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Extreme genetic diversity in asexual grass thrips populations

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Abstract

The continuous generation of genetic variation has been proposed as one of the main factors explaining the maintenance of sexual reproduction in nature. However, populations of asexual individuals may attain high levels of genetic diversity through within-lineage diversification, replicate transitions to asexuality from sexual ancestors and migration. How these mechanisms affect genetic variation in populations of closely related sexual and asexual taxa can therefore provide insights into the role of genetic diversity for the maintenance of sexual reproduction. Here, we evaluate patterns of intra- and inter-population genetic diversity in sexual and asexual populations of *Aptinothrips rufus* grass thrips. Asexual *A. rufus* populations are found throughout the world, whereas sexual populations appear to be confined to few locations in the Mediterranean region. We found that asexual *A. rufus* populations are characterized by extremely high levels of genetic diversity, both in comparison with their sexual relatives and in comparison with other asexual species. Migration is extensive among asexual populations over large geographic distances, whereas close sexual populations are strongly isolated from each other. The combination of extensive migration with replicate evolution of asexual lineages, and a past demographic expansion in at least one of them, generated high local clone diversities in *A. rufus*. These high clone diversities in asexual populations may mimic certain benefits conferred by sex via genetic diversity and could help explain the extreme success of asexual *A. rufus* populations.

Introduction

The evolution and maintenance of sexual reproduction has been a major focus in evolutionary biology for the last decades. Sex is the most widespread form of reproduction among eukaryotes, despite its profound costs relative to asexuality and other forms of female-producing parthenogenesis (reviewed in Bell, 1982; Lewis, 1987; Lehtonen *et al.*, 2012).

Many different hypotheses have been proposed to help explain the evolution and maintenance of sex. A number of them propose that sex is beneficial because it affects genetic variation (through recombination, segregation and mixis). For example, different theoretical approaches have shown that sex may be favoured indirectly, because it reduces interference between selected loci (e.g. Fisher, 1930; Hill & Robertson, 1966; Barton & Charlesworth, 1998; Otto & Lenormand, 2002; Agrawal, 2006) and/or allows populations to better adapt to certain temporal or spatial environmental changes (e.g. Peck, 1994; Orr, 2000; Roze & Barton, 2006). Several 'ecological models' further proposed mechanisms that would favour sex because it can generate higher levels of genetic diversity than asexuality. Under such genetic diversity-based mechanisms, individuals with rare genotypes (i.e. those generated by sex) have an advantage (Hurst & Peck, 1996). For example, under many scenarios of host–parasite coevolution, genotype-specific infection of hosts generates negative frequency-dependent selection for host genotypes (e.g. Buckling & Rainey, 2002; Decaestecker *et al.*, 2007; Jokela *et al.*, 2009). Similarly, rare genotypes can also be favoured under certain resource limitation scenarios if rare genotypes can exploit thinly occupied niche space (reviewed in Bell, 1982; Lam *et al.*, 2011; Song *et al.*, 2011).

Although sex can certainly contribute to the generation of genetic diversity within populations, high levels

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of diversity can also be present in asexual lineages. In fact, theory suggests that putative advantages of sex decrease as the genetic diversity of coexisting asexual lineages increases, to the point where the maintenance of sex can no longer be explained by negative frequency-dependent selection (Ladle *et al.*, 1993; Judson, 1997; Lively, 2010). Such scenarios are especially likely in assemblages of asexual lineages that were generated from multiple, independent transitions from sexual to asexual reproduction, given that independently derived asexuals often feature different ecologies (e.g. Lively & Howard, 1994; Janko *et al.*, 2008; Neiman *et al.*, 2014).

Here, we evaluate patterns of intra- and interpopulation genetic diversity in sexual and asexual populations of *Aptinothrips rufus* grass thrips. Species such as *A. rufus* which comprise both sexual and asexual lineages represent a unique opportunity to assess how different reproductive modes affect the generation and distribution of genetic diversity in natural populations. A previous study revealed that asexual *A. rufus* lineages are characterized by a much broader distribution range than the sexual lineages. Asexual populations are distributed throughout the world, whereas sexual lineages appear to be confined to few locations in the Mediterranean area, with only four known sexual populations (Palmer, 1975; van der Kooi & Schwander, 2014) (Fig. 1). We used all four known locations with sexual individuals, including the only currently known location where sexual and asexual females co-occur, and chose five locations with asexual females from three different continents. We then estimated genetic diversity and population differentiation using a combination of mitochondrial (COI, COII) and nuclear sequence markers (H3, EF) with 11 newly developed microsatellite markers. Furthermore, we investigate the mechanisms that might have generated the observed diversity patterns, including ecological processes and demographic scenarios (population expansions and post-glaciation colonization events), to ultimately understand what contributes to the success of asexual *A. rufus* grass thrips.

Materials and methods

Study species and sampling locations

Aptinothrips rufus is a small, wingless insect (1–2 mm) that occurs in various types of grasslands. All species in the *Aptinothrips* genus are specialist herbivores, feeding and breeding exclusively on grasses (Palmer, 1975). Whether there is further specialization within the grass family is not known. *Aptinothrips rufus* features mixed reproduction: some females reproduce sexually and are characterized by haplodiploid sex determination as typical for all thrips (Lewis, 1973); other females reproduce via parthenogenesis that is induced by infection with a currently nonidentified bacterial endosymbiont (van der Kooi & Schwander, 2014).

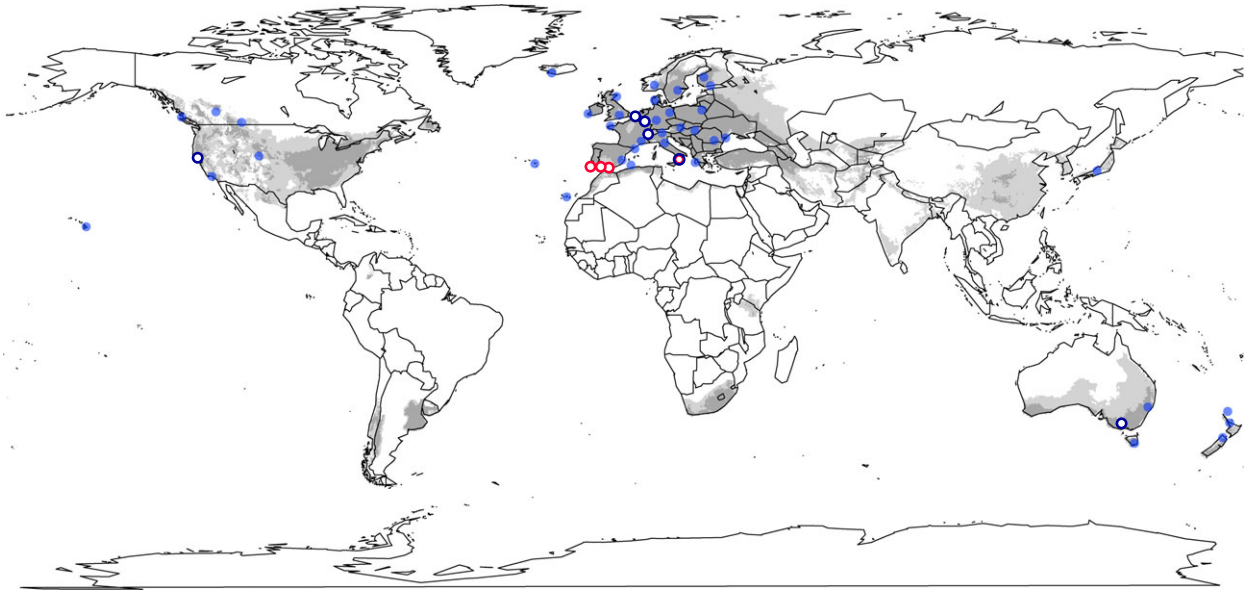

Fig. 1 Distribution of *Aptinothrips rufus* populations with known reproductive modes and sampling populations. The grey areas indicate the likely distribution of *A. rufus* (Palmer, 1975; van der Kooi & Schwander, 2014). Points indicate locations where the reproductive mode of A. rufus has been assessed (van der Kooi & Schwander, 2014), and conspicuous rings indicate locations used in the present study. Red points correspond to locations with sexual females only, blue points to locations with asexual females. Red-blue ring indicates the only location known so far where sexual and asexual females co-occur.

We sampled sexual and asexual thrips from nine different locations. Five of the nine locations were previously shown via breeding experiments to comprise only asexual *A. rufus* (Bex, La Roche, Groningen, Saratoga, Creswick), and three to comprise only sexual *A. rufus* (Lagos, Tavira, Malaga) (van der Kooi & Schwander, 2014; Z. Dumas & T. Schwander, unpublished; see Table S1 for additional information on the locations). The remaining location (Agropoli) consists of a mixture of sexual (~42%) and asexual females (~58%). Sexual and asexual females from Agropoli were separated for all analyses such that we had a total of ten populations stemming from nine locations: six asexual and four sexual (Table S1, Fig. 1). While four sexual populations represent a very limited sample, these four populations comprise all currently known sexual *A. rufus* populations. Furthermore, for the sexual Malaga population, only two individuals were available for genetic analyses. For the remaining locations, we used 20–24 field-collected individuals (Table S1).

Genotyping

We used a combination of mitochondrial (COI, COII) and nuclear sequence markers (H3, EF) with 11 newly developed microsatellite markers to estimate patterns of genetic diversity within and between thrips populations. To develop *Aptinothrips* microsatellite markers, a library of 6142 microsatellite-containing sequences was generated by GenoScreen, Lille, France (www. genoscreen.fr), by coupling multiplex microsatellite enrichment techniques with the 454 GS-FLX Titanium pyrosequencing (Malausa *et al.*, 2011). The library was based on pooled genomic DNA extractions from 200 *A. rufus* individuals collected from five different locations in four countries (40 individuals per location; two locations from the Netherlands, and one from the UK, Italy and Switzerland). DNA extractions were performed with the DNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. From the raw microsatellite-containing sequences, polymerase chain reaction (PCR) primer pairs were designed for 159 sequences, using the open access program QDD (Meglécz *et al.*, 2010). Of those, the 26 best ones were tested on a sample of three to four *A. rufus* individuals from diverse origins.

Of the 48 microsatellites markers tested, eleven were finally retained for further amplification based on the proportion of perfect repeats, amplification success and polymorphism level (Table S2). These 11 marker sequences were deposited in GenBank (Numbers KT768099–KT768109). Individual genotypes for the microsatellite loci were obtained with multiplex amplification. The PCR mix contained 4 μL of DNA template, 3 μL of Multiplex PCR Master Mix (Qiagen 2X), 2 μL of primer mix (0.2 μM of each primer; see Table S2 for primer sequences), 0.5 μL of $MgCl_2$ (1.25 mM) and 0.5 μL of nanopure water. Cycling conditions for all markers were as follows: 95 °C × 15 min; 40 cycles of 94 °C × 30 s, 58 °C × 90 s, 72 °C × 1 min; and 60 °C × 30 min. Amplification products (10× dilutions) were run on a ABI_3100 sequencing machine (Applied Biosystem, Foster city, CA), and alleles were scored with GeneMapper® v.3.7 (Applied Biosystems). We further carefully inspected the raw data for all individual genotype calls (peaks for each locus and individual) to identify potential cases of polyploidy (peaks consistent with more than two alleles per locus).

The amplification success of microsatellite markers was highly lineage-specific, most likely because the individuals used for marker development all belonged to the same asexual lineage of *A. rufus*. Of the 11 microsatellites, only one (TH41) successfully amplified across all *A. rufus* lineages. The remaining 10 microsatellites could only be amplified in one or two of the four sexual populations. We therefore used only one microsatellite for comparisons of genetic diversity and isolation by distance between sexual and asexual populations. All eleven microsatellites were used for estimating clonal diversities and heterozygosity in asexual populations (without comparisons with sexual populations).

In addition to the microsatellite marker(s), we amplified four DNA sequence markers to estimate within and between population diversities: two mitochondrial genes, cytochrome oxidases 1 and 2 (COI and COII, respectively, 266 and 327 bp) (GenBank accessions: KT699214–KT699396 for COI and KT699397–KT699476 for COII) and two nuclear genes, histone 3 (H3, 281 bp) (GenBank KT699577–KT699772) and elongation factor (EF, 96 bp). EF sequences could not be deposited in GenBank because they were too short and are instead deposited on Dryad (http://dx.doi.org/10.5061/dryad.h8017) along with the remaining raw data. Primer sequences and amplicon lengths for each sequence marker are specified in Table S3. DNA amplification for each marker was carried out in a 20 μL volume, including 5 μL of extracted DNA template, 2.0 μL of buffer (Qiagen 10x), 2.0 μL dNTP (2.5 μM), 1 to 2.5 μL of $MgCl_2$ (2.5 μM for COII and H3, 2 μM for EF, 1.5 μM for COI), 0.5 μL of each primer (0.4 μM), 0.6 μL Taq (Qiagen 5U/μL) and nanopure water. PCR cycling conditions were 1 × 95 °C, 38 × 95 °C-50 °C-72 °C, 1 × 72 °C, with each phase duration specified in Table S3. Five microlitres of the PCR product was run on an ethidium bromide-stained, 1.5% agarose gel. Five μL PCR product of each successfully amplified marker was purified using 4 μL mix of ExoI (20U/μL) (Thermo Scientific Waltham, MA, USA) and FastAP Thermosensitive Alkaline Phosphatase (1U/μL) (Thermo Scientific). After addition of 5 μL of forward primer, purified PCR products were sent to *GATC Biotech, Germany (*www.gatc-biotech.com*),* for Sanger sequencing.

DNA sequences were edited and aligned using CODON CODE ALIGNER (Codon Code Corporation, www.codoncode.com). Chromatograms were checked by eye to trim poor-quality regions and ensure correct base calling. As all mitochondrial markers are linked in both sexual and asexual lineages, we concatenated the two mitochondrial sequences (COI and COII) of each individual with SEQUENCE MATRIX (Vaidya *et al.*, 2011) and analysed them as a single marker. Mitochondrial haplotype networks were built with HAPLOVIEWER (Salzburger *et al.*, 2011) based on maximum parsimony inference as implemented in MEGA version 6 (Tamura *et al.*, 2013).

In contrast to mitochondrial loci, nuclear markers are diploid and PCRs amplify both alleles. As a consequence, direct Sanger sequencing of PCR products results in heterozygous positions that are displayed in chromatograms as two overlapping peaks of approximately equal heights. For the two sequenced nuclear markers (H3, EF), overlapping peaks with 60 : 40 (or more similar) relative heights were considered as heterozygous positions and assigned the corresponding IUPAC code. To phase multiple heterozygous positions into two 'pseudohaplotypes' (i.e. alleles), we used the Bayesian methods implemented in PHASE v2.1 (Stephens *et al.*, 2001) via the program DnaSP v5 (Librado & Rozas, 2009). A flexible recombination model was considered, and the algorithm was iterated 5 times. To test the robustness of the pseudohaplotype inferences, several runs were performed for each marker with variable parameters, which all yielded the same final most probable pseudohaplotype sets (data not shown). We used these pseudohaplotypes for all further analyses of genetic diversity and population differentiation described below.

Statistical analyses

We used DnaSP v5 and MEGA v6 to determine the number of haplotypes, the number of polymorphic sites and the genetic diversity for every marker and population. Intrapopulation genetic diversity was calculated both at the haplotype and nucleotide levels. Gene or haplotype (H) diversity is the probability that two random haplotypes in a sample are different (Nei, 1987), and nucleotide diversity (π) is the average proportion of different nucleotides between pairs of sequences (Nei, 1987).

To determine the number of different genotypes in asexual populations, we estimated clonal richness using the 'poppr' package in R (Kamvar *et al.*, 2014). Clonal richness is a measure of the number of clones in a population, corrected by rarefaction to the smallest sample size (Hurlbert, 1971). Clones were defined as a unique combination of genotypes across all studied loci (COI-COII, H3, EF and all 11 microsatellite markers).

To test whether gene and nucleotide diversities differed significantly between reproductive modes, we used mixed-effects models in the 'lme4' package (Bates *et al.*, 2014) in R (R core team, 2014). Three factors were considered: *reproductive mode* (sexual or asexual, fixed effect), *marker type* (mitochondrial or nuclear, fixed effect) and *marker* (H3, EF and TH41, nested in marker type). Analysis of deviance of the model with a Wald chi-square test showed significance of the interaction between *marker type* and *reproductive mode* (see results), and thus, we split the data for nuclear and mitochondrial markers for the remaining analyses. For the mitochondrial marker (COI-COII), gene and nucleotide diversities were compared between sexual and asexual populations with a Wilcoxon rank-sum test. For nuclear markers, we used a mixed-effects model including the fixed factor *reproductive mode* and the random factor *marker*. The significance of the factor *reproductive mode* was assessed with a Wald chi-square test and confirmed using a bootstrap ANOVA with 5000 repetitions because parametric conditions for the linear model were not met (Appendix S1).

The level and significance of genetic differentiation between pairs of populations were assessed by two different methods: Cavalli-Sforza distances were calculated in Populations 1.2.30 (Langella, 1999) and ΦST distances were calculated in Arlequin 3.5 (Excoffier & Lischer, 2010) based on Kimura 2p distances. Cavalli-Sforza distances are based on the population allele frequencies, whereas ΦST takes the molecular sequence of haplotypes into account. Correlation of population differentiation with geographic distances was tested separately for sexual and asexual populations using Mantel tests implemented in R.

Demographic expansion tests

Our results revealed the presence of three different asexual *A. rufus* lineages of which one was characterized by a very broad geographic distribution (see Results). We tested for signatures of a historical demographic expansion in this lineage via three complementary approaches. In the first approach, mismatch distributions were constructed with Arlequin 3.5 for mitochondrial DNA. Mismatch distributions are the distribution of the number of nucleotide differences between all pairs of sequences. Their shape allows inferring the mechanisms that created genetic diversity in a pool of sequences. For example, if a mismatch distribution has a bell-like shape, genetic diversity was generated either by a past demographic expansion, directional selection or purifying selection. A multimodal shape represents demographic equilibrium or balancing selection. Expected distributions in a demographic expansion scenario were calculated with 1000 bootstrap replicates in Arlequin 3.5. Estimation of the age of the demographic expansion was made based on the within-lineage mean number of pairwise distances and considering a general insect molecular clock of 2.3 My^{-1}, and 3.5 My^{-1} (Papadopoulou *et al.*, 2010).

For the second approach, we applied different neutrality tests to the sequence markers with enough polymorphism (COI-COII and H3) in order to disentangle the effects of demography and selection in the mismatch distribution. Tajima's D (Tajima, 1989). Fu's Fs (Fu, 1997) and F* (Fu & Li, 1993) statistics are all used to detect departures from neutral sequence evolution, due to demographic history or other evolutionary phenomena. However, Fu (1997) suggested that Tajima's D and F* were more sensitive to detect selection processes and Fs was more sensitive to detect demographic expansion. Fs and D were calculated with Arlequin and F* in DnaSP.

Finally, two demographic expansion tests were applied on microsatellite data: the *k* intralocus variability statistic and the *g* interlocus variability statistic (Reich *et al.*, 1999) as implemented in the Microsoft Excel application 'kgtests macro' (Bilgin, 2007).

Results

DNA polymorphism

We obtained sequences for 149–178 individuals for each of the nuclear sequence markers (H3, EF) and the combined mitochondrial markers COI-COII, as well as genotypes for 84–166 individuals for each of the eleven microsatellite loci (Table 1).

For mitochondrial DNA, there were 200 polymorphic sites in 592 base pairs (bp), for a total of 57 haplotypes (Table 1). There were no individuals with more than one allele, and no stop codons were found in any of the sequences, indicating that we solely amplified mitochondrial loci as intended and not potential nuclear pseudogene copies of mitochondrial genes ('numts'). The two nuclear sequence markers featured completely different levels of polymorphism. H3 was highly polymorphic, with 57 segregating sites in 283 bp. After phasing, 98 H3 pseudohaplotypes were resolved. Consistent with sequences generated in a previous study (van der Kooi & Schwander, 2014), all *A. rufus* H3 sequences were intron free, although an intron occurs in another species of *Aptinothrips* (*Aptinothrips stylifer*) (van der Kooi & Schwander, 2014) and was also discovered in *Heliothrips haemorrhoidalis* by Nguyen *et al.* (2015). Elongation factor had only four segregating sites in 96 bp sequence, giving six pseudohaplotypes. In all the three sequence markers, the vast majority of polymorphic sites comprise synonymous changes (see Table 1). For the microsatellites, all eleven loci were polymorphic. The number of different alleles at every locus ranged from 4 to 31, with a mean of 11.36 alleles per locus (Table 1). Careful analysis of all nuclear markers confirmed the absence of polyploid individuals (there was no individual with more than two alleles at any marker) and the complete lack of gene flow/allele sharing between sexual and asexual lineages reported previously (van der Kooi & Schwander, 2014).

Table 1 Polymorphism in sequence and microsatellite markers.

Marker	Marker type	*n*	*h*	*L*	*S*	AA	Aa v.	Aa v.
COI-COII	mtDNA	172	57	597	200	197	158	39 (2)
H3	Nuclear	178	98	281	57	91	78	14 (5)
EF	Nuclear	149	6	95	4	31	30	1
TH41	msat	166	31	163–246				
TH27	msat	84	6	170–184				
TH29	msat	106	4	115–126				
TH32	msat	161	27	261–349				
TH33	msat	88	9	122–148				
TH02	msat	145	13	179–191				
TH10	msat	139	5	106–122				
TH22	msat	155	13	113–153				
TH04	msat	87	6	187–201				
TH15	msat	146	6	188–203				
TH45	msat	122	5	135–152				

n, number of individuals sequenced; *h*, number of haplotypes for sequence markers or alleles for microsatellites; *L*, sequence length (bp) for sequence markers or allelic size range for microsatellites; *S*, number of polymorphic sites; AA, total number of amino acids; Aa c, number of conserved amino acids; Aa v, number of variable amino acids; in brackets, portion of those that are singletons.

Genetic characterization of populations

We used gene (H) and nucleotide diversities to compare intrapopulation genetic variation between sexual and asexual populations. Sexual and asexual populations displayed contrasting patterns in mitochondrial and nuclear markers, with asexuals tending to be more diverse for mitochondrial DNA and sexuals for nuclear DNA (Table 2; Fig. 2). These contrasting patterns were reflected by a significant interaction effect of reproductive mode and marker type (mitochondrial vs. nuclear) on gene diversity ($X^2 = 15.9$, d.f. = 1, $P < 0.0001$; Fig. 2a) and nucleotide diversity ($X^2 = 4.5$, d.f. = 1, $P = 0.033$; Fig. 2b). Given this significant interaction, the two marker types were analysed separately.

Considering only mitochondrial DNA, asexual populations have significantly higher gene diversity than sexual populations ($W = 22$, $P = 0.042$). This difference was not due to asexual populations harbouring more segregating coding (i.e. most likely deleterious) mutations, as we found the same result using only synonymous mutations (mean asexual populations = 0.77, sexual populations = 0.22; $W = 26$, $P = 0.029$). The higher mtDNA gene diversity in asexual than sexual populations is also not solely driven by the sexual population with low sample sizes (Malaga) as the tendency is clearly maintained if this population is not included ($W = 16$, $P = 0.095$). Nucleotide diversities did not differ significantly between sexual and asexual populations independently of whether the Malaga population

Table 2 Intrapopulation genetic diversity at each marker. Gene diversity (H); nucleotide diversity at all sites (πt) and nucleotide diversity at synonymous sites. (πs). Nucleotide diversity is expressed as $\pi \times 10^3$.

	Gene diversity				Nucleotide diversity					
	mtDNA	nuclear DNA			mtDNA		nuclear DNA			
	COI-COII	H3	EF	TH41	COI-COII		H3		EF	
	H	H	H	H	πt	πs	πt	πs	πt	πs
Asexual										
Creswick	0.86 ± 0.051	0.91 ± 0.027	0 ± 0	0.61	4.9 ± 0.7	20.1	17.1 ± 3.3	56.9	0 ± 0	0
Saratoga	0.60 ± 0.113	0.55 ± 0.048	0 ± 0	0.47	57.1 ± 16.9	172.3	30 ± 2.3	113.2	0 ± 0	0
Groningen	0.94 ± 0.037	0.83 ± 0.039	0 ± 0	0.88	6.8 ± 1.1	23.7	7.6 ± 1.2	20.5	0 ± 0	0
Bex	0.89 ± 0.060	0.39 ± 0.086	0 ± 0	0.84	42.9 ± 19.4	123.7	16 ± 4.7	61.9	0 ± 0	0
La Roche	0.94 ± 0.029	0.69 ± 0.066	0 ± 0	0.84	3.8 ± 0.6	14.3	19 ± 3.2	69.4	0 ± 0	0
Agropoli asexual	0.26 ± 0.142	0.49 ± 0.112	0 ± 0	0.42	0.9 ± 0.6	3.7	7.5 ± 1.9	18.4	0 ± 0	0
Sexual										
Lagos	0.08 ± 0.070	0.98 ± 0.009	0.33 ± 0.125	0.88	0.5 ± 0.5	1.6	29.0 ± 0.9	119.5	3.4 ± 1.3	137
Tavira	0.84 ± 0.050	0.91 ± 0.033	0.57 ± 0.058	0.88	18.5 ± 6.3	61.8	30.0 ± 2.7	117.6	6.9 ± 0.9	28.2
Agropoli sexual	0 ± 0	0.92 ± 0.042	0.57 ± 0.094	0	0 ± 0	0	8.9 ± 0.7	15.8	12.0 ± 1.9	24.5
Malaga	0 ± 0	–	–	1.00	0 ± 0	0	–	–	–	–

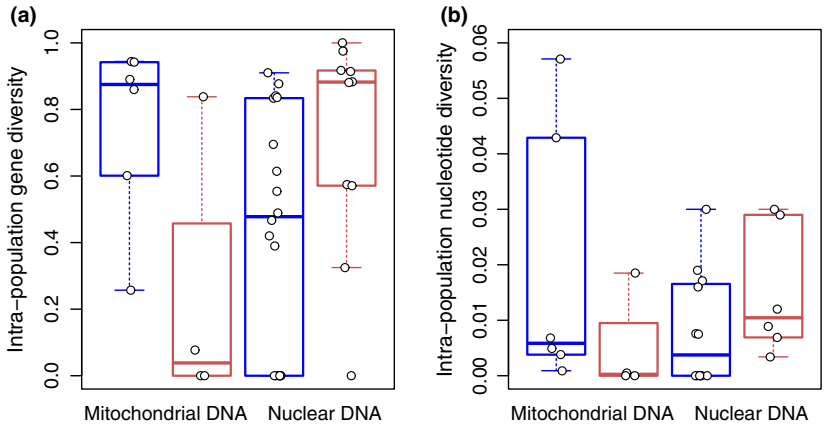

Fig. 2 Intrapopulation genetic diversity per marker type (nuclear or mitochondrial) and reproductive mode. Gene diversities (a) and nucleotide diversities (b) of sexual (red) and asexual (blue) populations are featured in split boxplots. Boxes are delimited by first and third quartiles, lines represent the medians, and whiskers denote minimum and maximum nonoutlier values (not further than 1.5 times the Interquartile range).

was included or not ($W = 20$, $P = 0.11$; without Malaga: $W = 14$, $P = 0.26$).

For nuclear DNA, sexual populations had significantly higher gene and nucleotide diversity than asexual populations (gene diversity: $X^2 = 7.44$, d.f. = 1, $P = 0.006$; nucleotide diversity: $X^2 = 4.59$, d.f. = 1, $P = 0.032$; bootstrap ANOVA $P = 0.006$ and $P = 0.032$, respectively). Observed heterozygosity was also significantly higher in sexual than asexual populations (Wilcoxon rank-sum test, $W = 0$, $P = 0.009$). Seventy to 100% sexual females in each population were heterozygous for at least one nuclear marker compared to four to 58% asexual females.

In many haplodiploid species with endosymbiont-induced parthenogenesis, the mechanism of parthenogenesis is gamete duplication, where an initially haploid egg divides once and the two products of this division then fuse to generate a diploid zygote (e.g. Stouthamer & Kazmer, 1994; Plantard *et al.*, 1998; Pannebakker *et al.*, 2004). Gamete duplication thus generates genomewide homozygosity. The finding of heterozygous asexual *A. rufus* females would therefore indicate that parthenogenesis does not occur via gamete duplication. However, because all asexual females that were heterozygous were heterozygous for the same marker (H3), we wanted to verify heterozygosity among

asexuals with the additional 10 microsatellite markers that cannot be amplified in all sexual populations. These markers confirmed heterozygosity in asexuals, as we found 19 asexual females from three different populations (and belonging to different asexual clades) with heterozygous loci. Heterozygosity occurred at the microsatellite loci TH10, TH27 and TH45.

Higher nuclear gene and nucleotide diversities in sexual than asexual populations are not surprising, given that recombination and segregation can generate many different haplotypes. Nevertheless, asexual populations still displayed considerable diversity. Indeed, clonal richness in each asexual population (based on the multilocus genotypes across all markers, including the ten microsatellite markers that do not amplify in sexuals) was extremely high. In a statistically standardized sample of 15 individuals, almost every individual is a different clone in four of the six asexual populations (clonal richness > 14), and clonal richness was 11 in a fifth population. The only asexual population with somewhat low clonal richness is Agropoli (clonal richness = 8), which coexists with a sexual population in the same location.

The high clonal diversity we found in asexual populations might stem from a mix of clones originating from a single transition to asexuality that subsequently diversified, and/or from a mix of clones from multiple transitions to asexuality. We thus assessed whether our study populations comprised individuals from different clades by constructing a mitochondrial DNA haplotype network. The network revealed that there were three asexual and two sexual clades (Fig. 3). The first of the asexual clades (asexual clade I in Fig. 3) is extremely diverse, and individuals of that clade occur in five populations on different continents. The only asexual population where we did not find individuals of clade I was Saratoga (USA). The second clade (clade II in Fig. 3) is only represented by individuals from the Saratoga (USA) population, and the last clade (clade III) comprises asexual individuals from the populations Bex (CH) and Saratoga.

Another important conclusion that can be drawn from the haplotype network is that asexuality arose at least twice independently in *A. rufus*, one transition generating asexual clades I and II and the other one generating asexual clade III.

Interestingly, the diverse asexual clade I shows a typical starlike shape (with a predominant, central haplotype and multiple rare ones differing little from the central one). Such a starlike shape is consistent with the expected pattern under a recent demographic expansion.

Demographic history of an asexual lineage

The hypothesis that asexual clade I has undergone a recent demographic expansion is supported by three lines of evidence. First, the mismatch distribution of mitochondrial DNA haplotypes displays a typical bell-like shape, which overlaps almost perfectly with the expected curve under a demographic expansion scenario (Fig. 4). There were on average 2.98 pairwise

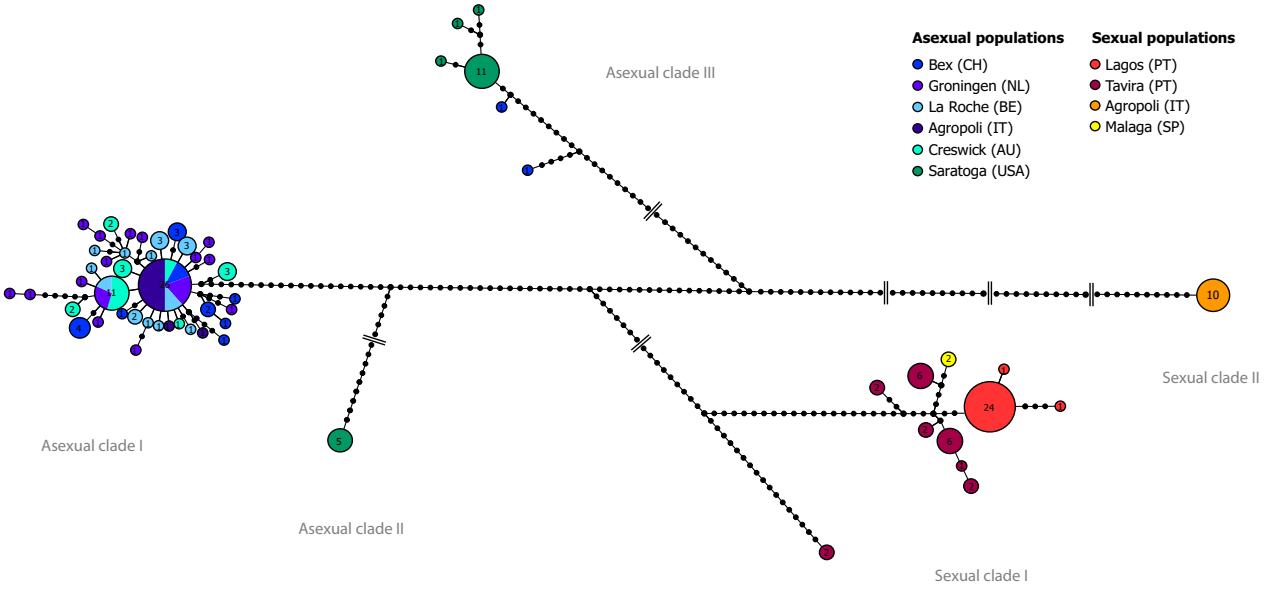

Fig. 3 Haplotype network of mitochondrial DNA. Every circle represents a different haplotype, and its size is proportional to its frequency. Numbers in circles indicate the number of individuals that present that haplotype. The length of the branches is proportional to the molecular distance among haplotypes: every point on the connection between haplotypes is a mutational step, and cuts on the branches represent 10 points. The asexual clade I presents a starlike shape.

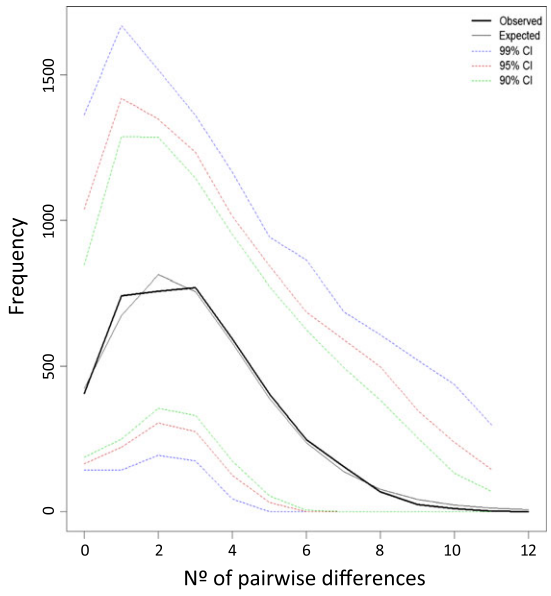

Fig. 4 Mismatch distribution for asexual clade I. Observed distribution of pairwise differences (black line) and the expected distribution under a model of sudden population expansion (dashed grey line) as calculated by Arlequin. Green, red and blue dashed lines are the 99%, 95% and 90% confidence intervals of the expected distribution.

nucleotide differences within the clade, corresponding to an expansion age of 141 800 and 210 000 years ago, assuming a molecular clock of 2.3 and 3.54 sequence divergence per million years, respectively.

Second, Fu's F, Tajima's D and Fu & Li's F* neutrality tests, with different sensitivity to demography or selection, all significantly rejected neutrality in COI-COII by an excess of rare alleles. These results therefore support a demographic expansion, but would also be consistent with directional or purifying selection in clade I. For nuclear markers, however, statistics sensitive to demography (Fs and D) rejected neutrality, while F*, sensitive to selection processes, was not significant. Consequently, these results suggest that a demographic expansion, rather than selection, caused the observed excess of rare alleles (Table 3).

Table 3 Tests for a demographic expansion of asexual clade I. Neutrality tests are ordered from left to right by high to low sensitivity to demographic expansion. Fs: Fu's Fs; Ho is Fs = 0, Fs significantly lower than 0 (α = 2%) indicates excess of rare alleles. D: Tajima's D; Ho is D = 0, D is significantly lower than 0 (α = 5%), indicating an excess of rare alleles. F*: Fu and Li's F. Ho is F* = 0, F* significantly lower than 0 (α = 2%) indicates excess of rare alleles.

Locus/ statistic	Fs	*P* Fs	D	*P* D	F*	*P* F*
COI-COII	−2.4	$P < 0.02$*	−26.5	$P < 0.05$*	−3.72	$P < 0.02$*
H3	−14.6	$P < 0.02$*	−0.8	0.19	0.25	$P > 0.10$

Finally, a demographic expansion in asexual clade I was also detected by the intralocus *k*-test on the eleven microsatellite loci, with 10 loci associated with negative k values ($P = 0.004$). By contrast, the interlocus *g*-test was not significant, as the observed g statistic was higher than the five-percentile cut-off ($g = 3.71$; g 5% = 0.18). However, g-tests are highly sensitive to variation in mutations rates among loci (King *et al.*, 2000). In our study, this could explain the nonsignificance of the *g*-test, given the extensive variability in allele size ranges and number of alleles among different microsatellites (Table 1).

Genetic differentiation and geography

The mitochondrial haplotype network also revealed that different asexual populations shared many haplotypes whereas not a single haplotype was shared among sexual populations. Population differentiation analyses confirmed these strikingly different patterns in sexual and asexual populations. Asexual populations are similar genetically considering that our sampling includes populations from different continents and with extensive geographic separation among them (mean geographic distance = 8007.9 km; mean Cavalli-Sforza distance = 0.63 ± 95% CI [0.57, 0.69]; Fig. 5a). By contrast, sexual populations are genetically more differentiated than asexual ones, even though sexual populations are on average almost eight times closer geographically (mean geographic distance = 1089.6 km; mean Cavalli-Sforza distance = 0.82 ± 95% CI [0.72,0.92]; Fig. 5a). However, isolation by distance among sexual populations was not statistically significant, most likely due to the small sample size of sexual populations ($n = 4$; $r = 0.59$, $P = 0.083$). Population differentiation among sexual populations appears to be less strong in nuclear DNA than in mitochondrial DNA, although there is no significant difference (mean Φst nuclear: 0.47; mitochondrial: 0.74, $t = 1.08$, d.f. = 4.3, $P = 0.34$; Fig. 5b,c).

Discussion

We found that in the thrips *Aptinothrips rufus,* sexual populations have higher genetic diversity in nuclear DNA than asexual populations. This is expected given that recombination, segregation and genetic exchange between individuals continuously generate new haplotypes. The pattern in mitochondrial DNA is the opposite: asexual populations are more diverse than sexual populations. *A priori*, asexual populations might be expected to harbour less mitochondrial diversity than their sexual counterparts. Because asexuals derive from sexual ancestors, an incipient asexual lineage represents

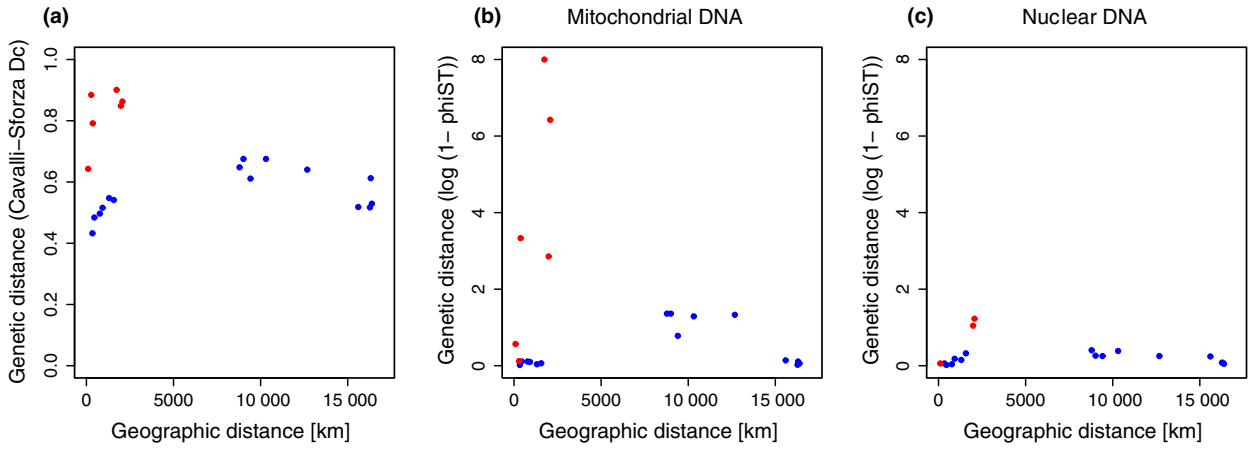

Fig. 5 Isolation-by-distance plots among sexual and asexual populations. Points represent genetic distance between pairs of sexual (red) and asexual (blue) populations. Cavalli-Sforza distances considering mitochondrial and nuclear markers for both sexual and asexual populations (H3, EF, TH41 and COI-COII) (a). Reynold's linearized Φst distances (log(1−Φst,)), based on kimura 2p molecular distance. In mitochondrial DNA (b), distance is calculated on the concatenated COI−COII markers. In nuclear DNA (c), the average distance of H3 and EF markers is presented.

a genetic bottleneck, capturing a small fraction of the standing genetic diversity present in the sexual ancestor. Nevertheless, mitochondrial diversities can increase following the initial bottleneck caused by the transition to asexuality, and such increase can be rapid owing to fast population growth rates and large population sizes known in many asexual species (Ross *et al.*, 2013). Furthermore, genetic diversities present at the species level may be distributed among populations is various ways, with specific demographic scenarios or migration patterns determining the local diversity found in sexual or asexual populations.

Asexual populations of *A. rufus*, with average mitochondrial gene diversities (H) of 0.75, are among the most diverse asexual populations described thus far. A detailed literature review allowed us to obtain mitochondrial genetic diversities for 25 different asexual species from 19 studies (Table S4; Table 4). Twenty of these 25 asexual species have lower diversity than *A. rufus* (H = 0.06–0.69), whereas only five species have equal or higher diversity than *A. rufus*: the weevil *Naupactus cervinus* (H = 0.84; Rodriguero *et al.*, 2010), the aphid *Aphis craccivora* (H = 0.79; Komazaki *et al.*, 2010), the two barklice *Liposcelis bostrychophila* (H = 0.88; Wei *et al.*, 2012) and *Echmepteryx hageni* (H = 0.98; Shreve *et al.*, 2011), and the tardigrade *Echiniscus testudo* (H = 0.86; Jørgensen *et al.*, 2007). Similar to asexual *A. rufus*, all five species have large distribution ranges and the first three are known for being pests for agriculture- or human-related wares. However, the genetic diversity is reported at the population level in only one of these five species (*L. bostrychophila;* Wei *et al.*, 2012). For the four remaining species, studies report diversity at the lineage or species level, with asexual individuals collected from distant locations, sometimes from different continents. Species-level diversities overestimate diversities present at local population scales, yet these four species-level diversities are almost matched by population-level diversities in asexual *A. rufus*.

Similar to mitochondrial diversities, clonal richness is also extremely high in five of the six asexual *A. rufus* populations studied, with almost every individual corresponding to a different clone. The sixth asexual population, Agropoli (Italy), only featured ~50% of this clonal diversity. Interestingly, this population is the only one that co-occurs sympatrically with a sexual population. The relatively low genetic diversity in Agropoli could indicate that asexuals have only recently colonized this location. If this is indeed the case and the colonization of Agropoli by asexuals is an ongoing process, the future of the sexual population is intriguing. Will the asexuals outcompete the sexuals? Monitoring of this location is a challenge for future studies as it could bring valuable insights into the relative advantage of sexual and asexual relatives in competition.

In addition to featuring high genetic diversity *per se*, asexual *A. rufus* populations are diverse compared with their sexual relatives and are even characterized by significantly higher genetic diversity at mitochondrial markers. How widespread this pattern is among other species is unknown, as most of the studies that characterize mitochondrial diversity in asexual populations do not provide a direct comparison with sexual populations. Among the studies we reviewed on 25 asexual species, only nine explicitly compare mitochondrial diversities in populations of asexuals and their sexual relatives (Table 4). Seven of those found higher mitochondrial diversity in sexual than in asexual populations. In the barklouse *L. bostrychophila* (Wei *et al.*, 2012), asexual and sexual populations feature similar

Table 4 Mitochondrial genetic diversity in asexual and sexual relatives of different species. Reported diversity corresponds to the mean across populations (when population diversity was available) or otherwise diversity measured at the species level (marked with stars). Genetic diversity is expressed as gene diversity (H), nucleotide diversity (π) or number of haplotypes per site (h/site). Comparisons refer to sexual and asexual populations within a species or to related sexual (s) and asexual (a) species.

Taxa	Species	Marker	Diversity measure	Diversity in asexuals	Diversity in sexuals	Asexual vs. sexual	Reference
Thysanoptera	*Aptinothrips rufus*	COI-COII	H	0.75	0.23	>	Present study
Psocoptera	*Echmepteryx hageni*	12S and ND5	H*	0.98	0.25	>	Shreve *et al.* (2011)
Psocoptera	*Liposcelis bostrychophila* (a), *L. entomophila* (s)	Cyt b	H	0.88	0.85	=	Wei *et al.* (2012)
Odonata	*Ischnura hastata*	COI	H	0.06	0.51	<	Lorenzo-Carballa *et al.* (2012)
Coleoptera	*Geodercodes latipennis*	COI	H	0.69	0.82	<	Polihronakis *et al.* (2010)
Coleoptera	*Polydrusus inustus* (a), *Centricnemus leucogrammus* (s)	COII, CytB, ND1, tRNA	H	0.24	0.75	<	Kajtoch *et al.* (2009)
Orthoptera	*Warramaba virgo* (a), *W. picta* (s), *P125* (s), *P169* (s), *P196* (s)	COI	π*	0.23	0.005; 0.042; 0.042; 0.22	<	Kearney & Blacket (2008)
Cladocera	*Daphnia pulex*†	mtDna RAPD	h/site*	4.0 ± 0.73	6.23 ± 1.17	<	Weider & Hobæk (1997)
Squamata	*Menetia greyi*i	COI	π*	0.002	0.015	<	Adams *et al.* (2003)
Nematoda	*Caenorhabditis elegans* (a), *C. briggsae* (a), *C. remanei* (s)	COII	π*	0.010; 0.009	0.023	<	Graustein *et al.* (2002)

*Diversity among individuals from different locations at regional or continental scale.
†Comparison between regions where all populations are asexual (glaciated areas) and region where some populations are sexual (unglaciated areas).

diversities. Only in the barklouse *E. hageni* (Shreve *et al.*, 2011), asexuals have higher mitochondrial diversity than sexuals, similar to our findings in *A. rufus*. However, diversity in asexual *E. hageni* was estimated among individuals from several locations from a large geographic area, with only few individuals per location, while all sexual individuals were sampled from a geographically restricted area. Thus, the only previous study that reported higher mitochondrial diversity in asexuals than in sexual relatives is not informative on the diversity at the intrapopulation level. Our study therefore represents an unprecedented case of small-scale diversity in asexuals, with asexual *A. rufus* populations featuring more than two times the amount of mitochondrial gene diversity in sexual populations.

An important factor contributing to the different levels of mitochondrial diversity in asexual and sexual populations is migration. We found that asexual populations are genetically diverse, but very similar among them, with the same set of clones found in geographically distant regions (Fig. 5). This pattern indicates that there is extensive migration across vast geographic ranges. By contrast, sexual populations are strongly differentiated even over short geographic distances (Fig. 5), which indicate that there is little or no current gene flow between very close sexual populations.

The factors that could generate these migration patterns remain elusive, as little is known about the dispersal mode of *Aptinothrips*. Given they are wingless and very small (~1 mm), they are most likely passively transported, for example by wind. Human-related transport of grains and pasture grasses might also have contributed to the spread of *A. rufus* across different continents (Mound, 1970; Palmer, 1975). As sexual and asexual *A. rufus* females are morphologically identical, it is unlikely that they have different dispersal modes. Nevertheless, it remains possible that certain differences in life-history traits between sexual and asexual females (for example lifespan or fecundity) or higher population densities in asexuals could result in asexuals being transported more often.

Alternatively, if sexual and asexual females would be dispersing at equal rates, there are at least two possible explanations for the unsuccessful establishment of sexual females in a new host population: strong local adaptation of cytoplasms in sexual (but not in asexual) populations, and limited reproduction between sexual females and males from different locations, either due to assortative mating or to some kind of cytonuclear incompatibility. Future studies are needed to determine what causes the strong differences in migration patterns between sexual and asexual *A. rufus*.

Independently of the mechanisms that generate the different population differentiation patterns for sexual and asexual lineages, the most widely distributed asexual *A. rufus* lineage (asexual clade I in Fig. 3) likely underwent a demographic expansion accompanying its worldwide spread. This demographic expansion strongly contributed to the current mitochondrial diversity in the clade I asexual *A. rufus* and thus partially accounts for the higher mitochondrial diversity in asexual than sexual *A. rufus*. We dated this expansion to between

142.000 and 210.000 years ago, corresponding to approximately one million *Aptinothrips* generations. This suggests that the colonization of Europe by asexuals occurred during the Pleistocene period (1.8 million to 14 000 y.a.), most likely during the interglacial period between the last and the previous Pleistocene glaciations, when no ice barrier was left between the Iberian peninsula and Central Europe (Horne & Martens, 1998). Range expansion during interglacial periods has occurred in several other asexual species (reviewed in Hörandl, 2009), eventually generating the characteristically distinct distributions of sexual and asexual relatives referred to as geographic parthenogenesis (Vandel, 1928).

Geographic parthenogenesis can, however, also be generated by mechanisms unrelated to post-glacial expansions. For example, asexual lineages could be characterized by a broader ecological niche than their sexual relatives, perhaps because of selection for 'general purpose genotypes' (Lynch, 1984). In the case of *Aptinothrips rufus*, niche breath might relate to the number of grass species that are suitable as host plants for sexual and asexual lineages, but there are currently no data available that would allow testing whether asexual *A. rufus* have a broader niche than sexual *A. rufus*.

Independently of the factors that generated geographic parthenogenesis in *A. rufus*, the extensive migration and potential for demographic expansion of asexual lineages have certainly facilitated the colonization of different continents. The *Aptinothrips* genus is native to Central Europe (Palmer, 1975) and our results suggest that two waves of secondary colonization occurred in North America: the first one corresponding to asexual clade III and the second one to asexual clade II (Fig. 3). These two asexual clades arose as independent transitions to asexuality from sexual ancestors (see also van der Kooi & Schwander, 2014). In Australia, the amount of identical haplotypes shared with several European populations suggests that diversity was generated by multiple, recent colonization events.

In summary, the higher mitochondrial DNA diversities in asexual than sexual *A. rufus* populations are caused by the combined effects of multiple transitions to asexuality, large asexual populations that underwent demographic expansions, and extensive exchange of individuals among asexual populations. An important remaining question is whether the genetic diversity in asexual populations includes adaptive variation. Importantly, adaptive variation among *A. rufus* clones could have contributed to the extreme success of this asexual species. For example, if different clones would use different ecological niches, high genetic variation among asexual clones may mimic certain types of benefits usually attributed to sexual reproduction (e.g. Lively, 2010; Neiman & Schwander, 2011).

Finally, population genetic patterns observed in the studied populations have to be considered in the light of the cytological mechanism of parthenogenesis present in asexual lineages. It is currently unknown what mechanism operates in *A. rufus.* Sixty-seven per cent of asexual *A. rufus* females are infected by *Wolbachia* (van der Kooi & Schwander, 2014) and the cytological mechanism in the majority of cases of *Wolbachia*-induced parthenogenesis is gamete duplication (e.g. Stouthamer & Kazmer, 1994; Plantard *et al.*, 1998; Pannebakker *et al.*, 2004). Under gamete duplication, a diploid zygote is produced by the duplication of a meiotic product, generating zygotes with two identical copies of the genome (i.e. genomewide homozygosity). However, we found that 17% of asexual females were heterozygous at least at one microsatellite marker, which excludes gamete duplication as the mechanism of parthenogenesis.

In conclusion, asexual populations of *Aptinothrips rufus* harbour an exceptionally high level of genetic diversity compared with their sexual relatives, but also in comparison with diversities reported in other asexual species. In some asexual populations, different clones belong to one diversified lineage, whereas in others, clones belong to two independently arisen asexual lineages. Little differentiation among very distant asexual populations suggests migration of asexuals over large geographic distances as a mechanism generating high local clone diversities. By contrast, geographically close sexual populations are strongly differentiated. We suggest that high migration ability of *A. rufus* asexuals has allowed for a demographic expansion during an interglacial period of the late Pleistocene, and to colonize localities across the world. *Aptinothrips rufus* is a great example of an extremely successful asexual lineage, which questions the widely held view that asexual metazoans are on an 'evolutionary dead-end' road to extinction.

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Supporting information

Additional Supporting Information may be found in the online version of this article:
Table S1 Thrips populations and sample sizes.
Table S2 Primer pairs and PCR conditions for amplification of microsatellites.
Table S3 Primer pairs and PCR cycling conditions for amplification of sequence markers.
Table S4 Asexual species and references used for comparison of genetic diversity with *A. rufus*.
Appendix S1 R script for Bootstrap ANOVA statistical testing.

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