EVOLUTION OF ASEXUALITY VIA DIFFERENT MECHANISMS IN GRASS THRIPS (THYSANOPTERA: Aptinothrips)

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Asexual lineages can derive from sexual ancestors via different mechanisms and at variable rates, which affects the diversity of the asexual population and thereby its ecological success. We investigated the variation and evolution of reproductive systems in Aptinothrips, a genus of grass thrips comprising four species. Extensive population surveys and breeding experiments indicated sexual reproduction in A. elegans, asexuality in A. stylifer and A. karnyi, and both sexual and asexual lineages in A. rufus. Asexuality in A. stylifer and A. rufus coincides with a worldwide distribution, with sexual A. rufus lineages confined to a limited area. Inference of molecular phylogenies and antibiotic treatment revealed different causes of asexuality in different species. Asexuality in A. stylifer and A. karnyi has most likely genetic causes, while it is induced by endosymbionts in A. rufus. Endosymbiont-community characterization revealed presence of Wolbachia, and lack of other bacteria known to manipulate host reproduction. However, only 69% asexual A. rufus females are Wolbachia-infected, indicating that either an undescribed endosymbiont causes asexuality in this species or that Wolbachia was lost in several lineages that remained asexual. These results open new perspectives for studies on the maintenance of mixed sexual and asexual reproduction in natural populations.

KEY WORDS: Asexual reproduction, parthenogenesis, haplodiploidy, hybridization, endosymbiont, Wolbachia.
may be caused by hybridization, documented mainly in vertebrates (Schultz 1973; Moritz et al. 1989; Neaves and Baumann 2011). Finally, asexuality may be induced through infection by endosymbionts, the best known being Wolbachia and Cardinium (Stouthamer and Werren 1993; Zchori-Fein et al. 2001; Weeks et al. 2003; Zchori-Fein and Perlman 2004; Werren et al. 2008). These endosymbionts are transmitted by females to their offspring through the egg cytoplasm. Because only females contribute cytoplasm to the next generation, the endosymbionts benefit from inducing hosts to allocate most or all resources to the production of daughters (reviewed in Breeuwer and Jacobs 1996; Werren et al. 2008). Endosymbiont-induced asexuality is best documented in parasitoid wasps, where bacterial infection has been linked to their haplodiploid sex determination system (e.g., Werren et al. 2008; Cordaux et al. 2011). Haplodiploidy is characterized by the development of males from unfertilized (haploid) eggs and females from fertilized (diploid) eggs. Unfertilized eggs laid by endosymbiont-infected females undergo diploidization in the absence of fertilization with sperm and develop into females.

Here, we investigate the evolution of asexuality in grass thrips (genus Aptinothrips). Similar to parasitoid wasps (and hymenopterans in general), sexual thrips species are characterized by haplodiploid sex determination (Lewis 1973). Asexual reproduction has been suggested to be widespread among thrips, as many species display strongly female-biased population sex ratios or are even only known from females (e.g., Lewis 1973; Mound and Moritz 2009). However, the potential for asexual reproduction has thus far only been investigated in a handful of species (Arakaki et al. 2001; Nault et al. 2006; Kumm and Moritz 2008). The genus Aptinothrips comprises four wingless species: A. rufus, A. elegans, A. stylifer, and A. karnyi (Palmer 1975). For A. rufus, A. stylifer, and A. elegans males have been reported but can be very rare locally (Sharga 1933; Palmer 1975), such that their role for reproduction remains unclear (Sharga 1933). For A. karnyi no males are known (Palmer 1975), suggesting this species is asexual.

We first conducted extensive Aptinothrips population surveys and breeding experiments using field samples from 16 different countries, to describe population sex ratios and to characterize the reproductive modes occurring in the genus. We then investigated the relationships between species and lineages with different reproductive modes via inference of molecular phylogenies based on nuclear and mitochondrial markers. Finally, to test for potential origins of asexuality through endosymbiont infection, we screened grass thrips for endosymbionts known to manipulate host reproduction and treated females of asexual grass thrips lineages with antibiotics.

**Material and Methods**

The grass thrips genus Aptinothrips is native to Europe, but the species A. rufus and A. stylifer have been reported throughout the world (Palmer 1975; Mound and Masumoto 2009). Aptinothrips species are small (< 1.5 mm) and occur on a variety of grasses (Poaceae family), with little or no information available on potential host specificity (Lewis 1973; Palmer 1975). To survey Aptinothrips field populations, we collected thrips from various grasslands from over 100 locations throughout Europe and the United States between February 2012 and June 2013. Details for the 100 locations at which we found Aptinothrips are given in the Supplementary Table 1. We used two different sampling strategies to determine the sex ratio of adult thrips in the field or to collect individuals for breeding experiments. Samples for determination of adult sex ratios were collected by beating grass tufts over a collecting tray and by preserving the content of the tray in alcohol. The alcohol-preserved material from the tray was then sorted in the laboratory, and all thrips (larvae and adults) were isolated from other arthropods and plant materials under a dissecting scope at 20–50× magnification. This strategy assures that no sex ratio bias is introduced artificially by preferentially collecting females, which are larger and more conspicuous than males. Among all the collected thrips, we isolated adult Aptinothrips, and identified species and sex for each individual following Palmer (1975) and Zur Strassen (2003) to determine the sex ratio among adults. Individuals for breeding experiments were either beaten from grass tufts onto a white collecting tray and picked up with a small paintbrush, or isolated from cut grass in the laboratory.

To determine the mode of reproduction characterizing each Aptinothrips species, Aptinothrips larvae of unknown sex (it is not possible to distinguish sexes or Aptinothrips species in larvae) collected in the field were isolated individually in a “cage” (a sealed 50 ml plastic tube or 3 dl cup), containing a wheat plantlet. This assured that all females used in breeding experiments were virgins—adult females collected in the field might have mated already. The larvae that reached maturity and turned out to be female were allowed to produce offspring. Asexual reproduction is then indicated by a virgin female producing only daughters. By contrast, sexual virgin females would produce sons, since under haplodiploidy, unfertilized eggs develop into males. The generation time for A. rufus was estimated to be approximately 40 days at room temperature (Sharga 1932); we therefore started checking for adult offspring in each cage after four weeks of maintaining the cages at 20°C ± 2 under a light regime of L:D = 16:8. When adults were spotted, cages were emptied completely to determine the number of larvae, males, and females present. Excluding all cages initiated with larvae that did not reach adulthood or that
EVOLUTION OF ASEXUALITY IN GRASS THRIPS

Fig. 1. Distribution and adult sex ratios of (A) Aptinothrips elegans (B) A. stylifer, and (C) A. rufus populations in the surveyed range. A. karnyi is not displayed as this species only occurred in three locations in Switzerland. Geographically close locations were fused for easier display; a complete list of locations with numbers of individuals sampled is provided in the Supplementary Table 1.

developed into males, we obtained 238 cages with virgin females for which we could determine the mode of reproduction.

Molecular Sequence Analyses
To infer phylogenetic relationships between different Aptinothrips lineages we used 1–6 individuals from 68 locations, for a total of 159 individuals (114 A. rufus, 26 A. stylifer, 14 A. elegans, and 5 A. karnyi, see Supplementary Table 1 for details). A subset of these individuals (58 in total; 15 A. stylifer females, 6 A. elegans males, and 37 A. rufus) came from locations where we did not directly infer the reproductive mode of individuals via breeding experiments. Males in these uncharacterized samples were considered sexual and A. stylifer females were considered asexual as there was no indication for sexual reproduction in this species (see results). Twenty-eight A. rufus females from Belgium and the Netherlands were also considered asexual because they were collected from female-only populations in a very densely surveyed region with many populations in which asexual reproduction was inferred via breeding experiments (see Fig. 1). For the remaining nine A. rufus females (two from Poland, two from Finland, three from Germany, and two from Denmark) the reproductive mode is unknown. DNA from each individual was extracted using the QIAGEN tissue extraction kit (QIAGEN Inc.) according to the manufacturer’s protocol. After extraction, the exoskeleton of each individual was stored in ethanol as a voucher.

We sequenced portions of one mitochondrial gene (COI, 650 bp), one nuclear gene (Histone H3, 454 bp), and the nuclear 28S rRNA (350 bp), using previously published primer pairs (Table 1). The following PCR conditions were used for all primer combinations: each reaction contained 5 μl extracted DNA template, 2.5 μl 10× buffer, 2.0 μl dNTP (2.5 μM), 2.5 μl MgCl2 (2.5 μM), 0.5 μl of each primer (10 μM), 0.6 μl Taq (250 units) and were completed to 25 μl using nanopure water. Cycling conditions were as follows: 1 × 5 min 95 C; 35 × 40 sec 95 C, 40 sec 50 C, 40 sec 72 C; 1 × 7 min 72 C. Nanopure water was always included as negative control. Five microliters of the PCR-product was run on an ethidiurnbromide-stained, 1% agarose gel. Products that showed the expected size were purified using ExoSap-IT according to the manufacturer’s protocol (Isogen Life Science B.V., De Meem, The Netherlands). Five microliters purified PCR product was combined with 5 μl forward or reverse primer (5 μM) and sent to GATC Biotech, Germany (www.gatc-biotech.com) for sequencing. The sequences have been deposited in GenBank with the accession numbers KJ459552–KJ459710 for COI, KJ459393–KJ459551 for H3, and KJ436391–KJ436566 for 28S.

Nucleotide sequences were aligned using ClustalX (Thompson et al. 1994) via the BioEdit Sequence Alignment Program version 7.1.3 (www.mbio.ncsu.edu/BioEdit/bioedit.html). Maximum likelihood phylogenetic analyses (with heuristic tree searches) and topology tests were carried out using PAUP*4.0b10 (Swofford 1993, 2000) on Bioportal (www.bioportal.uio.no), using Aeolothrips as an outgroup. We used the optimal model of sequence evolution identified with the Akaike information criterion (AIC) as implemented in jMODELTEST 0.1.1 (Posada 2008). Branch support in the full phylogenies was evaluated by a maximum-likelihood/parsimony bootstrapting analysis using Seqboot (500 replicates), DNAml respectively DNApars, and Consense within the Phylib 3.68 package (Felsenstein 1993). Bayesian posterior probabilities were inferred with MrBayes3.1.2.
(Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003), with Markov chains run for 10^6 generations, 10^4 generations burn-in values, and trees sampled every 100 generations. Trees were formatted with FigTree version 1.4 (Rambaut 2012).

### ANTIBIOTIC TREATMENT AND ENDOSYMBOIONT SCREENS

The population surveys and breeding experiments revealed asexual reproduction in three of the four Aptinothrips species (see Results). To test whether asexuality in these species is caused by infection with endosymbionts, a subset of thrips was treated with the broad-spectrum antibiotic tetracycline (Sigma, St. Louis MI) and the offspring sex ratio produced by treated thrips females was compared to the sex ratio produced by females of a control group. We used different strategies to treat the females, as we did not have any prior knowledge of what type of treatment would be effective in removing putative endosymbionts. First, females were isolated in groups of 5 for 18–24 h in a 1.5 ml eppendorf tube with a paper towel soaked in grass juice containing 50 mg/ml tetracycline. Females of the control group were isolated similarly but without tetracycline dissolved in the grass juice. Grass juice was obtained by blending wheat plants (grown in the laboratory) with demineralized water. The females were then transferred to cages (similar to those used in the breeding experiments), with five treated or five untreated females per cage. Twenty-four hours later, cages containing treated females were sprayed with tetracycline dissolved in water (50 mg/ml), control cages were sprayed with water. This treatment was repeated once per week for six weeks. Cages were opened 5–7 weeks after introducing the females to determine the number and sex ratio of adult individuals present. Out of 55 cages started with five females, 47 yielded offspring (30 A. rufus, 14 A. stylifer, 3 A. karnyi). For each species, we compared the production of males between treatment and control cages using GLM with a quasibinomial error distribution using R 2.13.0 (R development core team, 2011).

Given that the antibiotic treatments revealed evidence of endosymbiont-induced asexuality at least in some A. rufus females (see results), we screened the endosymbiont communities of two sexual (sexual A. rufus and A. elegans) and two asexual (asexual A. rufus and A. stylifer) lineages for bacteria associated with reproductive manipulation (reviewed in Duron et al. 2008; Kageyama et al. 2012): Wolbachia, Cardinium, Ricksettia, Spiroplasma, Flavobacteria, Arsenophonus (note that only the first three are known to induce asexuality). For each of the four lineages, we pooled 20–60 individuals from at least three separate populations and extracted genomic DNA as described above. Prior to DNA extraction, samples were surface sterilized by washing them in 10% bleach for ~30 sec and rinsed in 95% ethanol to remove residual bleach before DNA extraction. We then amplified ~520 bp of the bacterial 16S rRNA region with general primers (Uni9F: GAGTTTGATYMTGGCTC and BR534/18: ATTACCGCGCTGCCGTGC; Wilmotte et al. 1993) for each of the four population pools. The purified 16S rRNA amplicons were sent to a commercial facility (GATC Biotech, Konstanz, Germany) for FLX 454 pyrosequencing (1/16 plate). Sequencing reads were analyzed with the QIIME (Caporaso et al. 2010) core analyses script (core_qiime_analyses.py) using default parameters, OTU tables were summarized at different taxonomic levels using a second QIIME script (summarize.taxa.py).

The 16S amplicon screen revealed only Wolbachia as a candidate for asexuality induction in grass thrips; we did not find any of the other bacteria previously reported to manipulate reproduction in arthropod hosts (see Results). We therefore tested the grass thrips individuals used for phylogenetic inference, and additional 104 individuals (total 263), for Wolbachia infection, by amplifying a Wolbachia-specific surface protein (WSP). Since primers may vary in their ability to detect different Wolbachia strains (Baldo et al. 2006), two different sets of primers were used, following the published PCR conditions in each case: WSP-F and WSP-R from Jeyaprakash and Hoy (2000), amplifying ~600 bp, and WSP-F236 and WSP-R446 from Braig et al. (1998), amplifying ~210 bp. Five microliters of the PCR-product were run

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### Table 1. Information for primers used in molecular analyses.

<table>
<thead>
<tr>
<th>Locus: Primer pair (forward: reverse)</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>COI: (mtD7.2F; CI-N-2329)</td>
<td>ATTAGGAGCHCCHGAYATAGCATT</td>
<td>ACTGTAAATATATGAGCTCA</td>
<td>(Simon et al. 1994; Brunner et al. 2002)</td>
</tr>
<tr>
<td>COI: (HCO-2198; LCO-1490)</td>
<td>TAAACTTCAAGGTGACAAAAATCA</td>
<td>GGTCACAAATCTCAAGATATTGG</td>
<td>(Simon et al. 1994)</td>
</tr>
<tr>
<td>Histone H3: (H3NF; H3R)</td>
<td>ATGGCTCTGTACAAAAAGACAGAC</td>
<td>ATATCCCTTGGCATATRGTGAC</td>
<td>(Glover et al. 2010)</td>
</tr>
<tr>
<td>28S rRNA: (28SS; 28SA)</td>
<td>GACCGTCTTTGAAMCAMGGA</td>
<td>TCGGARGGAACCAGCTACTA</td>
<td>(Inoue and Sakurai 2007)</td>
</tr>
</tbody>
</table>

(LJC and TSC)
on an ethidiumbromide stained 1% agarose gel. A sample was considered positive for Wolbachia if a PCR with one of the two WSP primer pairs generated a band of the expected size on the gel. Positive controls (initially, the asexual A. rufus extraction used for the 16S screen, later samples tested positive in previous PCRs) and negative controls (nanopure water) were always included. Wolbachia-negative samples were tested at least twice and up to six times with each primer pair.

**Results**

**POPULATION SURVEYS AND REPRODUCTIVE MODES**

We found different Aptinothrips species at 100 locations spread over 16 countries (Supplementary Table 1, Fig. 1A–C). Samples from most locations (82%) comprised only one Aptinothrips species, but we also found two species (15% of the locations), or even three (3%), co-occurring together. The most widespread species is A. rufus, which we found at 87 locations (15 countries). A. elegans occurred at nine southern locations (Fig. 1A). A. karnyi was very rare in the surveyed area as it was only found in Switzerland (three locations). Aptinothrips is a genus of European origin (Palmer 1975), but we found introduced populations in the United States of A. rufus, A. stylifer, and A. elegans, whereby A. elegans was thus far not known to occur in the United States.

We found no evidence for asexual reproduction in A. elegans at the nine locations where this species occurred. Males occurred at all nine locations, with the sex ratio among field-collected adults ranging from 0.17 to 0.5 (Supplementary Table 1, Fig. 1A). All 29 female larvae reared in isolation produced only sons after reaching adulthood, as expected under sexual reproduction and haplodiploidy. While it is possible that this species may reproduce asexually at other locations, or that some locations comprise rare asexual lineages among the sexual ones, the predominant mode of reproduction in the surveyed area appears to be sexual. Sexuality in A. elegans is in contrast to the three other Aptinothrips species that, as explained below, appear to reproduce mostly (A. rufus) or exclusively (A. stylifer and A. karnyi) asexually.

We did not find a single male at any of the locations comprising A. stylifer (22 locations, 233 adult females) or A. karnyi (three locations, 28 females). For both species, females collected as larvae and reared in isolation produced only daughters (40 A. stylifer females and six A. karnyi females tested), corroborating asexual reproduction suggested by the female-only natural populations.

For A. rufus, females at 83 out of the 87 locations were found to reproduce exclusively asexually. Not a single adult male was found in the field among over 3000 females and all 137 virgin females reared in isolation produced only daughters. One additional location (“Plateau des Tailles,” Supplementary Table 1) most likely also comprises only asexual females. We found only two males among 150 females at this location, but it is currently not possible to test whether these males are indicative of rare sexual lineages or whether they represent developmental “accidents”—rare males are known to be produced by many obligately asexual lineages (see Discussion). A. rufus females at the three remaining locations, clustered at the south-western end of the surveyed area (southern Spain and Portugal, Fig. 1C), reproduced exclusively sexually. Adult males were found at each of the locations (sex ratios respectively 0.20, 0.26, and 0.33) and all 26 females reared in isolation produced only sons.

**APTINOTHIRPS SPECIES RELATIONSHIPS AND TRANSITIONS TO ASEXUALITY**

Our genetic analyses supported the distinction of the four Aptinothrips species as they were described using morphological criteria (Palmer 1975). However, the relative grouping of the species, while consistent between the mitochondrial gene COI and the nuclear gene H3 (Kishino–Hasegawa topology test: P = 0.48), differed significantly for the noncoding 28S locus (Kishino–Hasegawa topology test: P < 0.001). The different topologies were due to A. stylifer sharing similar or even identical 28S sequences with A. elegans individuals, while this species was clearly differentiated from A. elegans at the two other markers and also more closely related to A. rufus than to A. elegans (Fig. 2).

Independently of whether the Aptinothrips species tree is better represented by the topology of the COI-H3 gene tree or the 28S tree, both topologies indicate that transitions to asexuality within A. rufus would have occurred independently of the transition leading to A. stylifer and A. karnyi. In both cases, a tree for which we constrained all asexual groups to be monophyletic was a significantly poorer fit to the data than the unconstrained tree (Templeton tests, all P < 0.0001; Shimodaira–Hasegawa tests, all P < 0.0001).

Within A. rufus, the tree topologies obtained with each individual or concatenated loci were very similar (Kishino–Hasegawa topology test: all P > 0.45) and revealed three differentiated clades, one comprising only individuals from the sexual populations and two reciprocally monophyletic clades of asexual individuals (Fig. 2; topology tests conducted with all three loci concatenated: Templeton test, P < 0.001; Shimodaira–Hasegawa test, P < 0.001). Eight of the nine A. rufus females for which the mode of reproduction was not known clustered together with 84 asexual individuals in A. rufus clade I, the ninth individual clustered with six asexual individuals in clade III (Fig. 2). The topology of the A. rufus subtree thus indicates either at least two independent transitions to asexuality in A. rufus, or one transition to asexuality followed by a reversal back to sexual reproduction.
Figure 2. *Aptinothrips* phylogeny based on (A) concatenated COI and Histone H3 sequences and (B) 28S sequences. Asexual species and lineages are displayed in red, the size of the triangles is proportional to the genetic diversity in each clade. For each clade, the total number of individuals (ind) and the percentage of *Wolbachia*-infected individuals (infected) is indicated in square brackets (for *A. rufus*, percentages only refer to individuals used in the phylogeny, for the other species percentages are based on all tested individuals). Numbers at branches indicate bootstrap support for maximum likelihood and parsimony and Bayesian posterior probabilities. Clade II of *A. rufus* comprises one female, from Nawa, Poland for which the reproductive mode was not tested in breeding experiments (Supplementary Table 1). Clade I comprises eight such females, including one from Nawa. (C) Minimum spanning network based on COI sequences indicating the genetic divergence within and between groups, inferred in Arlequin 3.5.1.3 (Excoffier and Lischer 2010). The length of the branches separating haplotypes is proportional to the number of mutations separating them (scale indicates length for 40 mutations).
**ANTIBIOTIC TREATMENT AND ENDOSYMBIONT SCREENS**

Antibiotic treatments revealed that asexuality is induced by bacterial endosymbionts in *A. rufus* but most likely not in *A. stylifer* or *A. karnyi*. All groups of *A. rufus* females fed with antibiotics produced at least some male offspring (14 out of 14 cages), no males were produced by untreated groups of females (0 out of 16 cages), with treatment cages being significantly more likely to yield males than control cages ($t = 135$, $P < 0.0001$). The proportion of males produced by females in the treatment cages varied widely (0.09–0.81), but offspring comprised individuals of both sexes in each cage. For the two other species, no males were found among the offspring of either the treated or untreated female groups (*A. stylifer*: seven control and seven test cages, $t = 0.63$, $P = 0.54$; *A. karnyi*: one control and two test cages, $t = 0.03$, $P = 0.98$).

Given the evidence for endosymbiont-induced asexuality in *A. rufus*, we screened bacterial communities of asexual and sexual *A. rufus* population pools, as well as of *A. stylifer* and *A. elegans* populations, for the presence of endosymbionts known to manipulate reproduction in other arthropods. *A. karnyi* was not included because we did not have enough individuals available. 16S amplicon sequencing of each of the four samples produced combined 45,842 reads longer than 200 bp, representing 919 different bacterial strains. Blast searches revealed that 42 of these strains corresponded to *Wolbachia*. No similarity to other bacterial endosymbionts associated with reproductive manipulation was found for the remaining strains.

Forty of the 42 *Wolbachia* strains were from the pooled *A. rufus* asexual populations, where *Wolbachia* accounted for the majority (71%) of reads. This indicates that *Wolbachia* is the dominant endosymbiont in asexual *A. rufus*. The two remaining *Wolbachia* strains were from the pooled *A. stylifer* (one strain, 0.01% of the reads) and *A. elegans* populations (one strain, 0.02% of all reads). Not a single *Wolbachia* sequence was found among the 11,965 reads from the pooled sexual *A. rufus* populations.

The abundance of *Wolbachia* in the endosymbiont communities of asexual *A. rufus* prompted us to screen a subset of 263 individuals (including the 159 used for phylogenetic analyses) from different locations for *Wolbachia* infection via PCR assays. These screens revealed variable infection with *Wolbachia*, both between and within species, that were loosely associated with reproductive mode. For the sexual species *A. elegans*, four out of 23 tested females were infected with *Wolbachia* (17%), all four from the same location (La Sarraz). Interestingly, this population corresponds to the one with the most strongly female-biased sex ratio among all *A. elegans* populations surveyed (Supplementary Table 1). Two additional females from the same location were not infected. Only one of the five tested *A. karnyi* females and 16 out of 45 *A. stylifer* females (36%) were infected with *Wolbachia* without any particular geographic pattern relative to the distribution of infected or noninfected individuals. For the asexual *A. rufus*, 67% of the individuals were infected (111 out of 166) with infection rates varying among clades. Asexual *A. rufus* of clade I had the highest infection rate (70%, Fig. 2A). Given this clade is characterized by very low genetic diversity (Fig. 2C), the incomplete infection suggests either recent infection with *Wolbachia* or recent loss of infection. Interestingly, none of the seven asexual *A. rufus* individuals of the clade most closely related to the sexual *A. rufus* was infected with *Wolbachia* (clade III, Fig. 2). Similarly, not a single one of the 24 tested sexual *A. rufus* females were infected. The partial (or complete lack of) infections are unlikely to stem from technical errors. The systematic use of positive controls in PCR assays and repeated tests for *Wolbachia*-negative individuals (4–6 independent assays, including tests with different primer sets) make false negatives as a consequence of PCR failures highly unlikely.

In summary, sexual populations of *Aptinothrips* are generally not infected with *Wolbachia* (except for *A. elegans* in La Sarraz, where it remains to be confirmed whether there could be a mix of sexual and infected asexual individuals). The majority of asexual *A. rufus* females are infected with *Wolbachia* (67%), and antibiotic treatment reveals that asexuality is induced by endosymbionts in at least a subset of these females. Only a minority of asexual *A. karnyi* (1 out of five) and *A. stylifer* (36%) females are infected with *Wolbachia* and antibiotic treatment of females in these species did not induce the production of males. No other endosymbionts known to manipulate reproduction in arthropods were uncovered in the endosymbiont communities of *A. rufus*, *A. stylifer*, and *A. elegans*.

**Discussion**

Our study shows that the genus *Aptinothrips* comprises both sexual and asexual species and populations. Across the surveyed locations, *A. elegans* and the southern Spain and Portugal *A. rufus* populations are characterized by exclusively sexual reproduction, even though sex ratios are generally female biased. Female-biased population sex ratios are characteristic of many thrips species, and have largely been explained by males being short lived (Lewis 1973). In the remaining *A. rufus* populations, as well as in *A. karnyi* and *A. stylifer*, females reproduce asexually. The distribution of *A. karnyi* appears to be very limited, with records confined to the Alps and one report from Nepal (Palmer 1975, this study). The three remaining *Aptinothrips* species are geographically very widespread. The sexual species *A. elegans* occurs at least throughout Europe and in one location in the United States, whereas *A. stylifer* and especially *A. rufus* have as a minimum a Holarctic, if not worldwide, distribution (Palmer 1975; Zur Strassen 2003; Hoddle et al. 2012; this study). Given this widespread occurrence...
of *A. rufus*, the very limited distribution of sexual *A. rufus* populations, confined to southern Spain and Portugal, indicates that asexuality is a very successful strategy in this species. Asexuality may either have allowed the colonization of a vast geographic range inaccessible to sexual *A. rufus* lineages, or asexual lineages would have largely or completely outcompeted their sexual counterparts across most of the range. Indeed we found no evidence for sexual and asexual *A. rufus* lineages forming locally mixed populations. Asexual *A. rufus* lineages co-occur, however, with the asexual *A. stylifer* as well as with the sexual species *A. elegans*, raising the question of what mechanisms allow for the maintenance of reproductive polymorphisms among these closely related species.

We cannot formally exclude the hypothesis that that rare sexual lineages persist in largely asexual populations as we found occasional males in otherwise asexual populations of *A. rufus* and as males have also been reported for *A. stylifer* (Sharga 1933; Palmer 1975; this study). However, given the very sporadic occurrence of such males in populations with no evidence for sexual reproduction among females, it appears more likely that these males are produced “accidentally” by asexual females. Such accidental production of males is known for many, if not most, obligately asexual lineages, where males represent vestiges of a formerly sexual lifestyle and typically have no mating opportunities or success in natural populations (recently reviewed in van der Kooi and Schwander 2014).

The production of occasional males by asexual females is especially widespread among lineages in which asexuality is induced by infection with endosymbionts (van der Kooi and Schwander 2014), possibly because of incomplete endosymbiont transmission to offspring or failed manipulation of host reproduction (Reumer et al. 2012). Consistent with this pattern, asexuality in *A. rufus* is caused by endosymbionts. Indeed, treatment of asexual *A. rufus* females with the antibiotic tetracycline induced the production of males, as reported for other species with haplodiploid sex determination. For example, endosymbiont-induced asexuality is well-known among parasitoid wasps (e.g., Stouthamer et al. 1990; Zchori-Fein et al. 1992; Werren et al. 1995; Pijls et al. 1996; Kremer et al. 2009) and has also been documented in mites (e.g., Breeuwer and Jacobs 1996; Groot and Breeuwer 2006; Ros et al. 2008). Among thrips, it has previously been reported for *Frankliniella vespiformis* (Arakaki et al. 2001) and *Hercinothrips femoralis* (Kumm and Moritz 2008). Antibiotic treatment generally causes asexual females to produce only sons. In *A. rufus* however, antibiotic-treated females still produced daughters in addition to sons, as previously reported in *Hercinothrips femoralis* (Kumm and Moritz 2008). Mixed progeny may indicate incomplete removal of the endosymbionts, whereby only individuals with very few or no endosymbionts develop into males. However, because we treated and maintained females in groups of five, it is currently not possible to say whether all females produced daughters and sons, or whether antibiotic treatment does not induce male production in a subset of females.

The combination of male induction via antibiotic treatment and evidence for infection with *Wolbachia* (or another endosymbiont known to induce asexuality in some species) is usually interpreted as evidence for the endosymbiont inducing asexuality in a species (e.g., Pijls et al. 1996; Arakaki et al. 2001; Zchori-Fein et al. 2001; Kumm and Moritz 2008; Kremer et al. 2009). Such interpretations should be considered with caution, as *Wolbachia*-infection is very widespread, including among species with sexual reproduction (Duron et al. 2008; Hilgenboecker et al. 2008; Zug and Hammerstein 2012). In *A. rufus*, the characterization of endosymbiont communities via 16S sequencing revealed evidence for *Wolbachia* infection, and lack of infection with any other endosymbiont known to induce asexuality. This may suggest that *Wolbachia* also induces asexuality in *A. rufus*. However, in contrast to previous studies where typically only one or a few strains were tested for endosymbiont infection, we screened many asexual *A. rufus* individuals from a broad geographic range. This screen revealed that less than 70% asexual *A. rufus* females are infected, indicating that either a different, currently unknown, endosymbiont causes asexuality in *A. rufus* or that *Wolbachia* has been lost in a number of strains that remained asexual in the absence of infection. Consistent with the latter hypothesis, one clade of *A. rufus* (clade III consisting of seven individuals, Figure 2) was completely uninfected. However, most asexual *A. rufus* females belong to clade I (*n = 93*), in which 30% of individuals were also uninfected, indicating that there would have been multiple losses of endosymbionts with maintenance of asexuality. Such losses would have occurred very recently given this clade is characterized by very low genetic diversity (Fig. 2C). The possibility that asexuality has been maintained, for example via transfer of asexuality-inducing elements from the *Wolbachia* genome into the host genome, is currently being investigated. Independently of the mechanism accounting for incomplete *Wolbachia* infection in asexual *A. rufus*, the geographic distributions of infection prevalence or asexual lineage representation had no apparent pattern. Most locations comprised individuals from different asexual clades with a subset of them being uninfected, indicating extensive dispersal for all categories of asexual *A. rufus*.

The reciprocal monophyly of the two *A. rufus* clades consisting of asexual individuals, and their positioning relative to sexual *A. rufus*, indicates either two independent transitions to asexuality from a sexual ancestor, or one transition to asexuality followed by a reversal to sexual reproduction. These two alternatives cannot be distinguished with the currently available data. Reversals from asexual to sexual reproduction are generally considered to be impossible, since sexuality is assumed to be a complex trait which is
highly unlikely to reevolve once it is lost (Bull and Charnov 1985). However, cases of endosymbiont-induced asexuality appear to be an exception to this rule (Stouthamer et al. 1990; van der Kooi and Schwander 2014). This may be because in contrast to other forms of asexuality, endosymbiont-induced asexuality does not require genetic changes in the host, such that reversals may be possible, at least in recently infected lineages (van der Kooi and Schwander 2014).

In contrast to A. rufus, there is no evidence that asexuality in A. stylifer and A. karnyi is caused by infection with endosymbionts. Treatment with broad-spectrum antibiotics did not induce the production of males, and we found low infection rates with Wolbachia, and no evidence for any other endosymbiont known to induce asexuality. Consistent with different mechanisms leading to asexuality in A. rufus versus A. stylifer and A. karnyi, phylogenetic analyses indicate that the transition to asexuality in A. stylifer and A. karnyi is independent from transitions to asexuality in A. rufus (Fig. 2). While we cannot formally exclude the hypothesis that other, unknown, endosymbionts that are insensitive to the applied treatments induce asexuality in A. stylifer and A. karnyi, the current evidence suggests that asexuality in these two species has genetic causes. Furthermore, the sharing of closely related 28S haplotypes between A. stylifer and A. elegans (Fig. 2B) suggests recent gene flow from A. elegans into A. stylifer that may be indicative of hybridization contributing to the split between A. stylifer and A. karnyi. Independently of the mechanisms that caused this split, the fact that there are two asexual sister species suggests that speciation would have occurred after the transition to asexuality, a rarely observed pattern in asexual lineages (Fontaneto et al. 2007).

In conclusion, our study reveals multiple transitions to asexuality that occurred via different mechanisms in a genus consisting only of four species. Endosymbiont-induced asexuality in A. rufus but incomplete infection with Wolbachia suggests either recurrent losses of infection or that a yet unknown endosymbiont may cause asexuality in this species, and highlights the importance of conducting species-wide screens for endosymbiont-infection when identifying bacterial candidates underlying the induction of parthenogenesis. Finally, Aptinothrips species and lineages with different reproductive modes co-occur together locally or at least at geographically close locations, raising the question of the mechanisms maintaining reproductive polymorphism in the genus Aptinothrips and opening new perspectives for studies on the maintenance of mixed sexual and asexual reproduction in natural populations.

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LITERATURE CITED


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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Table S1. Information on sampling locations.

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