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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[®], 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[®], 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- μ l final volume reaction containing 200 μ M dNTP, 10 pM
208 primers, 0.5 IU HotStarTaq[®] DNA polymerase, and 1.5 μ 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, <3.10⁻⁵ 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
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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	Paelearctic

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>
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. 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 [*])	% 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 (\pm SE) produced do not differ significantly using a Kruskal-Wallis test (P<0.05).

Figure 1

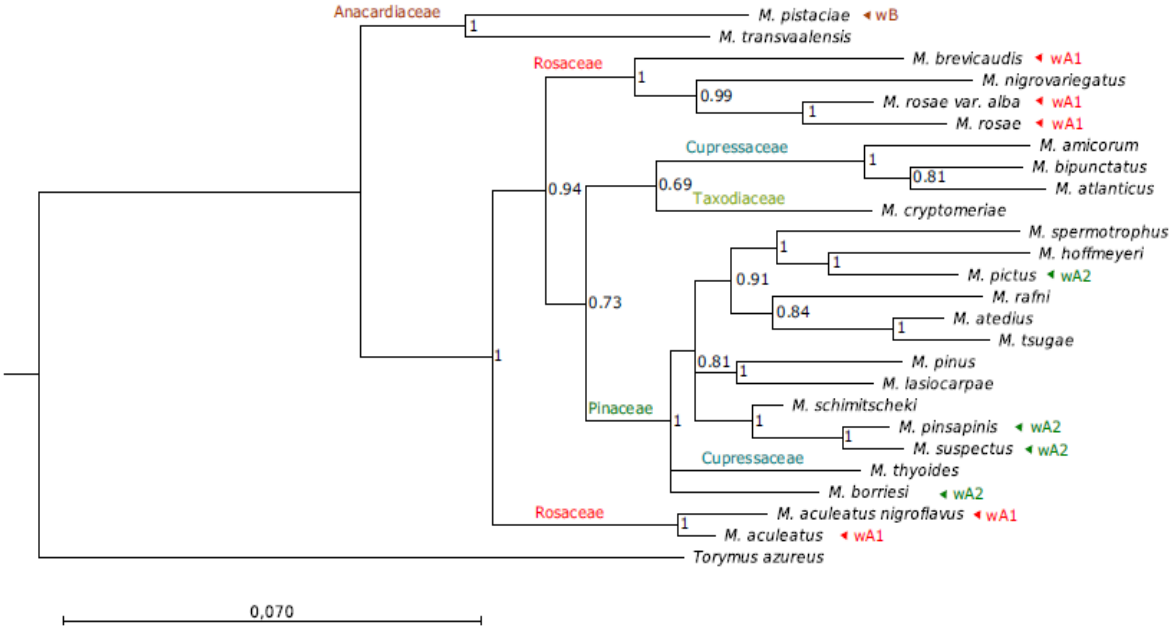


Figure 2

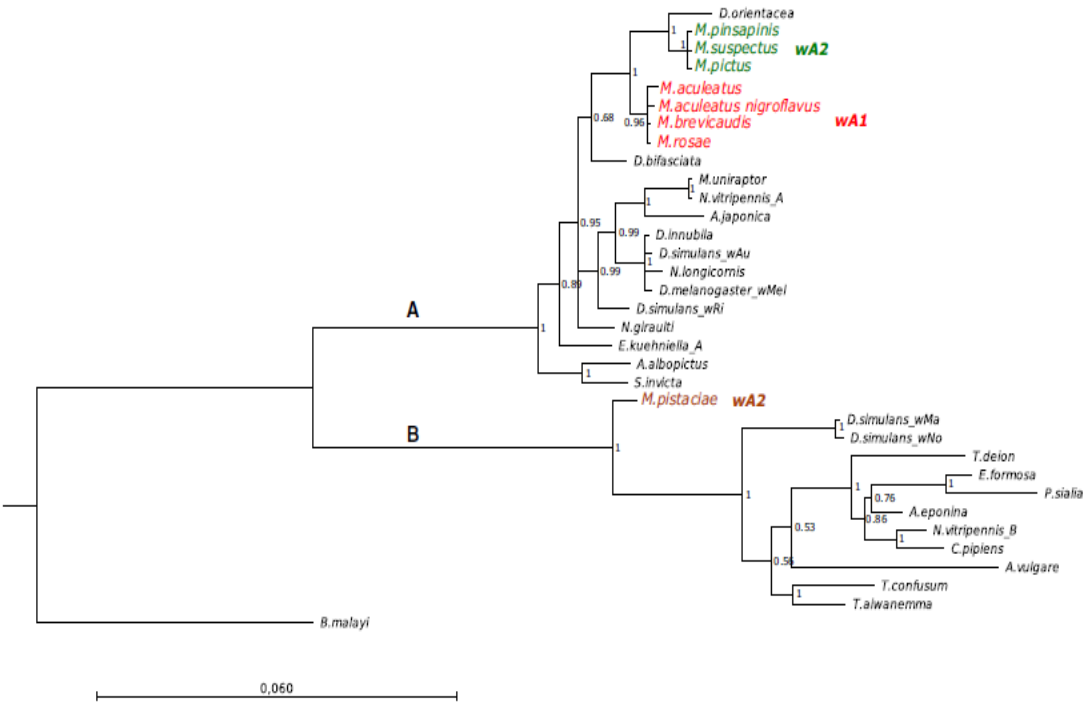


Table S1. Distribution of arrhenotoky, thelytoky and host-plant specialization among the seed-feeding wasps of the *Megastigmus* genus. Data were compiled from reviews of Grissell (1999), Roques and Skrzypczynska (2003), Roques et al. (2003) and Auger-Rozenberg et al. (2006). Species in bold were specifically 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
Gymnosperms					
<u>Cupressaceae</u>	<i>M. chamaecyparidis</i>	A	balanced	<i>Chamaecyparis</i>	Palaeartic
	<i>M. thyoides</i>	A	balanced	<i>Chamaecyparis</i>	Nearctic
	<i>M. atlanticus</i>	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
	<i>M. amicorum</i>	A	balanced	<i>Juniperus</i>	Palaeartic
	<i>M. bipunctatus</i>	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
	<i>M. lasiocarpae</i>	A	balanced	<i>Abies</i>	Nearctic
	<i>M. milleri</i>	A	balanced	<i>Abies</i>	Nearctic
	<i>M. pinus</i>	A	0.4-0.5	<i>Abies</i>	Nearctic
	<i>M. rafni</i>	A	0.4	<i>Abies</i>	Nearctic
	<i>M. specularis</i>	A	balanced	<i>Abies</i>	Nearctic
	<i>M. schimitscheki</i>	A	0.4-0.5	<i>Cedrus</i>	Palaeartic
	<i>M. laricis</i>	A	balanced	<i>Larix</i>	Nearctic
	<i>M. atedius</i>	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
Angiosperms					
<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)
wsp81F TGGTCCAATAAGTGATGAAGAAAC ^a wsp691R AAAAATTAAACGCTACTCCA ^a	<i>Wolbachia pipientis</i>	<i>wsp</i> (610 bp)	59
Ch-F TACTGTAAGAATAAGCACCGGC ^b Ch-R GTGGATCACTTAACGCTTTCG ^b	<i>Cardinium</i> sp.	16S rRNA (~900 bp)	57
Ars23S-1 CGTTTGATGAATTCATAG TCAAAC ^c Ars23S-2 GGTCCTCCAGTTAGTGTTACCCAAC ^c	<i>Arsenophonus nasoniae</i>	23S rDNA (650 bp)	60
27f GAGAGTTTGATCCTGGCTCAG ^d 1495r CTACGGCTACCTTGTTACGA ^d	<i>Rickettsia</i> sp.	16S rRNA (1500 bp)	50
LCO1490 GGTCAACAAATCATAAAGATATTGG ^e HCO2198 TAAACTTCAGGGTGACCAAAAAATCA ^e	Arthropods	COI (658 bp)	48

^aBraig *et al.* (1998)

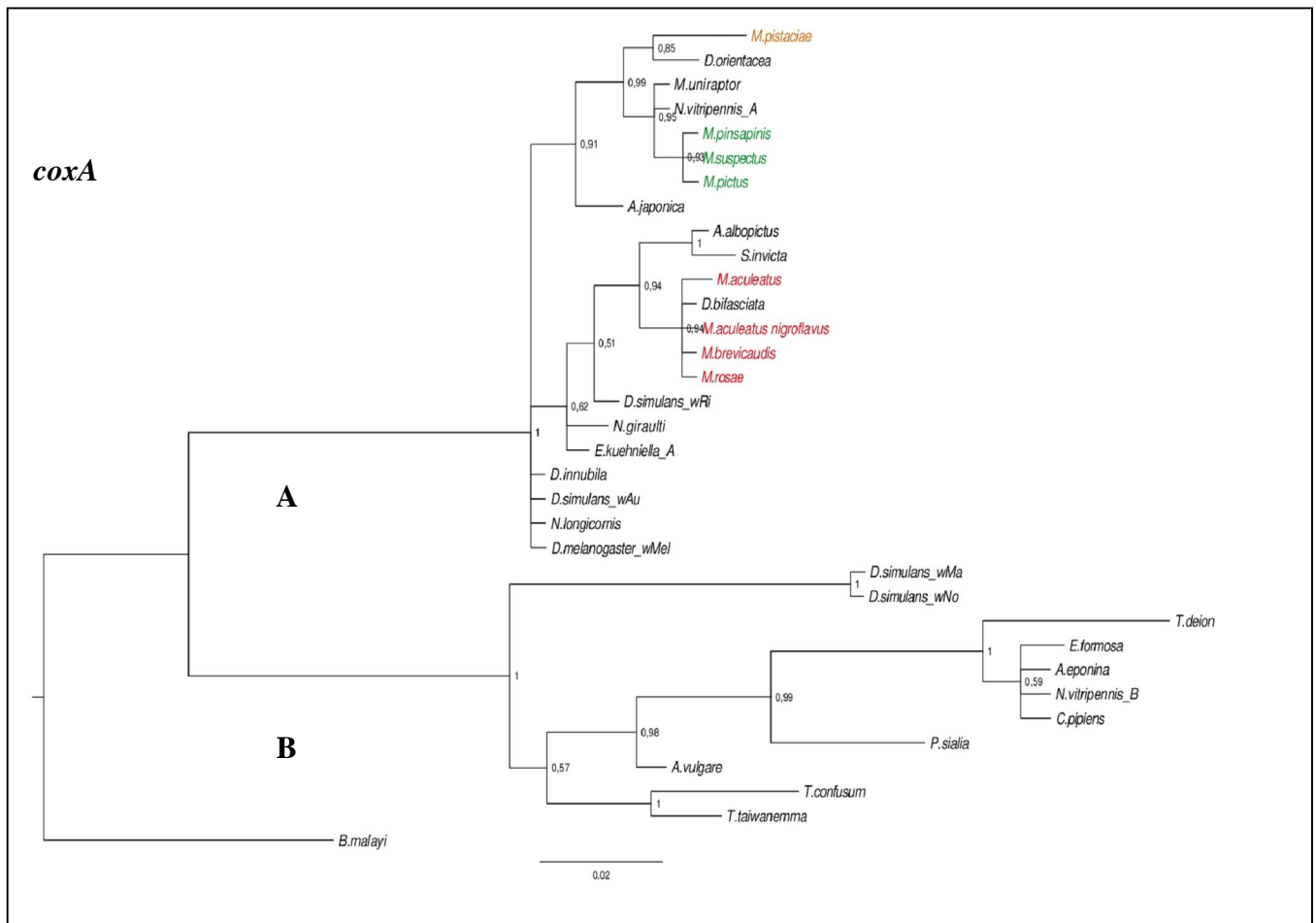
^bZchori-Fein and Perlman (2004)

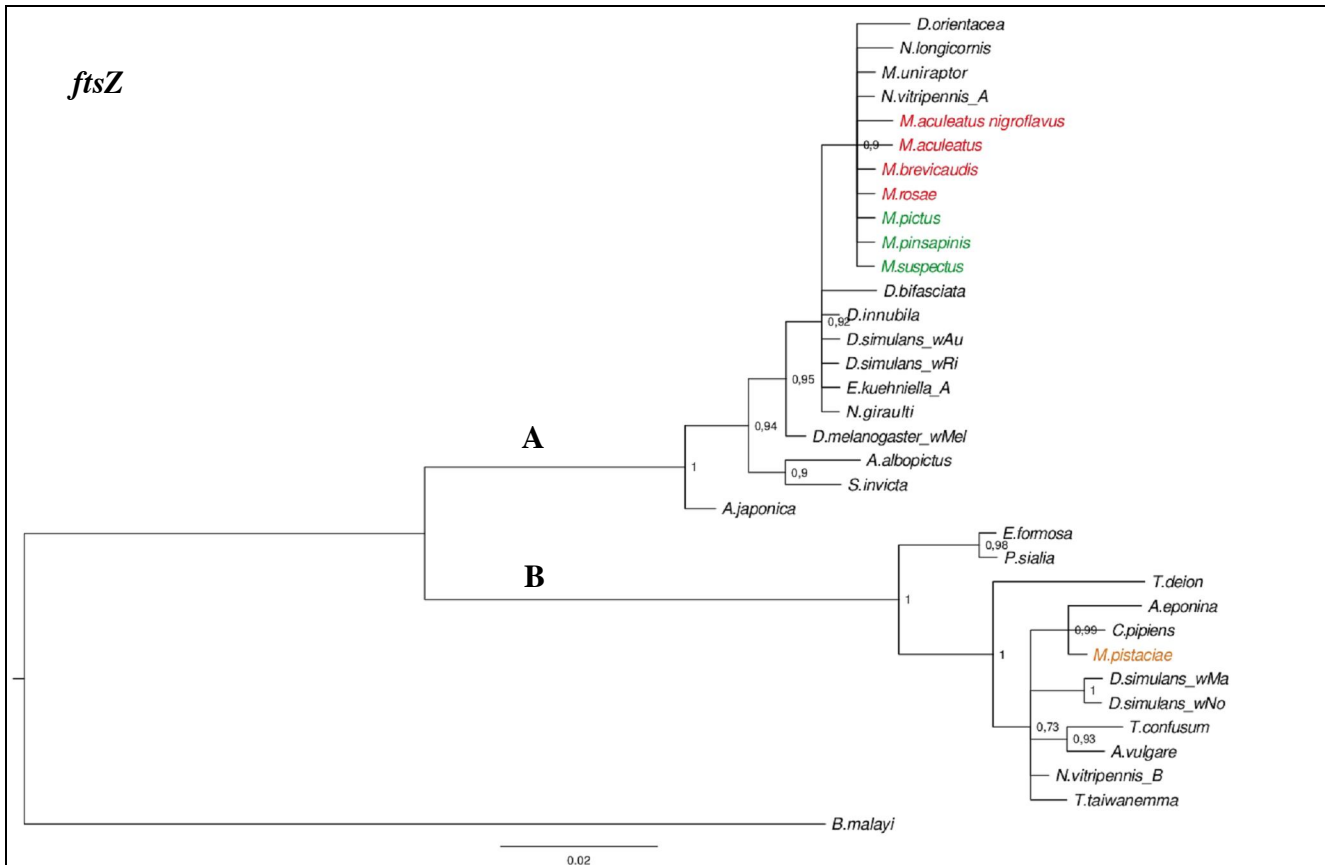
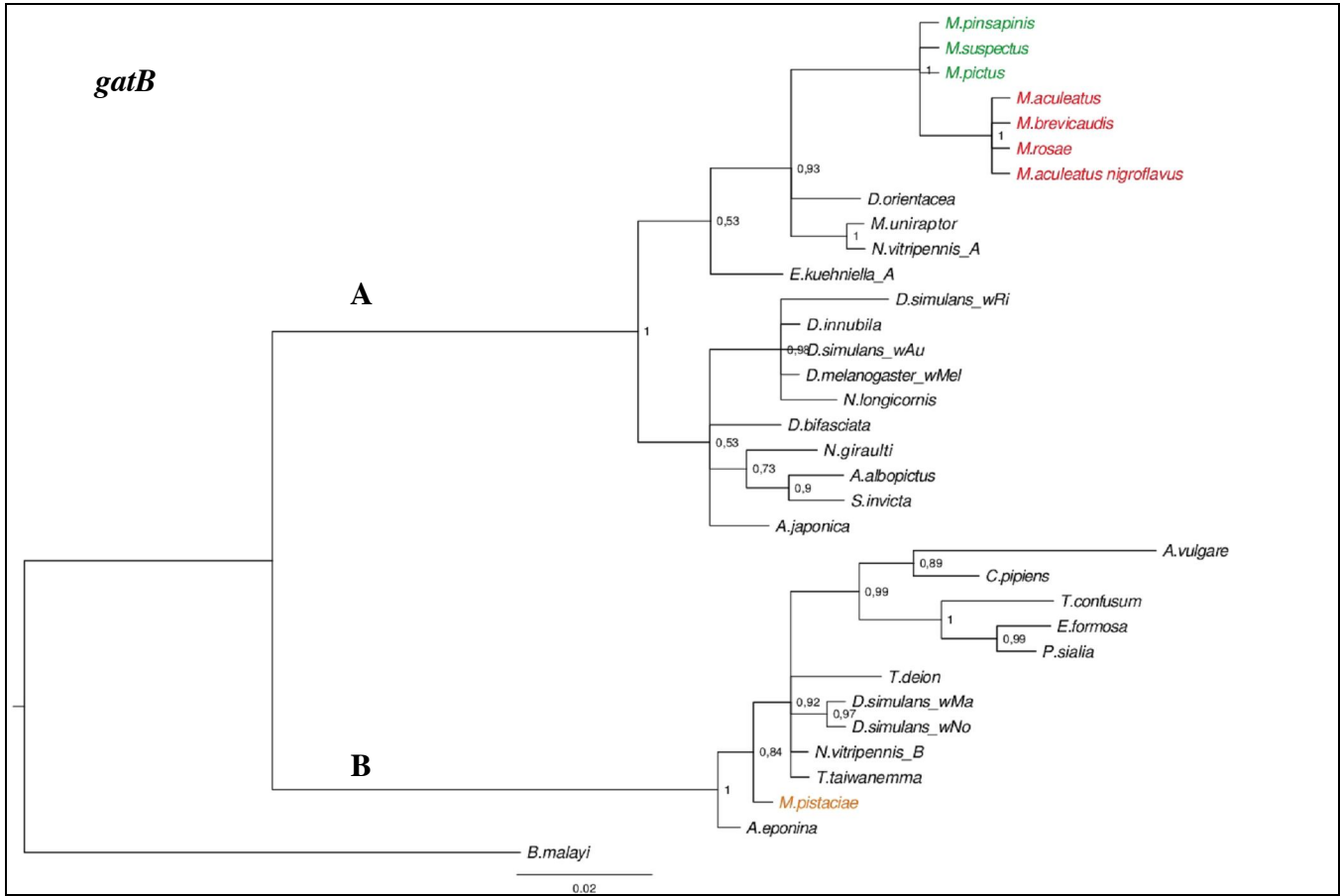
^cThao and Baumann (2004)

^dHagimori *et al.* (2006)

^eFolmer *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

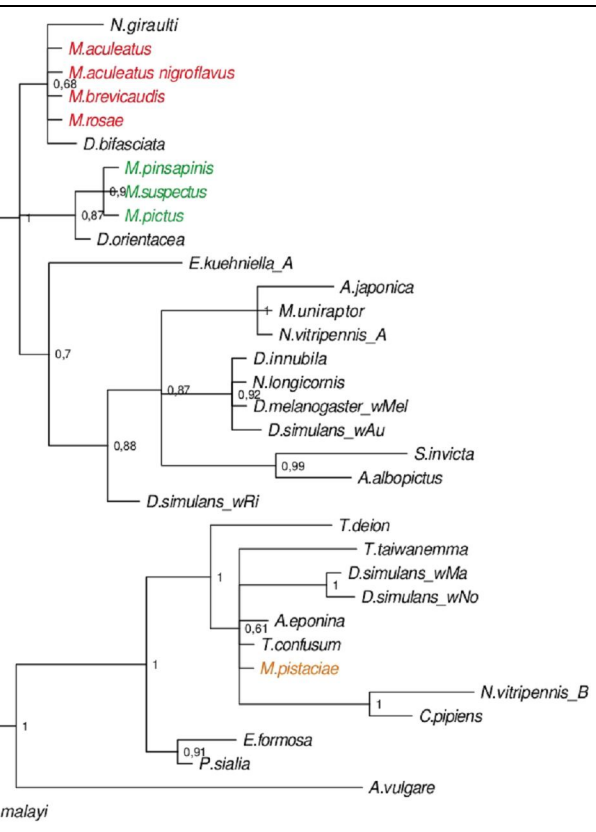
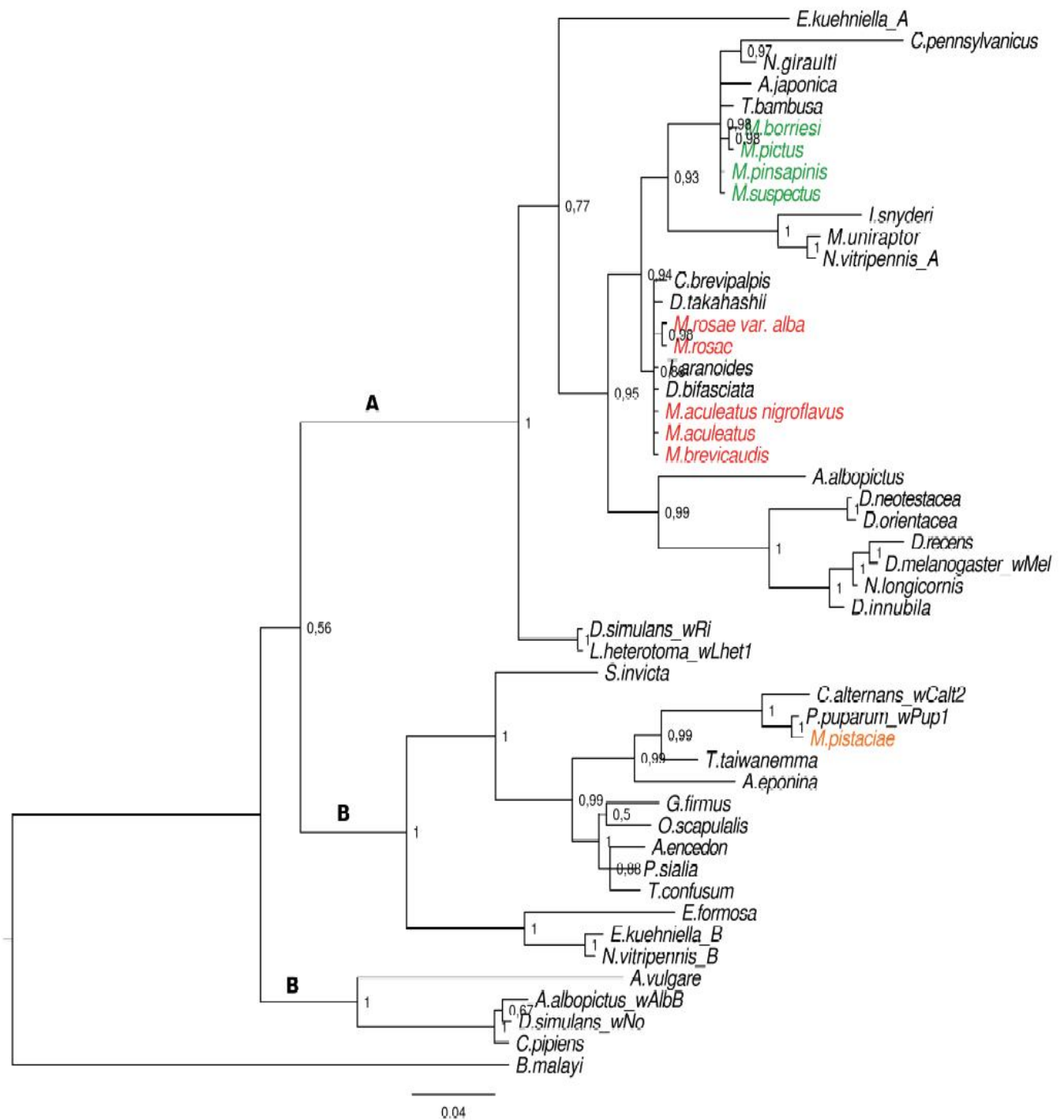


Figure S2. Phylogenetic placement of the *Wolbachia* strains infecting the seed-specialized wasps *Megastigmus* spp., among other *Wolbachia* strains belonging to A and B supergroups, based on wsp sequences of 49 strains (530 bp). Bayesian likelihood inference phylogenies are shown, while maximum likelihood 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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