky, in which  
62 fertilized eggs develop as diploid females and unfertilized eggs develop as haploid males.  
63 However, numerous species display thelytoky, which is a form of complete parthenogenesis  
64 where unfertilized eggs develop into diploid females. Thelytoky can be under the control of  
65 the insect itself or its endosymbionts (Stouthamer *et al.*, 1990; Vavre *et al.*, 2004).  
66 Parthenogenesis inducers include bacteria of the genera *Wolbachia* (Werren, 1997),  
67 *Cardinium* (Zchori-Fein and Perlman, 2004) and *Rickettsia* (Hagimori *et al.*, 2006). While

68 induction of male production is impossible in genetically-based thelytoky, endosymbiotic  
69 thelytoky can be characterized by its reversibility, as antibiotic or heat treatment of females  
70 leads to bacteria elimination and production of males in their progeny (Stouthamer *et al.*,  
71 1990). Generally, infection by parthenogenesis-inducing (PI) *Wolbachia* is fixed within  
72 species or populations and results in an obligate parthenogenesis associated with a loss of  
73 sexual function in females (Jeong and Stouthamer 2005; Kremer *et al.*, 2009), or in both  
74 sexes (Gottlieb and Zchori-Fein, 2001), making *Wolbachia* indispensable. Only in very rare  
75 cases, polymorphism of infected and uninfected females is maintained (REF).

76         Despite vertical transmission via the egg cytoplasm being the predominant  
77 transmission mode of PI endosymbionts (Werren, 1997), phylogenetic incongruence  
78 between host and endosymbiont histories strongly indicates occasional horizontal  
79 transmission events (O'Neill *et al.*, 1992; Stouthamer *et al.*, 1993; Baldo *et al.* 2006; Perlman  
80 *et al.*, 2010). Horizontal transmissions of *Wolbachia* have been successfully demonstrated  
81 experimentally by injection (Braig *et al.*, 1994; Grenier *et al.*, 1998), introgression (Jaenike,  
82 2007), maintenance of close contacts between conspecifics (Rigaud and Juchault, 1995) or in  
83 host-parasitoid associations (Vavre *et al.* 1999). Effective horizontal transmission of  
84 endosymbionts depends (i) on intimate ecological associations which provide within-  
85 community horizontal transmission opportunities (Vavre *et al.*, 1999; Sintupachee *et al.*,  
86 2006; Stahlhut *et al.*, 2010) and (ii) on the phylogenetic similarity of donor and recipient host  
87 species because internal defence mechanisms against infections are likely to be more similar  
88 between closely related hosts (Stahlhut *et al.*, 2010). Studies addressing how insect  
89 phylogeny and ecology affect patterns of similarity between strains of PI endosymbionts  
90 would help gain critical insights on how these two forces affect shifts in reproduction modes.

91 In particular, study systems involving several groups of closely related thelytokous species  
92 but displaying different levels of ecological proximity due to habitat specialization can be of  
93 critical interest to define the possible ecological boundaries to the evolutionary trajectory of  
94 thelytoky in insects.

95 In this paper, we address the potential for phylogeny, ecology and infection by PI  
96 endosymbionts to drive the spread of asexuality within insects. For this purpose, we focused  
97 on the genus *Megastigmus* (Hymenoptera: Torymidae), which counts more than 125  
98 arrhenotokous and thelytokous wasp species worldwide (Grissell, 1999). Half of these  
99 species are specialist seed feeders, the other half are presumed to be parasitoids, gall-  
100 makers or to have unknown hosts. Within the seed-specialized group, two thirds of species  
101 exploit gymnosperm hosts (Pinaceae and Cupressaceae families), whereas one third exploits  
102 preferentially angiosperms, especially the Rosaceae and Anacardiaceae families (Roques &  
103 Skrzypczynska, 2003). Being highly specialized in the seed resource, several wasp species  
104 exploiting the same host strictly require the same ecological niche, which is then particularly  
105 propitious to direct intra- or interspecific interactions within this insect group (Boivin *et al.*,  
106 2008; Auger-Rozenberg and Roques, 2012). In the genus *Megastigmus*, the dominant and  
107 ancestral form of parthenogenesis is by far arrhenotoky as thelytoky characterizes only a  
108 minority of species (Grissell, 1999), but both arrhenotokous and thelytokous species can  
109 occur in sympatry on gymnosperm or on angiosperm hosts (Boivin *et al.*, 2008; Auger-  
110 Rozenberg and Roques, 2012). Thelytoky has only been established from records of highly  
111 female-biased sex ratios in field populations, but neither its determinism nor its evolutionary  
112 trajectory have been elucidated yet. We first performed a large literature survey to examine  
113 the distribution of thelytoky in *Megastigmus* across their known obligate host-plant families.

114 Second, we tested for thelytoky caused by endosymbionts by screening in a large sample of  
115 arrhenotokous and thelytokous species for endosymbionts and by performing antibiotic  
116 treatments. Finally, we performed phylogenetic reconstructions to examine the evolution of  
117 endosymbiont-mediated thelytoky in *Megastigmus* and its possible connections to host-  
118 plant specialization. This study strongly suggests that thelytoky is caused by *Wolbachia*  
119 endosymbionts, and interestingly, we show that host plant specialization is a key  
120 determinant of *Wolbachia* infection and thelytoky.

121

## 122 **Material and Methods**

### 123 *Distribution of thelytoky among phytophagous Megastigmus species*

124 Parthenogenesis, *i.e.* arrhenotoky or thelytoky, is the only mode of reproduction in the  
125 *Megastigmus* genus. It has never been formally studied and it was generally deduced from  
126 sex ratio estimates in field sampled wasp populations. Here, we used extensive catalogs of  
127 seed-feeding *Megastigmus* species to gather the current knowledge on the relative  
128 prevalence of arrhenotoky and thelytoky in the genus *Megastigmus* (Grissell, 1999; Roques  
129 & Skrzypczynska, 2003; Roques *et al.*, 2003; Auger-Rozenberg *et al.*, 2006). A bibliographic  
130 search of non-redundant articles in the Web of Science (2003-2012) and CAB abstracts  
131 (2003-2012) was also performed on key terms '*Megastigmus* and seed\*' to check for  
132 potential new species descriptions or increase knowledge in the biology of the species  
133 described in the above catalogs. We considered only wasp species for which arrhenotoky or  
134 thelytoky were ascertained by authors, or for which male frequencies estimated at  
135 emergence from sampled seed lots provided unambiguous support for arrhenotoky (high  
136 male abundance) or thelytoky (absence or <1%). Species for which the parthenogenesis

137 mode was not mentioned or for which sample sizes were insufficient (<10) to estimate sex  
138 ratios were not included. The prevalence of thelytoky was assessed as the percentage of  
139 thelytokous species in all of the selected wasp species. The distributions of both arrhenotoky  
140 and thelytoky relative to host plant specialization was assessed by assorting both  
141 arrhenotokous and thelytokous wasp species according to their host plant group  
142 (gymnosperm or angiosperm), family and genus.

143

#### 144 *Wasp sampling*

145 *Megastigmus* species were collected across the Northern Hemisphere (Nearctic and  
146 Palearctic) by seed samplings on diverse host plants. Insect-infested seeds were separated  
147 from non-infested ones by X-ray radiography (using Faxitron-43855<sup>®</sup>, 15 kV, 3 mA, 3'30" to  
148 4'30" depending on seed species with X-ray-sensitive films Kodak 'Industrex M', and  
149 Faxitron-MX20<sup>®</sup>, 20 kV, 0.3 mA, 1'45" with an EZ20 digital scanner). The insect-infested seeds  
150 were placed in individual rearing boxes stored in outdoor insectaries located at INRA,  
151 Orléans, France and at INRA, Avignon, France. Adult emergence was recorded over the 3  
152 years following seed maturation because of a possible prolonged diapause (Roques &  
153 Skrzypczynska, 2003). After emergence, insect species determination was ascertained using  
154 the morphological keys of Grissell (1999) and Roques and Skrzypczynska (2003). Emerged  
155 insects were then sorted by species and collection site and preserved in 100% alcohol at -  
156 20°C. A total of 25 *Megastigmus* species were used in this study, 10 were thelytokous and 15  
157 were arrhenotokous. These species, their reproduction mode, their host-plants, collecting  
158 localities and distributions are summarized in Table I.

159

#### 160 *DNA extraction*



161 Total genomic DNA was isolated by crushing individually whole adult *Megastigmus* females  
162 using two different procedures, depending on sample size and further DNA use. Screening  
163 for PI endosymbiont infection, which involves large sample sizes, was performed using the  
164 Chelex method. This fast and cheap method consisted of digesting tissues kept for 2 h at  
165 56°C in a 200 µl 10% Chelex solution (Biorad). After 30 min at 100°C, samples were  
166 centrifuged and supernatants were used as DNA sources. For sequencing and further  
167 phylogenetic analyses, which requires smaller sample sizes and long term conservation of  
168 DNA, we used the DNeasy extraction Tissue Kit (Qiagen). Total genomic DNA was eluted in  
169 200 µl of AE buffer in this case. In addition, a PCR was systematically carried out on Qiagen  
170 extractions with *Wolbachia* primers in order to confirm the lack of bias in the detection of  
171 the symbiont due to different methods of DNA extraction.

172

### 173 *Wasp sequences*

174 We used Sigma RedTaq for PCR amplification. The forward and reverse primers used were  
175 1775-COI-F (Clyde, 5'-CGAATAAATAATATAAGATTTTG-3'), and 2773-COI-R (Bonnie, 5'-  
176 GGATAATCTCTATATCGACGAGGTAT-3') (Scheffer & Grissell 2003) for the segment of  
177 the cytochrome oxidase I (COI) gene. Cycling programs were as follows: a denaturation  
178 step at 94°C for 1 min, annealing for 1 min at 48°C, and extension at 72°C for 1 min with 30  
179 cycles being performed. All PCR products were then purified with a QIAquick PCR  
180 purification kit (Qiagen) and were directly sequenced with the amplification primers.  
181 Sequencing was performed using the big-dye terminator sequencing kit (PE Applied  
182 Biosystem) and carried out with an ABI 3100 automatic sequencer. The gene fragment was  
183 sequenced on two to four individuals for each species, except for cryptic and rare species  
184 because of the small number of specimens available. When individuals were identified as

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

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

196

#### 197 *Population screenings for PI-endosymbiont infection*

198 In a wide range of insect species, sex ratio distortion has been associated with infection by  
199 diverse bacterial symbionts such as *Wolbachia* (Werren, 1997), *Cardinium* (Zchori-Fein and  
200 Perlman, 2004), *Arsenophonus* (Thao and Baumann, 2004) and *Rickettsia* (Hagimori et al,  
201 2006). As a first test for an association between such endosymbionts and thelytoky in the  
202 *Megastigmus* genus, we screened for their presence in both arrhenotokous and thelytokous  
203 species (listed in Table 1). Separate Polymerase Chain Reactions (PCR) were performed on  
204 each *Megastigmus* female using specific primers for *Wolbachia* surface protein (wsp) (Braig  
205 *et al.*, 1998), *Cardinium* 16S rRNA (Zchori-Fein and Perlman, 2004), *Arsenophonus* 23S rDNA  
206 (Thao and Baumann, 2004) and *Rickettsia* 16S rRNA (Hagimori et al, 2006) gene fragments.  
207 PCR reactions were done in a 25- $\mu$ l final volume reaction containing 200  $\mu$ M dNTP, 10 pM  
208 primers, 0.5 IU HotStarTaq<sup>®</sup> DNA polymerase, and 1.5  $\mu$ l DNA solution. All reactions were

209 run in a ABI thermal cycler (PE Applied Biosystems PCR System 9700) with an initial  
210 denaturing step at 94°C for 4 min, an annealing step for 1 min, an elongation step at 72°C for  
211 1 min 30s and a final extension step of 72°C for 1 min 30 s. Annealing temperatures and  
212 primer pairs used for *Wolbachia*, *Cardinium*, *Arsenophonus* and *Rickettsia* PCR screening are  
213 detailed in Table S2. All PCR reactions included a positive control of *Asobara tabida*  
214 (*Wolbachia* infected) and *Bemisia tabaci* biotype Q (*Cardinium*, *Arsenophonus* or *Rickettsia*  
215 infected) samples. For each bacterial gene, 5 µl of amplified reaction product was run on a  
216 1% agarose gel stained with ethidium bromide after the PCRs in order to determine the  
217 presence of an amplified DNA fragment. If a sample of a *Megastigmus* species was negative  
218 for any bacterial-specific amplification, but COI amplification succeeded, the insect was  
219 considered uninfected. When amplification with a bacterial-specific primer yielded visible  
220 bands, the insect was considered infected, and samples were kept for sequencing and strain  
221 characterization.

222

### 223 *Wolbachia* strain characterization

224 Only *Wolbachia* was detected in our samples (see Results section). We used multilocus  
225 sequence typing (MLST) (Baldo *et al.*, 2006) to characterize *Wolbachia* strain similarity  
226 among *Megastigmus* host species. A *wsp* sequence was also obtained from each infected  
227 insect. MLST was based on the methods of Baldo *et al.* (2006) using the standard primers  
228 that amplify the five conserved *Wolbachia* genes *hcpA*, *ftsZ*, *gatB*, *coxA* and *fbpA*. These are  
229 degenerate primers designed to amplify sequences from diverse *Wolbachia* strains. Despite  
230 repeated attempts, we could not amplify the *fbpA* gene. Thus, four MLST loci (*hcpA*, *ftsZ*,  
231 *gatB* and *coxA*), plus the *wsp* gene, were sequenced in both directions to provide double

232 coverage of the region of interest, with the exception of both *M. borriesi* and *M. rosae* var.  
233 *alba*, which could be sequenced for the *wsp* gene only.

234

#### 235 *Phylogenetic analyses*

236 *Wolbachia*. Two samples of each species were sequenced and revealed no polymorphism  
237 between individuals. A single sequence was therefore chosen for alignment to a set of  
238 database sequences for phylogenetic analysis. Our multi-locus gene phylogeny was based on  
239 the four successfully amplified loci (*coxA*, *gatB*, *hcpA*, *ftsZ*) used by Baldo *et al.* (2006) and  
240 includes many *Wolbachia* strains identified as from A and B supergroups. Sequences are  
241 accessible from the GenBank database under accession numbers KF531859 to KF531890.  
242 *Wolbachia* from *Brugia malayi* was used as an outgroup. As more sequences are available  
243 for *wsp* gene (in our sampling as well as in Genbank database) the analysis was conducted  
244 separately for *wsp* sequences for fine scale genotyping of *Wolbachia* harboured by  
245 *Megastigmus* species since this gene often evolves rapidly (Genbank accession numbers  
246 KF531891 to KF531900).

247 For all datasets, alignments were initially generated using the MUSCLE software (Edgar,  
248 2004) implemented in CLC Main Workbench v6.7.1 (CLC Bio). Phylogenetic analyses were  
249 performed using maximum likelihood (ML) inference with PhyML v3.0 (Guindon *et al.*, 2010).  
250 The appropriate model of evolution was evaluated with jModeltest v0.1.1 (Posada, 2008).  
251 The models selected were TIM1+G for *coxA* and *gatB*, TIM3+G for *ftsZ* and *hcpA* and  
252 GTR+I+G for the concatenated MLST data set (*coxA* + *gatB* + *ftsZ* + *hcpA*) and for the *wsp*  
253 gene. The robustness of the nodes was assessed with 100 bootstrap replicates. Additionally,  
254 bayesian inferences were also used to reconstruct phylogenies with MrBayes v3.1.2

255 (Ronquist & Huelsenbeck, 2003) using appropriate settings leading to convergence between  
256 two independent runs. Finally, trees were edited with Figtree v1.4.0 (A. Rambaut,  
257 <http://tree.bio.ed.ac.uk/software/figtree>).

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

261 *Analysis of congruence*. The Shimodaira-Hasegawa (SH) and approximately unbiased (AU)  
262 tests were used to evaluate the significance of topological incongruence among trees. First,  
263 to check for some methodological artifacts, topologies obtained with the two methods (ML  
264 and bayesian inferences) were compared and showed no significant difference. Second, for  
265 *Wolbachia* phylogenies, we compared topologies obtained with the different loci and also  
266 with the concatenated data set. These comparisons were performed on the entire dataset  
267 (i.e. the *Wolbachia* from *Megastigmus* species and other sequences retrieved from  
268 Genbank) and for the *Wolbachia* infecting *Megastigmus* only. Differences in log likelihood  
269 between the best ML topology and an alternative topology were used as values of the AU  
270 test and the SH test and then p-values were calculated with Consel program (v0.1i)  
271 (Shimodaira & Hasegawa, 2001).

272

273 *Analysis of host-symbiont associations*

274 ParaFit statistic was used to test whether hosts and symbionts evolution were independent  
275 or if there was a pattern of co-cladogenesis between them (Legendre *et al.*, 2002). The  
276 AxParaFit program implemented in the Copycat software v2.00.02 (Meier-Kolthoff *et al.*,  
277 2007) was used with patristic distances as input matrix and 999 permutations both for *COI*

278 sequences data and for 28S sequences compared with the *Wolbachia* sequences  
279 concatenated data set.

280

### 281 *Antibiotic treatments*

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

290 Two hundred *M. pinsapinis* females within 24h of emergence from seeds of *Cedrus atlantica*  
291 (Pinaceae) were split into two groups of 100, one dedicated to antibiotic treatment and the  
292 other used as a control. The 100 females to be treated with antibiotics were placed in  
293 ventilated plastic tubes and were provided with cotton soaked in a sugared solution  
294 containing tetracycline hydrochloride (Sigma) at a final 0.2% concentration in the diet. These  
295 females were referred to as being cured. The 100 control females were provided with  
296 sugared water only. Both cured and control females were left for 5 days at 19°C and natural  
297 daylight. Fifty females from each of the cured and control groups were then removed and  
298 stored at -20°C in 100% ethanol for subsequent DNA tests for the presence of *Wolbachia*  
299 with PCR tests using the *wsp* gene. The remaining females of each group were allowed to lay  
300 eggs on young conelets of *C. atlantica* after their introduction in insect-proof bags. Because

301 emergence of a cohort of this species may require up to 5 years due to prolonged diapause  
302 and because sex is indistinguishable at the larval stage using morphological characters,  
303 proportions of males and females in the progeny of both cured and control females were  
304 estimated directly on larvae using flow cytometry (Boivin and Candau, 2007). Antibiotic  
305 treatments, *Wolbachia* detection and male proportion estimations in the *M. schimitscheki*  
306 progeny strictly followed the same procedure as that in *M. pinsapinis*, using 160 newly  
307 emerged females to constitute two groups of 80 for the establishment of both cured and  
308 control groups, and 30 and 50 females of each group for PCR and progeny production,  
309 respectively.

310

## 311 **Results**

### 312 *Distribution of thelytoky among phytophagous Megastigmus species*

313 A review of the current knowledge of parthenogenesis in the seed-feeding *Megastigmus*  
314 group showed a low prevalence of thelytoky (15%) as only 10 out of 69 species for which the  
315 reproduction mode could be unambiguously established are thelytokous (Table S1).  
316 Thelytoky tends to be more frequently associated with species developing on angiosperms  
317 (26%) than on gymnosperms (9%), but the difference was not statistically significant (Fisher's  
318 exact test,  $P=0.0729$ ). Seed wasps of gymnosperms count 4 thelytokous species, all  
319 specialized on Pinaceae, specifically in the genus *Abies*, *Larix* and *Cedrus* (Table S1).  
320 Arrhenotokous species of *Megastigmus* are also found on the same host plant genus. Among  
321 seed wasp of angiosperms, thelytoky occurs almost exclusively in species specialized on the  
322 *Rosa* genus (Rosaceae) except for one on Anacardiaceae (Table S1).

323 In order to study the evolutionary history of thelytoky in *Megastigmus*, thelytokous species  
324 must be placed on the phylogenetic tree of the *Megastigmus* genus. COI reconstruction was  
325 performed on sequences obtained from the 25 species sampled in this study (Figure 1).  
326 There was an ancestral split between the species related to Anacardiaceae and the others.  
327 Wasp species related to Rosaceae form two clades, supported by high bootstrap values, with  
328 an initial split between the *aculeatus* group on the one hand and all others species  
329 associated with Rosaceae on the other hand. This latter clade is sister to a monophyletic  
330 clade comprising all species exploiting gymnosperms (posterior probability 0.73). In  
331 accordance with previous studies, *Megastigmus* developing on gymnosperms also show  
332 specialization, with for example, species exploiting the Pinaceae forming a monophyletic  
333 group (except for *M. thyoides*, the only species related to Cupressaceae in the Nearctic region,  
334 which could rather reflect a secondary change of host family than an ancestral association).  
335 Replacing species reproducing through thelytoky on that tree does not show any strong  
336 phylogenetic signal: thelytokous species occur sporadically on the tree. While the ancestral  
337 mode of reproduction is difficult to assess, one clear result is that transitions from one mode  
338 of reproduction to the other have regularly occurred during the evolutionary history of seed-  
339 specialized *Megastigmus*.

340

#### 341 *Screening for PI-endosymbiont infection*

342 A total of 25 arrhenotokous and thelytokous *Megastigmus* species (15 and 10 species,  
343 respectively) were sampled and screened for the presence of *Wolbachia*, *Cardinium*,  
344 *Arsenophonus* and *Rickettsia* using specific primers. None of the arrhenotokous species was  
345 found infected by any of the targeted endosymbionts (Table 2). On the contrary, *Wolbachia*



346 was found fixed in all of the thelytokous samples tested, while *Cardinium*, *Arsenophonus* and  
347 *Rickettsia* were not detected in any of these species (Table 2). The correct amplification of  
348 both positive controls and arthropod COI indicated true lack of infection and not a failure in  
349 the set-up of the PCR reactions. These results demonstrate the strong association of  
350 *Wolbachia* infection with thelytokous parthenogenesis in *Megastigmus* (Fisher's Exact Test's  
351 p-value<0.001).

352

### 353 *Antibiotic treatments*

354 Tetracycline treatments led to an almost complete loss of *Wolbachia* infection in *M.*  
355 *pinsapis* females and a significant recovery of male production, whereas control ones  
356 remained infected and produced only females (Table 3). As expected for arrhenotokous  
357 species, both control and treated *M. schimitscheki* females were not infected by *Wolbachia*  
358 and produced exclusively males. In addition, for each species, the mean brood size was not  
359 significantly altered by tetracycline treatment (Table 3). These data suggest that arrhenotoky  
360 restoration in treated *M. pinsapis* was due to symbiont curing rather than direct toxicity of  
361 antibiotics to insects, and that *Wolbachia* is probably the causative agent of thelytoky.

362

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

364 Topologies obtained with the four MLST genes were not congruent among each other on the  
365 entire dataset (34 taxa; all p-values <0.01 for SH test, <3.10<sup>-5</sup> with AU test), as already shown  
366 (Baldo *et al.* 2006). In contrast, for the eight *Wolbachia* infecting *Megastigmus* for which  
367 sequences have been obtained on the four MLST genes, topologies were globally congruent  
368 as all SH tests and most AU were not significant. The only consistent exception was for the

369 *coxA* gene, for which topology was significantly different from the other genes based on AU  
370 tests. In relation to that, a closer look at the *Wolbachia* strain infecting *M. pistaciae* suggests  
371 this strain has undergone a recombination event between A and B *Wolbachia*. Indeed, this  
372 strain belongs to the B supergroup based on all sequenced genes, except for *coxA* for which  
373 it falls within the A supergroup (Figure S1). In summary, despite the complex history of  
374 *Wolbachia* strains at a global scale, the tree based on the concatenated dataset is a good  
375 mean representation of the history of *Wolbachia* infection in *Megastigmus* (Figure 2) and is  
376 also consistent with the tree obtained on the *wsp* gene (Figure S2).

377 Three highly homogeneous lineages of *Wolbachia* infecting *Megastigmus* species were  
378 identified (namely WA1, WA2 and WB). We refer to these as lineages as opposed to strains,  
379 because although they are closely related, some substitutions were observed. A strict  
380 association between *Wolbachia* lineage and host plant is observed. The WB lineage is  
381 restricted to *M. pistaciae*, the only thelytokous species developing on Anacardiaceae. A  
382 second lineage, named WA1, was only found in thelytokous species developing on Rosaceae,  
383 i.e. *M. brevicaudis*, *M. rosae* (both subtypes) and *M. aculeatus* (both subtypes). The third  
384 lineage, named WA2, is closely related to WA1 and grouped the *Wolbachia* infecting *M.*  
385 *borriesi*, *M. pictus*, *M. pinsapinis* and *M. suspectus*, which all develop on Pinaceae.

386 Importantly, and despite this strict association between host plant and *Wolbachia* lineage,  
387 no significant association was detected between *Wolbachia* and host lineages using Parafit  
388 test. In addition, *Wolbachia* (concatenated dataset) and *Wolbachia*-infected *Megastigmus*  
389 (COI) topologies were not congruent (SH test,  $p < 0.014$  and AU test,  $p < 0.003$ ). These analyses  
390 exclude co-cladogenesis, suggesting horizontal transfers have played a major role in the  
391 distribution of the *Wolbachia* strains.

392 The distribution of *Wolbachia* strains among *Megastigmus* species suggests that  
393 horizontal transmission routes are constrained by the plant family, with only one plant  
394 family per strain. Our tree in Fig. 1 shows no exception to this rule, but this could  
395 nevertheless be obtained by chance, at least in principle. To evaluate how likely this  
396 is, we performed a simple test, stating as our null hypothesis that a strain can appear  
397 in an insect irrespective of the plant it parasitizes. Because infection with PI-*Wolbachia*  
398 rapidly leads to the irreversible loss of sexual reproduction, rendering *Wolbachia*  
399 indispensable for the insect, infection loss is highly improbable (Stouthamer et al. 2010).  
400 Under this hypothesis, from the 10 infected species, at least 7 independent *Wolbachia*  
401 acquisitions have occurred during *Megastigmus* evolution. Indeed, in three cases, it is not  
402 possible to distinguish between independent acquisition or ancestral infection (e.g. in the  
403 two *M. rosae*). In all other cases, the presence of an uninfected species within the clade  
404 excludes ancestral acquisition. Using the constraint solver clingo [1], we first  
405 enumerated all possible scenarios with 7 independent *Wolbachia* acquisitions (at  
406 least one by strain) possibly occurring at each node of the actual *Megastigmus*  
407 phylogeny and leading to 10 infected extant species. Among all these scenarios, we  
408 reported the proportion  $p$  of cases where a strain is strictly specific of a plant family  
409 and obtained a value for  $p$  of 0.0051. This result indicates that the probability of  
410 getting such a plant specific distribution by chance is exceptional, which constitutes a  
411 reasonable evidence that the events of horizontal transmission are not occurring  
412 randomly, but are rather influenced by the host plant.

413

414 **Discussion**

415 *Distribution and endosymbiotic origin of thelytoky in phytophagous Megastigmus species*

416 A few hundred species in all of the major animal groups are characterized by thelytokous  
417 parthenogenesis; its patchy taxonomic distribution in Hymenoptera is consistent with  
418 several independent evolutionary origins from ancestral arrhenotokous species (Cook,  
419 1993). The relatively high prevalence of thelytoky in Hymenoptera might reflect that some  
420 mechanisms of their haplodiploid sex determination are rather easily redirected to thelytoky  
421 by mutations (Lattorff *et al.* 2005) or bacteria (Cordaux *et al.*, 2011). Our literature review of  
422 parthenogenesis in the *Megastigmus* genus estimated the prevalence of thelytoky to 15%,  
423 which supported the assumption that arrhenotoky is the dominant and ancestral form of  
424 parthenogenesis in these phytophagous wasps. Thelytoky was unambiguously described in  
425 species exploiting diverse habitats, although it appears to occur in only three out of the ten  
426 host plant families currently known to host *Megastigmus* species in both gymnosperms and  
427 angiosperms (Pinaceae, and Rosaceae and Anacardiaceae, respectively). Interestingly,  
428 several thelytokous species share congeneric host plants (e.g. on the *Rosa* genus), but each  
429 groups of thelytokous species associated with Pinaceae, Rosaceae or Anacardiaceae remains  
430 confined at the host family taxonomic level. This observation supports the potential for  
431 intimate ecological connections within a given host plant family, but not between different  
432 host plant families.

433 Thelytoky may be under the control of the insect itself or its endosymbionts (Stouthamer *et*  
434 *al.*, 1990; Vavre *et al.*, 2004). Strict association between thelytoky and *Wolbachia* infection  
435 and reversibility of thelytoky through antibiotic treatments provide a particularly strong  
436 support for an endosymbiotic origin of thelytoky in *Megastigmus* wasps. Within the  
437 Hymenoptera, *Wolbachia*-induced thelytoky is found in three super-families: the Cynipoidea

438 (Rokas *et al.*, 2002), the Braconidae (Kremer *et al.*, 2009) and the Chalcidoidea (Stouthamer  
439 1997), within which only entomophagous families were concerned. To our knowledge, we  
440 document here the first case of *Wolbachia*-induced thelytoky in phytophagous chalcids.

441

#### 442 *Host plant specialization matters in the spread of thelytoky*

443 The phylogenetic tree of the *Megastigmus* genus including all main ecological groups  
444 (angiosperm- and gymnosperm-specialized seed wasps) indicated that thelytokous  
445 *Megastigmus* species do not form a monophyletic group, but occur sporadically on the COI  
446 phylogenetic tree (Figure 1). This pattern is consistent with the hypothesis of independent  
447 evolutionary origins of thelytokous species from ancestral arrhenotokous species (Cook,  
448 1993), and thus that transitions from arrhenotoky to thelytoky have repeatedly occurred.  
449 While transition from arrhenotoky to thelytoky is possible, the reverse is much more  
450 constrained and even impossible after sexual traits have decayed (Jeong and Stouthamer  
451 2005; Kremer *et al.*, 2009).

452 The phylogenetic tree obtained on the concatenated MLST dataset provided critical insights  
453 on the history of *Wolbachia* infection in *Megastigmus*. Three homogeneous lineages of  
454 *Wolbachia* (WA1, WA2 and WB) were identified and shown to be associated with the host  
455 plant families of their hosts (Figure 1). Although both lineages were found closely related,  
456 WA1 infected exclusively thelytokous wasp species exploiting the Rosaceae while WA2 was  
457 detected only in those exploiting the Pinaceae. The third lineage WB was restricted to the  
458 only currently known thelytokous wasp species exploiting Anacardiaceae (*M. pistaciae*).  
459 More interestingly, there was no statistical support for an association between *Wolbachia*  
460 and host lineages. For example, WA1 is found in five species developing on Rosaceae, but

461 belonging to highly divergent clades (10% COI). Similarly, WA2 is only found in four species  
462 exploiting Pinaceae, despite a divergence of as high as 5-6% on COI among these species. On  
463 the opposite, we found a non-random association between *Wolbachia* strains and the host  
464 plant used by the insects. Altogether, this led us to postulate on the one hand that thelytoky  
465 may have spread across the *Megastigmus* genus through horizontal transmission of  
466 *Wolbachia* among wasp species, and on the other hand that these transfers occurred  
467 preferentially between species exploiting similar host plants.

468 Within-community horizontal transmission of *Wolbachia* is likely to result from ecologically  
469 mediated pathways such as host-parasitoid associations (Vavre *et al.* 1999; Huigens *et al.*,  
470 2004). Shared feeding and breeding sites may also act as a route through which *Wolbachia*  
471 can be transmitted from one host species to another (Sintupachee *et al.* 2006). Primary  
472 infection by *Wolbachia* has to face two main filters (Vavre *et al.* 2003). First, *Wolbachia* must  
473 come into contact with the recipient species (i.e. has to pass the encounter filter). Second,  
474 *Wolbachia* has to evade the host immune system and to develop in the new host (i.e. has to  
475 pass the compatibility filter), which is facilitated by the relatedness of the donor and  
476 recipient species. Community structure may affect both filters and thus impact the  
477 epidemiology of *Wolbachia* across species, as recently showed theoretically using small-  
478 world networks (Zug *et al.*, 2012). Whether communities are composed of generalist or  
479 specialist species, and of distantly or closely related species will clearly impact these two  
480 filters. On the one hand, interspecific horizontal transmission of *Wolbachia* may be favoured  
481 in particular habitats that are attractive for a wide set of generalist host species, which will  
482 simultaneously feed and develop on it and/or which can be connected by shared parasitoids  
483 (Stahlhut *et al.*, 2010). The situation encountered in *Trichogramma* may be representative of

484 such case with the existence of a specialized PI-*Wolbachia* clade infecting these generalist  
485 wasps, but within which horizontal transmission is rampant (Schilthuizen and Stouthamer,  
486 1997). On the other hand, intimate interspecific connections between host species may also  
487 arise from host plant specialization, which may favour *Wolbachia* transmission due to  
488 narrow shared ecological niches. This situation opens widely the encounter filter, but also  
489 restricts the host spectrum to the few species exploiting this particular resource. When  
490 species within the community are moreover closely related species exploiting the same  
491 resource, the compatibility filter will also be open and facilitates symbiont infection. This  
492 latter situation is probably the one encountered in the seed-feeding *Megastigmus* analysed  
493 here, revealing two essential features of the spread of thelytoky in the context of insect  
494 ecological specialization.

495 First, the COI reconstruction of the present 25 *Megastigmus* species strongly confirmed host  
496 plant specialization in this genus (Figure 1). Within the species on angiosperms, the clade of  
497 species attacking Anacardiaceae first diverged. Within one of the sister groups, wasp species  
498 exploiting gymnosperms form a monophyletic group and even show specialization, as  
499 depicted by the monophyletic group developing on the Pinaceae (except for *M. thyoides*).  
500 The existence of a common *Wolbachia* lineage in all wasps exploiting the Rosaceae (WA1)  
501 and another one in all those developing on the Pinaceae (WA2) suggests that host ecological  
502 specialization can promote the spread of thelytoky through seed use. All *Megastigmus*  
503 females oviposit during a rather narrow period of the development of the host reproductive  
504 structures and progeny develop exclusively within seeds at a final density of one larva per  
505 seed, while several eggs can be found within the same seed (Turgeon *et al.*, 1994; Rouault *et*  
506 *al.* 2004; T. Boivin, personal observation). On both wild roses and Pinaceous trees, many

507 *Megastigmus* species show some restricted behavioral plasticity allowing shifts onto  
508 different congeneric host species or even onto a new host genus (Grissell 1999; Auger-  
509 Rozenberg & Roques 2012). The possibility of short-range host shifts may have favoured  
510 novel wasp assemblages and novel opportunities for them to interact and potentially  
511 exchange symbionts. Although shared parasitoids between phytophagous *Megastigmus* spp.  
512 can not be ruled out (Mailleux *et al.*, 2008; M.-A. Auger-Rozenberg, personal observation),  
513 host-parasitoid associations in this genus remain too poorly documented to formally invoke  
514 this *Wolbachia* horizontal route at this point. We rather suggest that if *Wolbachia* infection  
515 occurs mainly via ingestion during the early larval stages, both seed tissues and/or  
516 cannibalism by larvae competing for the seed tissues may facilitate interspecific horizontal  
517 transfers of the endosymbiont. A similar pattern of endosymbionts sharing among sibling  
518 species competing for a common resource have recently been shown in weevils (Merville *et*  
519 *al.* 2013).

520 Another key result of this study is that *Megastigmus* ecological specialization at the host  
521 plant family level probably constrained the invasion of *Wolbachia* lineages throughout the  
522 whole host genus. Indeed, diverging strategies in the use of angiosperms and gymnosperms  
523 may be an essential feature of the strict host radiation depicted here in the evolutionary  
524 history of *Megastigmus*. For this reason, intimate ecological connections promoting  
525 horizontal transfers of *Wolbachia* (see above) are thus unlikely to arise at this community  
526 scale. More interestingly, thelytoky appears to have still not invaded wasps developing on  
527 both the Cupressaceae (Fig. 1, Table S1). According to Auger-Rozenberg *et al.* (2006), wasps  
528 developing on the Cupressaceae exhibit a higher level of host specificity, because they seem  
529 host species specific, while wasps on Pinaceae or Rosaceae are specialized to particular host



530 genera, but frequently attack several congeneric species if they occur in sympatry. This is  
531 consistent with a taxonomic radiation following initial host adaptation, which might have  
532 constituted an efficient barrier to the spread of thelytoky in this group, due to unlikely  
533 opportunities for interspecific horizontal transfers of *Wolbachia*.

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

539

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548

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708

709 **Data accessibility**

710 *Wolbachia* and wasp DNA sequences: GenBank accession numbers KF531859-KF531890 and  
711 KF531891-KF531900, and KF531833-KF531858, respectively.

712

713

714 **Author contributions**

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

719

720 **Figure Legends**

721 **Figure 1.** Bayesian likelihood inference phylogeny based on COI sequences in seed-  
722 specialized wasps of the *Megastigmus* genus (26 taxa, 962 bp). *Torymus azureus* was used as  
723 an outgroup. Posterior probability values are indicated at each node. Host plant families are  
724 indicated at each branch forming a monophyletic group (excepted for the Cupressaceae).  
725 Anarcadiaceae and Rosaceae are angiosperms, and Pinaceae, Cupressaceae and Taxodiaceae  
726 are gymnosperms. *Megastigmus* species infected by *Wolbachia* are indicated by arrowheads  
727 followed by the name of the *Wolbachia* lineage.

728

729 **Figure 2.** Phylogenetic placement of the *Wolbachia* strains infecting the seed-specialized  
730 wasps *Megastigmus* spp. among other *Wolbachia* belonging to A and B supergroups.

731 Bayesian likelihood inference phylogenies are shown, while maximum likelihood analyses  
732 gave substantially the same results. *Wolbachia* sequences are labelled with the name of  
733 their host. *Wolbachia* of *Brugia malayi* was used as an outgroup. Posterior probability values  
734 are indicated at each node. This phylogeny is based on concatenated sequences data set for  
735 the four MLST loci *coxA*, *gatB*, *ftsZ* and *hcpA* (34 strains, 1650 bp).

736

737

### 738 **Supplementary material**

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

743

744 **Figure S2.** Phylogenetic placement of the *Wolbachia* strains infecting the seed-specialized  
745 wasps *Megastigmus* spp., among other *Wolbachia* strains belonging to A and B supergroups,  
746 based on *wsp* sequences of 49 strains (530 bp).

747

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

750

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

**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 COI phylogenetic tree of the *Megastigmus* genus presented in Fig. 1. Specimens collected outside of their native range belonged to invasive 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 rugosa</i>	Krasnoyarsk, Russia	Palearctic
<i>M. aculeatus nigroflavus</i>	T	Angiosperm	Rosaceae	<i>Rosa</i> sp.	Iowa, 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</i> sp.	Ojcow, Poland	Palearctic
<i>M. pictus</i>	T	Gymnosperm	Pinaceae	<i>Larix gmelini</i>	Loiret, France	Palearctic
<i>M. pinsapinis</i>	T	Gymnosperm	Pinaceae	<i>Cedrus atlantica</i>	Vaucluse, France	Palearctic
<i>M. pistaciae</i>	T	Angiosperm	Anacardiaceae	<i>Pistacia terebenthus</i>	Exocori, Greece	Palearctic
<i>M. rosae</i>	T	Angiosperm	Rosaceae	<i>Rosa</i> sp.	Hautes-Alpes, France	Palearctic
<i>M. rosae alba</i>	T	Angiosperm	Rosaceae	<i>Rosa majalis</i>	Vilnius, Lithuania	Palearctic
<i>M. suspectus</i>	T	Gymnosperm	Pinaceae	<i>Abies alba</i>	Gard, France	Palearctic
<i>M. amicum</i>	A	Gymnosperm	Cupressaceae	<i>Juniperus phoenicea</i>	Ericera, Portugal	Palearctic
<i>M. atedius</i>	A	Gymnosperm	Pinaceae	<i>Picea</i> sp.	British Columbia, Canada	Nearctic
<i>M. atlanticus</i>	A	Gymnosperm	Cupressaceae	<i>Cupressus atlantica</i>	Idni, Morocco	Afro-tropical
<i>M. bipunctatus</i>	A	Gymnosperm	Cupressaceae	<i>Juniperus communis</i>	Hautes-Alpes, France	Palearctic
<i>M. cryptomeriae</i>	A	Gymnosperm	Cupressaceae	<i>Cryptomeria fortunei</i>	Zeihjang, China	East-Asia
<i>M. hoffmeyerii</i>	A	Gymnosperm	Pinaceae	<i>Tsuga canadensis</i>	Ontario, Canada	Nearctic
<i>M. lasiocarpae</i>	A	Gymnosperm	Pinaceae	<i>Abies amabilis</i>	British Columbia, Canada	Nearctic
<i>M. nigrovariegatus</i>	A	Angiosperm	Rosaceae	<i>Rosa</i> sp.	British Columbia, Canada	Nearctic
<i>M. pinus</i>	A	Gymnosperm	Pinaceae	<i>Abies procera</i>	California, USA	Nearctic
<i>M. rafni</i>	A	Gymnosperm	Pinaceae	<i>Abies alba</i>	Aude, France	Nearctic
<i>M. schimitscheki</i>	A	Gymnosperm	Pinaceae	<i>Cedrus atlantica</i>	Luberon, France	Palearctic
<i>M. spermotrophus</i>	A	Gymnosperm	Pinaceae	<i>Pseudotsuga mensiezii</i>	California, USA	Nearctic

<i>M. thyoides</i>	A	Gymnosperm	Cupressaceae	<i>Chamaecyparis sp.</i>	North Carolina, 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>	British Columbia, Canada	Nearctic
<i>Torymus azureus</i>	A	Gymnosperm	Pinaceae	<i>Picea abies</i>	Suchora, Poland	Palaearctic

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**Table 2.** *Megastigmus* species screened for bacterial infection (+) or non-infection (-) 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. N: number of females tested for infection. +: amplification of the primer. -: no amplification of the primer tested. NA: non available data.

Parthenogenesis and species (N)	<i>Wolbachia</i> (infection %)	<i>Cardinium</i>	<i>Arsenophonus</i>	<i>Rickettsia</i>	<i>Arthropod COI</i>
<b>Thelytoky</b>					
<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)	-	-	-	
<b>Arrhenotoky</b>					
<i>M. amicorum</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)	-	-	-	-	+

**Table 3.** *Wolbachia* infection rates in control and tetracycline-treated females of *M. pinsapinis* (thelytokous) and *M. 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 <sup>*</sup> )	% of <i>wsp</i> positives <sup>**</sup>	Number of mothers	Mean brood size <sup>***</sup>	Mean % of males
<i>M. pinsapinis</i>	control (44)	100	44	21.4 ± 5.3 <sup>a</sup>	0
	tetracycline (42)	4.7	42	18.7 ± 7.8 <sup>a</sup>	92 ± 1.1
<i>M. schimitscheki</i>	control (30)	0	30	30.3 ± 4.5 <sup>a</sup>	100
	tetracycline (28)	0	28	32.1 ± 7.5 <sup>a</sup>	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 ( $\pm$ SE) produced do not differ significantly using a Kruskal-Wallis test (P<0.05).

Figure 1

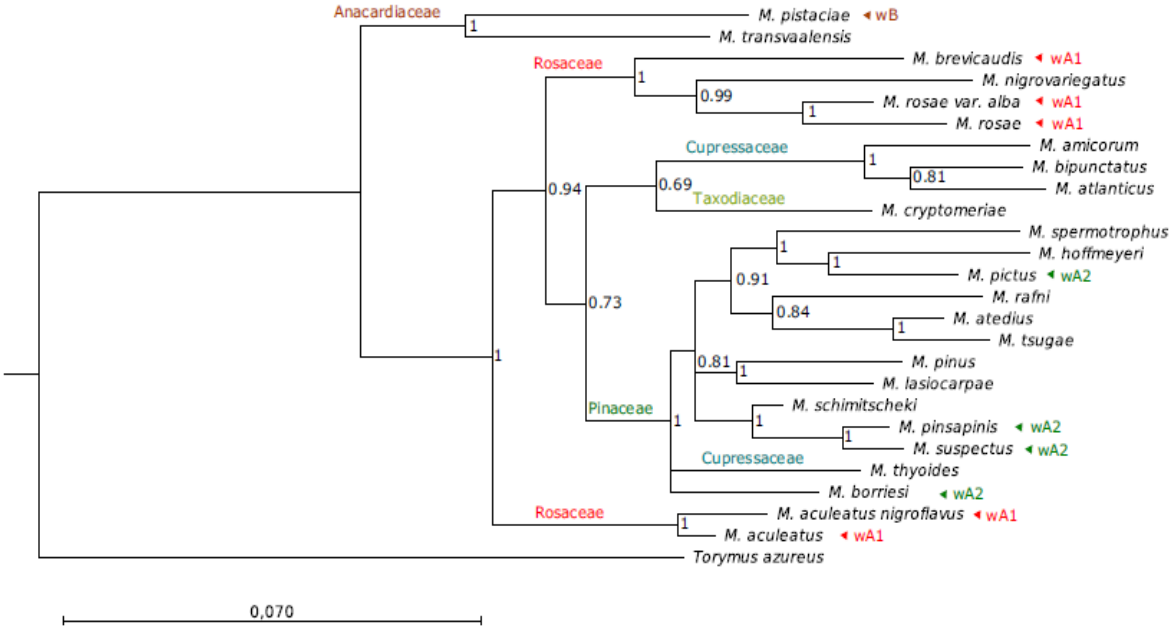
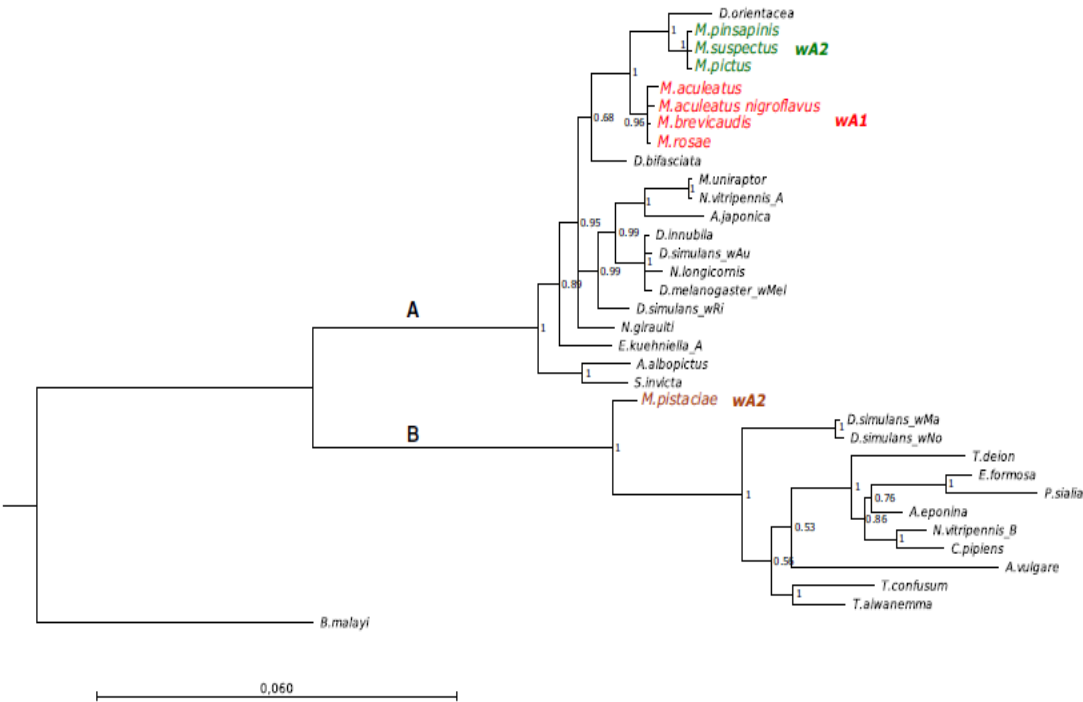


Figure 2





**Table S1.** Distribution of arrhenotoky, thelytoky and host-plant specialization among the seed-feeding wasps of the *Megastigmus* genus. Data were compiled from reviews of Grissell (1999), Roques and Skrzypczynska (2003), Roques et al. (2003) and Auger-Rozenberg et al. (2006). Species in bold were specifically studied in this paper. A: arrhenotokous parthenogenesis. T: thelytokous parthenogenesis. \* Ratio of males to females; balanced: consideration of similar frequencies of females and males by authors; NA: no specific mention on sex ratio by authors but numbers of males mentioned in samples (thelytoky being associated with “males unknown” or “males are scarce” specific mentions).

Host-plant taxon	Species	Reproduction	Sex ratio*	Main host-plant genus	Native area
<b>Gymnosperms</b>					
<u>Cupressaceae</u>	<i>M. chamaecyparidis</i>	A	balanced	<i>Chamaecyparis</i>	Palaeartic
	<b><i>M. thyoides</i></b>	A	balanced	<i>Chamaecyparis</i>	Nearctic
	<b><i>M. atlanticus</i></b>	A	balanced	<i>Cupressus</i>	Palaeartic
	<i>M. carinus</i>	A	NA	<i>Cupressus</i>	Palaeartic
	<i>M. cupressi</i>	A	NA	<i>Cupressus</i>	Oriental
	<i>M. duclouxiana</i>	A	NA	<i>Cupressus</i>	Palaeartic
	<i>M. watchli</i>	A	0.5-1.7	<i>Cupressus</i>	Palaeartic
	<b><i>M. amicorum</i></b>	A	balanced	<i>Juniperus</i>	Palaeartic
	<b><i>M. bipunctatus</i></b>	A	balanced	<i>Juniperus</i>	Palaeartic
	<i>M. certus</i>	A	NA	<i>Juniperus</i>	Palaeartic
	<i>M. formosana</i>	A	balanced	<i>Juniperus</i>	East-Asia
	<i>M. fidus</i>	A	balanced	<i>Juniperus</i>	Palaeartic
	<i>M. gravis</i>	A	balanced	<i>Juniperus</i>	Palaeartic
	<i>M. juniperi</i>	A	NA	<i>Juniperus</i>	Palaeartic
	<i>M. pingii</i>	A	NA	<i>Juniperus</i>	East-Asia
	<i>M. procerae</i>	A	NA	<i>Juniperus</i>	Palaeartic
	<i>M. rigidae</i>	A	NA	<i>Juniperus</i>	Palaeartic
	<i>M. sabinae</i>	A	balanced	<i>Juniperus</i>	Palaeartic
	<i>M. somaliensis</i>	A	NA	<i>Juniperus</i>	Afrotropical
	<i>M. thuriferana</i>	A	balanced	<i>Juniperus</i>	Palaeartic
<i>M. validus</i>	A	balanced	<i>Juniperus</i>	Palaeartic	
<i>M. thuyopsis</i>	A	NA	<i>Thuyopsis</i>	Palaeartic	
<u>Pinaceae</u>	<i>M. firmae</i>	A	NA	<i>Abies</i>	Palaeartic
	<b><i>M. lasiocarpae</i></b>	A	balanced	<i>Abies</i>	Nearctic
	<i>M. milleri</i>	A	balanced	<i>Abies</i>	Nearctic
	<b><i>M. pinus</i></b>	A	0.4-0.5	<i>Abies</i>	Nearctic
	<b><i>M. rafni</i></b>	A	0.4	<i>Abies</i>	Nearctic
	<i>M. specularis</i>	A	balanced	<i>Abies</i>	Nearctic
	<b><i>M. schimitscheki</i></b>	A	0.4-0.5	<i>Cedrus</i>	Palaeartic
	<i>M. laricis</i>	A	balanced	<i>Larix</i>	Nearctic
	<b><i>M. atedius</i></b>	A	0.19-0.66	<i>Picea</i>	Palaeartic
	<i>M. ezomatsuanus</i>	A	0.5	<i>Picea</i>	Palaeartic
	<i>M. likiangensis</i>	A	NA	<i>Picea</i>	East-Asia
	<i>M. strobilobius</i>	A	0.4-0.6	<i>Picea</i>	Palaeartic
	<i>M. albifrons</i>	A	balanced	<i>Pinus</i>	Nearctic
	<i>M. strobiformis</i>	A	NA	<i>Pinus</i>	Nearctic

	<i>M. pseudotsugae</i>	A	NA	<i>Pseudotsuga</i>	East-Asia
	<i>M. spermotrophus</i>	A	balanced	<i>Pseudotsuga</i>	Nearctic
	<i>M. hoffmeyeri</i>	A	balanced	<i>Tsuga</i>	Nearctic
	<i>M. tsugae</i>	A	balanced	<i>Tsuga</i>	Nearctic
	<i>M. tsugaphilus</i>	A	NA	<i>Tsuga</i>	Palaeartic
	<i>M. borriesi</i>	T	♂<0.1%	<i>Abies</i>	East-Asia
	<i>M. suspectus</i>	T	♂<0.2%	<i>Abies</i>	Palaeartic
	<i>M. pinsapinis</i>	T	♂<0.1%	<i>Cedrus</i>	Palaeartic
	<i>M. pictus</i>	T	♂<1%	<i>Larix</i>	Palaeartic
<u>Taxodiaceae</u>	<i>M. cryptomeriae</i>	A	balanced	<i>Cryptomeria</i>	East-Asia
<b>Angiosperms</b>					
<u>Anacardiaceae</u>	<i>M. thomseni</i>	A	NA	<i>Heeria</i>	Afrotropical
	<i>M. ozoroae</i>	A	NA	<i>Ozoroae</i>	Afrotropical
	<i>M. rhusi</i>	A	NA	<i>Rhus</i>	Afrotropical
	<i>M. transvaalensis</i>	A	0.5	<i>Schinus</i>	Afrotropical
	<i>M. pistaciae</i>	T	♂<0.1%	<i>Pistacia</i>	Palaeartic
<u>Fabaceae</u>	<i>M. albizziae</i>	A	NA	<i>Albizia</i>	Oriental
<u>Hamameliaceae</u>	<i>M. distylii</i>	A	NA	<i>Distylium</i>	Palaeartic
<u>Myrtaceae</u>	<i>M. ophelinii</i>	A	NA	<i>Eucalyptus</i>	Australasia
<u>Proteaceae</u>	<i>M. hakeae</i>	A	NA	<i>Hakea</i>	Australasia
<u>Rhamnaceae</u>	<i>M. helianthae</i>	A	NA	<i>Helinius</i>	Afrotropical
<u>Rosaceae</u>	<i>M. amelanchieris</i>	A	NA	<i>Amelanchier</i>	Nearctic
	<i>M. cotoneastri</i>	A	NA	<i>Cotoneaster</i>	Palaeartic
	<i>M. mali</i>	A	NA	<i>Malus</i>	Palaeartic
	<i>M. pourthiaceae</i>	A	0.6	<i>Photinia</i>	Palaeartic
	<i>M. physocarpus</i>	A	NA	<i>Physocarpus</i>	Nearctic
	<i>M. fangii</i>	A	NA	<i>Rosa</i>	East-Asia
	<i>M. nigrovariegatus</i>	A	balanced	<i>Rosa</i>	Nearctic
	<i>M. yunnanensis</i>	A	NA	<i>Rosa</i>	East-Asia
	<i>M. aculeatus</i>	T	♂<4%	<i>Rosa</i>	Palaeartic
	<i>M. aculeatus nigroflavus</i>	T	♂<1%	<i>Rosa</i>	Palaeartic
	<i>M. rosae</i>	T	♂<1%	<i>Rosa</i>	Palaeartic
	<i>M. rosae var. alba</i>	T	♂<1%	<i>Rosa</i>	Palaeartic
	<i>M. brevicaudis</i>	T	♂<1%	<i>Sorbus</i>	Palaeartic

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

Primer pair (5'-3')	Target group	Target gene (fragment size)	Annealing temperature (°C)
<b>wsp81F</b> TGGTCCAATAAGTGATGAAGAAAC <sup>a</sup> <b>wsp691R</b> AAAAATTAAACGCTACTCCA <sup>a</sup>	<i>Wolbachia pipientis</i>	<i>wsp</i> (610 bp)	59
<b>Ch-F</b> TACTGTAAGAATAAGCACCGGC <sup>b</sup> <b>Ch-R</b> GTGGATCACTTAACGCTTTCG <sup>b</sup>	<i>Cardinium</i> sp.	16S rRNA (~900 bp)	57
<b>Ars23S-1</b> CGTTTGATGAATTCATAG TCAAAC <sup>c</sup> <b>Ars23S-2</b> GGTCCTCCAGTTAGTGTTACCCAAC <sup>c</sup>	<i>Arsenophonus nasoniae</i>	23S rDNA (650 bp)	60
<b>27f</b> GAGAGTTTGATCCTGGCTCAG <sup>d</sup> <b>1495r</b> CTACGGCTACCTTGTTACGA <sup>d</sup>	<i>Rickettsia</i> sp.	16S rRNA (1500 bp)	50
<b>LCO1490</b> GGTCAACAAATCATAAAGATATTGG <sup>e</sup> <b>HCO2198</b> TAAACTTCAGGGTGACCAAAAAATCA <sup>e</sup>	Arthropods	COI (658 bp)	48

<sup>a</sup>Braig *et al.* (1998)

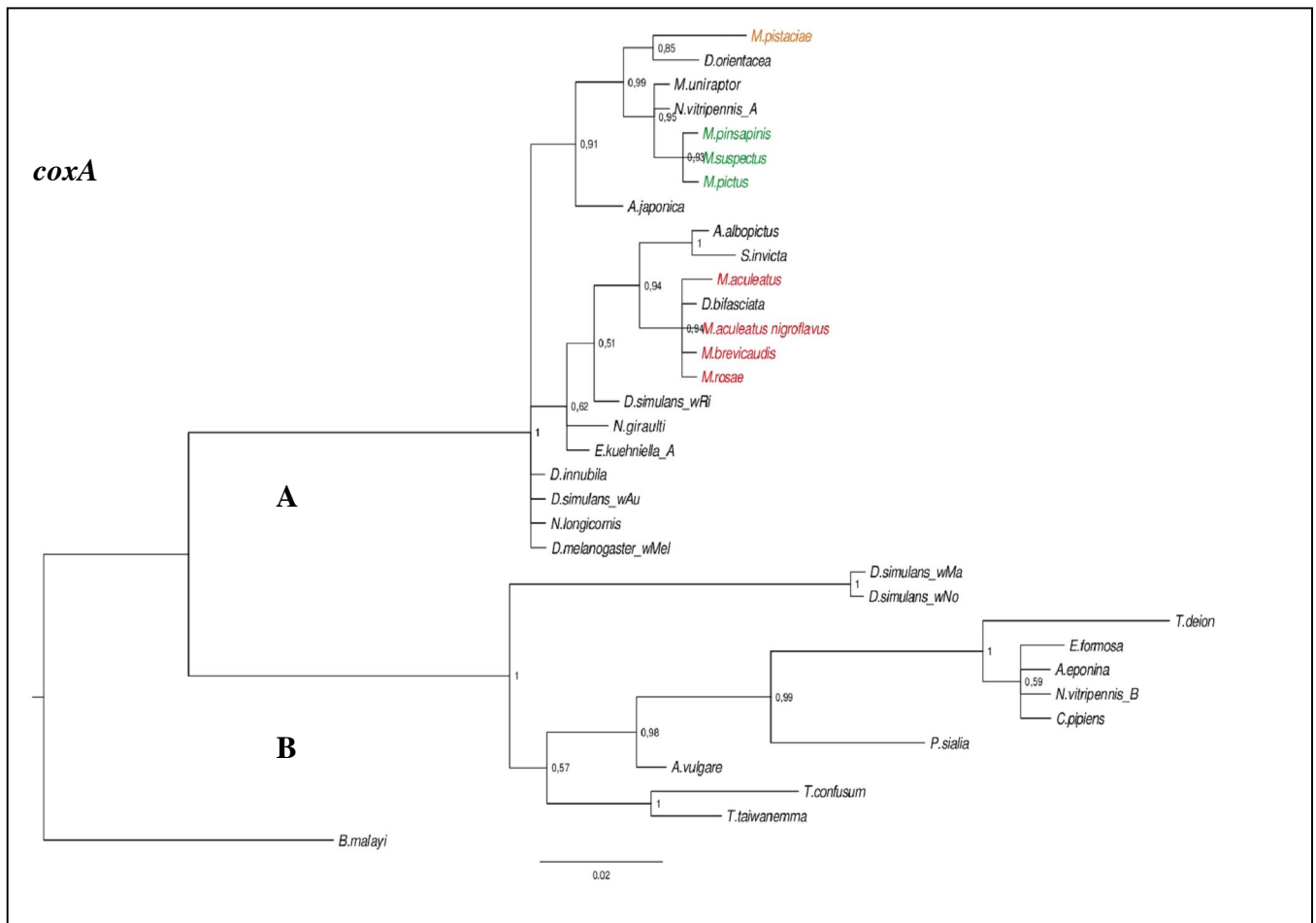
<sup>b</sup>Zchori-Fein and Perlman (2004)

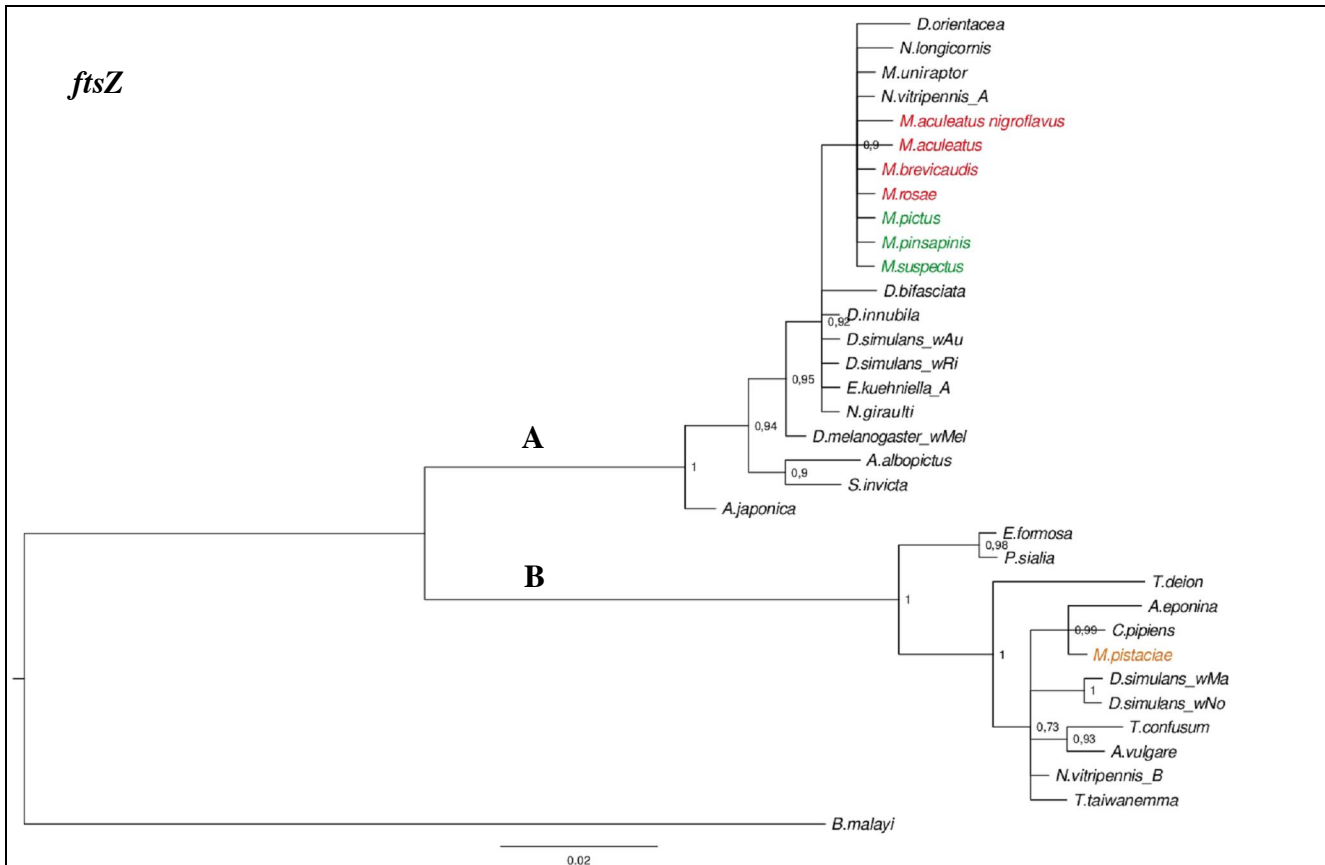
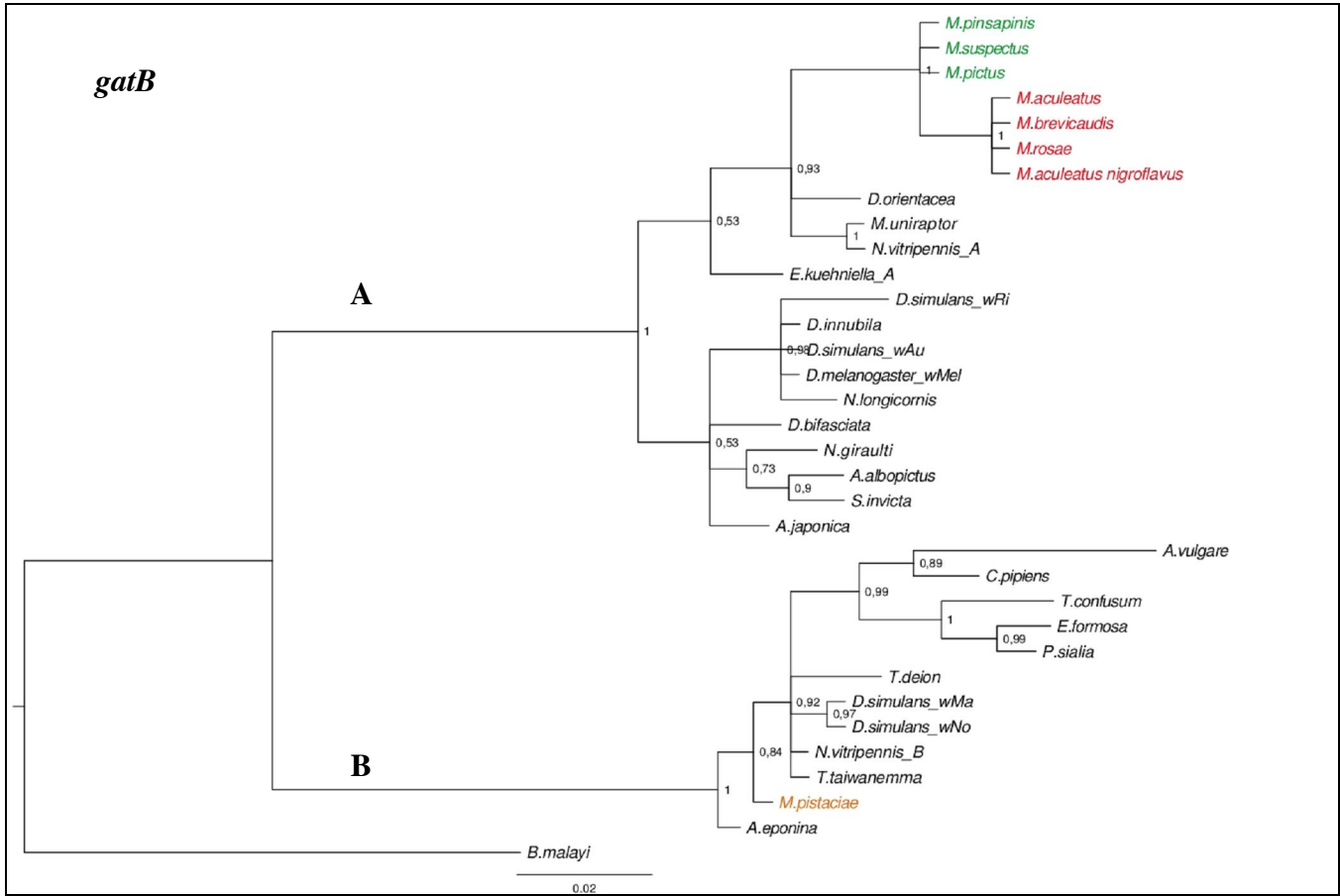
<sup>c</sup>Thao and Baumann (2004)

<sup>d</sup>Hagimori *et al.* (2006)

<sup>e</sup>Folmer *et al.* (1994)

**Figure 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 MLST genes used in this study (*coxA*, *gatB*, *ftsZ* and *hcpA*). 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. Each tree represents phylogenetic reconstruction based on 34 *Wolbachia* strains for *coxA* (402 bp), *gatB* (369 bp), *ftsZ* (435 bp) and *hcpA* (444 bp). Three lineages are revealed (green, red and light brown), two belonging to the A supergroup while the position of the *Wolbachia* infecting *M. pistaciae* suggests a recombination event between A and B *Wolbachia* supergroups. Indeed, this strain belongs to the B supergroup based on all sequences genes except for *coxA* for which it is placed in the B supergroup.

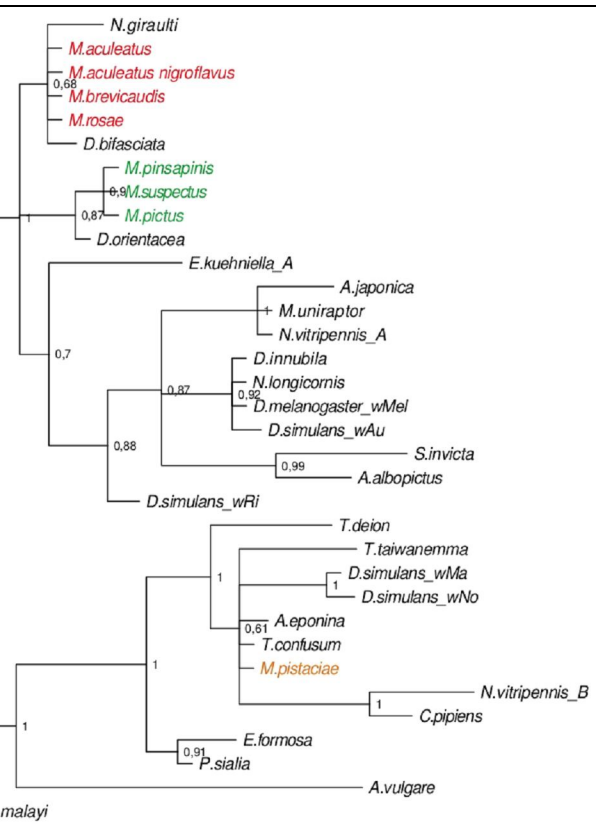




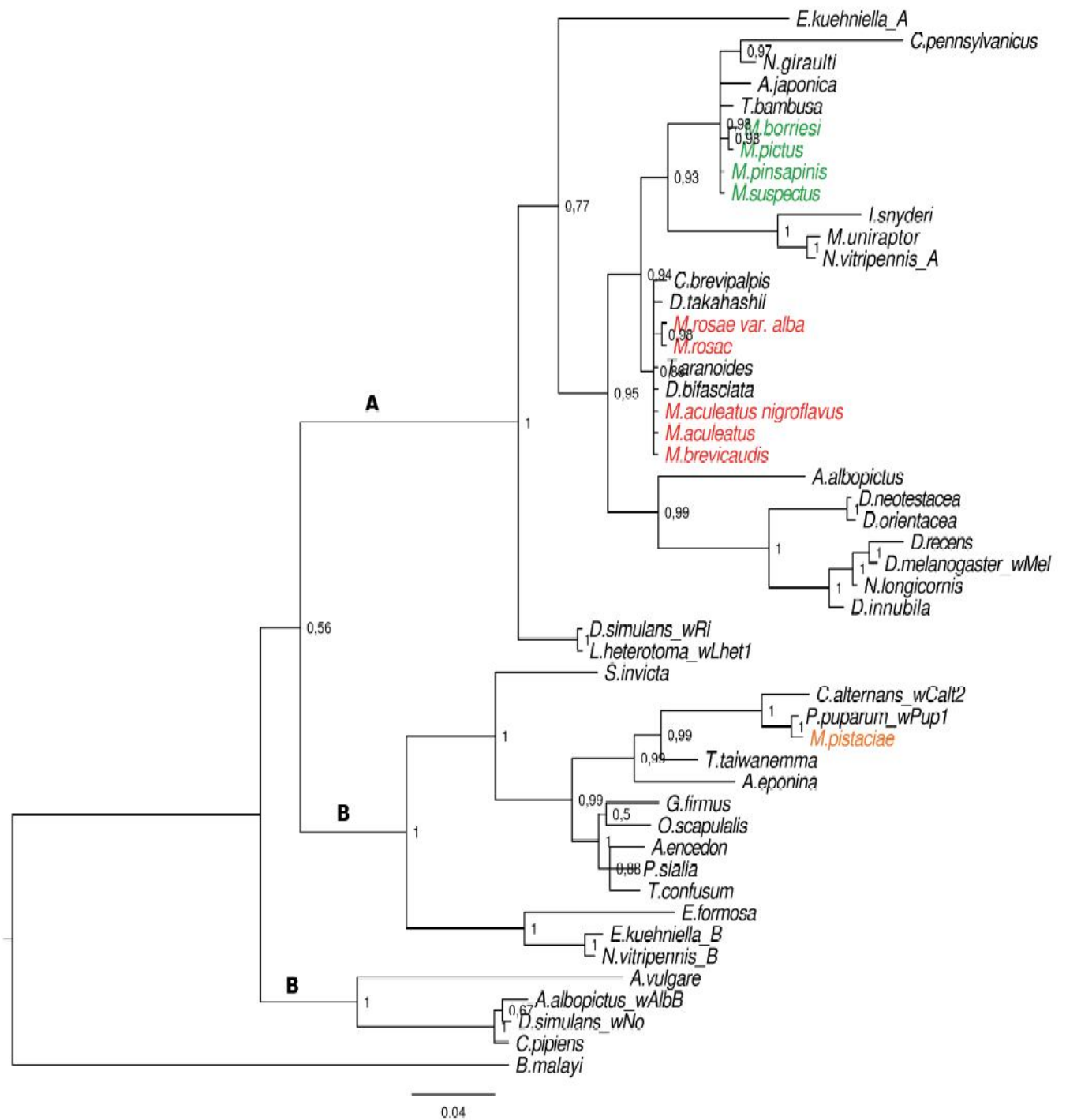
*hcpA*

**A**

**B**



**Figure 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). 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.



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