


# Genetic and phenotypic responses to habitat fragmentation in a European harvester ant

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## Abstract

1. Habitat fragmentation threatens wildlife populations and may cause reduced genetic variation, fitness, and even local extinction, but effects differ across species.
2. Here, we addressed effects of habitat fragmentation using the harvester ant *Messor structor*, a habitat specialist in natural xerothermic grassland, in 54 habitat islands of a fragmented 200 km<sup>2</sup> area in Austria analysing microsatellites, mitochondrial DNA, infection with the endocellular bacterium *Wolbachia*, and morphology.
3. We found (a) pronounced genetic diversity and two mitochondrial lineages that we infer to originate from separate glacial refugia. (b) *Wolbachia* infection rates were high, but the two strains detected, correlating with the mitochondrial lineages, do not seem to have induced a reproductive barrier. (c) Habitat islands with fewer ant nests had less allelic richness, and the ones with less allelic richness had reduced heterozygosity, as measured by mean locus heterozygosity; this indicated inbreeding. (d) Fluctuating asymmetry of workers, known to be low in fit individuals, positively correlated with heterozygosity, as measured by the interallelic distance of heterozygous loci; high levels of this distance are probably due to the mixing of lineages, and facing such a potential outbreeding depression, vulnerability for inbreeding may increase. (e) Gyne mesosoma size in less connected habitat was smaller than that of gynes in better connected habitat, indicating reduced flight ability.
4. Our work highlights the use of multiple approaches to evaluate species responses to habitat fragmentation, showcasing the importance of historical colonisation and current habitat connectivity to the maintenance of genetic and phenotypic diversity.

## KEYWORDS

conservation genetics, fluctuating asymmetry, gyne size, habitat island, inbreeding, outbreeding

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## INTRODUCTION

Habitat area and isolation are important to the population size of natural populations (Fahrig, 2003; see Valente et al., 2023 for a summary of the ongoing debate on this). Negative effects of habitat fragmentation on wildlife include reduced genetic variation, reduced fitness and local extinction (e.g., Haddad et al., 2015; Keller & Largiadèr, 2003; Mäki-Petäys & Breen, 2007). These effects, however, cannot be generalised (butterfly: Ugelvig et al., 2011; ant: Soare et al., 2014), because the threshold at which habitat fragmentation leads to reduced gene flow and loss of genetic diversity varies across species (Ortego et al., 2015). A series of traits – from area requirements to dispersal ability – define a species' sensitivity to habitat fragmentation. Moreover, the more specialised a species is to a certain habitat, the more likely it is to suffer from habitat fragmentation (as reviewed by Henle et al., 2004). However, for generalisation, further case studies are needed from additional taxa and ecosystems.

A good specialised biological system for such case studies can be found among harvester ants. These ants are ecosystem engineers (Bulot et al., 2016; Del Toro et al., 2012; Elmes, 1991) as they influence soil properties (Wagner et al., 2004) and plant communities (Azcárate et al., 2002; Azcárate & Peco, 2006; Wolff & Debussche, 1999). For example, harvester ants not only collect but also distribute plant seeds around their nest sites, increasing vegetation density (Bulot et al., 2016). In Central Europe, the recently red-described (Steiner et al., 2018) harvester ant *Messor structor* (Latreille, 1798) (Figure 1a,b) is a habitat specialist. This species is constrained to natural xerothermic grassy habitats (Schlick-Steiner et al., 2003) and does not occur in anthropogenic habitats, such as ornamental green spaces in urban areas. Thus, evaluating *M. structor*'s responses to habitat fragmentation can increase our understanding of how human changes can affect specialised species.

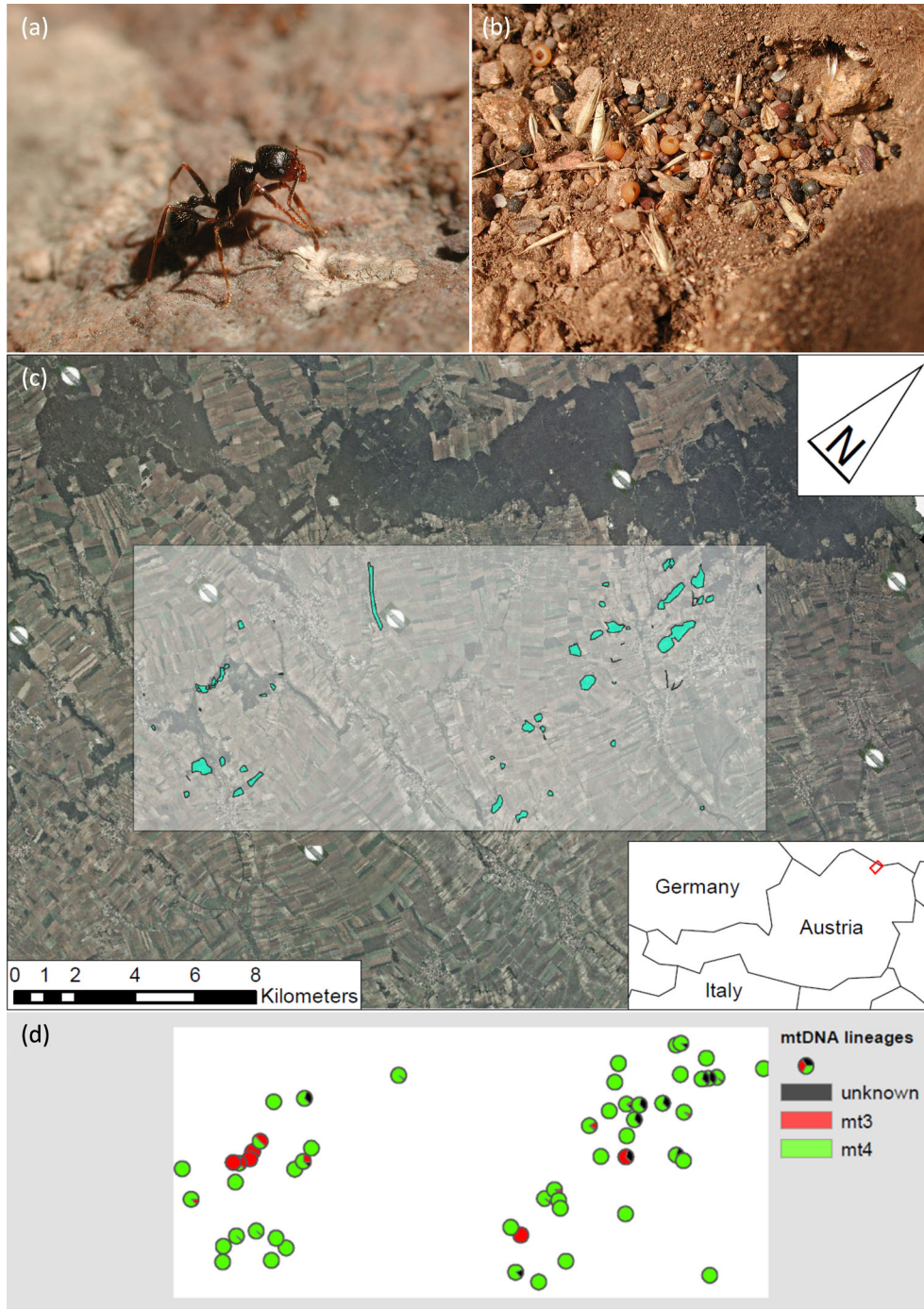
Here, we studied *Messor structor* in the face of habitat fragmentation. We sampled a 200 km<sup>2</sup> area near Retz, Austria, at the northern distribution boundary (Steiner et al., 2018) of this species in Central Europe. During the last glacial maximum (LGM, ca. 20,000 years ago, Clark et al., 2009), ice and permafrost in the region (Lindgren et al., 2016) made it hostile for xerothermophilous organisms. With rising temperatures, large forests increasingly covered the area (Davis et al., 2015), but hilltops with bare rock remained free of trees (Resch, 1936), representing natural habitat islands for *M. structor*. With Retz documented as a permanent settlement since 1180 (Resch, 1936), transformation of forest and scrubland into low-intensity pasture resulted in large habitats suitable for grassland specialists in the last millennium (Resch, 1936). However, during the 20<sup>th</sup> century, xerothermic grassland in the region was increasingly transformed into arable land and vineyards. To increase both mechanisation and economic yield of agricultural areas, consolidation of arable land took place since circa 1890; the process was intensified after World War II and even more so in the 1950s (Liebel et al., 1986; Wirtschaftsforschung Ölf, 1955). This consolidation included the merging of agricultural parcels, annihilating any area and hilltop among former parcel borders. All these actions resulted in strong

fragmentation of the grassland and thus a suitable habitat for *Messor structor*. The number and size of grassland habitat islands were reduced, and the distance among them increased.

Previous work on the variation of *M. structor* across its entire distribution range (Austria, Bulgaria, Czech Republic, France, Hungary, Romania, Slovenia), used here as background for the interpretation of the new findings, found several mitochondrial lineages within this species (Schlick-Steiner et al., 2006; Steiner et al., 2018). Two of these lineages, corresponding to Lineages 3 and 4 in Steiner et al. (2018), occur in the 200-km<sup>2</sup> study area. Supercolonial population structure with lack of aggression and possible exchange of workers among nests has been observed in the area, as has been a “lack of nuptial flight” (Schlick-Steiner et al., 2005), possibly indicating mating inside nests.

By combining multiple approaches, we studied the impact of habitat fragmentation on the genetic and phenotypic diversity of the habitat specialist *M. structor*. We specifically addressed the following questions:

- i. *From how many refugia did the ants originate that colonised the study area after the LGM?* To reconstruct postglacial recolonisation of the Retz population, we combined analyses of the maternally inherited mitochondrial DNA (mtDNA) and microsatellites as nuclear, codominant markers. Both markers can reflect the number of refugia from which the study area was colonised after the LGM. In mtDNA, the number of lineages can be used to infer the number of refugia (ant examples: Goropashnaya et al., 2007; Schlick-Steiner et al., 2007). Thus, from the mtDNA perspective, we hypothesise at least two refugia based on the previously recorded two lineages (Steiner et al., 2018), but the extensive sampling here could bring to light additional lineages. Following the logics of Chavez-Galarza et al. (2015), we expect microsatellite allele-size distribution to reflect the number of refugia, with one more-or-less continuous size range resulting from a single refugium and gap(s) between size ranges resulting from two or more refugia.
- ii. *Do reproductive barriers separate populations in the study area?* This inference should be feasible by evaluating whether congruence or incongruence arises at the level of individuals when mitochondrial and nuclear DNA data are combined (cf. Schlick-Steiner et al., 2010). Thus, given the maternal inheritance of and lack of recombination in mtDNA and our knowledge of at least two mtDNA lineages in the area (Steiner et al., 2018), we expect individuals to cluster in their microsatellite data congruently with their mtDNA data under reproductive barriers between the two lineages but incongruently under a lack of such barriers.
- iii. *Has Wolbachia influenced genetic diversity?* We here include *Wolbachia* infection for a better understanding of potential nuclear and mitochondrial structuring across our sample and thus of population history. The alphaproteobacterium *Wolbachia pipientis* Hertig, 1936 is found in many arthropod species (Werren et al., 2008) and is mainly inherited from females to their offspring. Ants are common hosts of *Wolbachia* (Russell et al., 2017), and recently, these endosymbionts have been used in species



**FIGURE 1** (a) Worker of *Messor structor* (© B.C. Schlick-Steiner & F.M. Steiner); (b) opened nest chamber of *M. structor* with stored seeds (© B.C. Schlick-Steiner & F.M. Steiner); (c) aerial photo of the study area near Retz, Austria, with surrounding land (orthophotograph from [www.geoimage.at](http://www.geoimage.at)) (©), cyan patches are habitat islands sampled positive for *M. structor*; (d) proportion of mitochondrial DNA lineages of all nests found per habitat island; unknown, mtDNA lineage unknown due to sequencing failure; mt3, mtDNA Lineage 3 in Steiner et al. (2018); mt4, mtDNA Lineage 4.

delimitation in *Messor* ants (Steiner et al., 2018). In various population-genetic studies of arthropods, *Wolbachia* data were needed for correctly interpreting nuclear and mitochondrial data (e.g., Jäckel et al., 2013). *Wolbachia* infection can, for instance, maintain mtDNA diversity despite nuclear gene flow between populations infected with different, incompatible strains but can

also homogenise mtDNA among different populations that are affected by the same endosymbiont sweep (Hurst & Jiggins, 2005).

iv. *Did habitat fragmentation change genetic diversity?* Reduced gene flow across habitat fragments can lead to inbreeding, and genetic drift can lead to lower genetic diversity in the habitat fragments

(Mäki-Petäys & Breen, 2007; Vitikainen et al., 2011; Vitikainen et al., 2015). For the grassland fragments (habitat islands) analysed here, which differ in size and connectivity, we expect reduced microsatellite allele numbers and reduced heterozygosity with increasing habitat fragmentation.

- v. *Does fluctuating asymmetry in workers correlate with genetic architecture?* Inbreeding can reduce fitness, but documenting inbreeding depression is challenging (e.g., Gooley et al., 2017). In Hymenoptera, inbreeding depression may be rare, as haplodiploidy may help to purge deleterious mutations as suggested by theory (Werren, 1993) and empirical studies (e.g., Kureck et al., 2012). Some ant studies, however, did find inbreeding depression (Haag-Liautard et al., 2009; Vitikainen et al., 2011; Vitikainen et al., 2015). Possibly less considered, also interbreeding among different lineages can result in depression, termed outbreeding depression (ant example: Schrempf et al., 2015). Fluctuating asymmetry, that is, random deviation from symmetry of paired morphological traits, can indicate developmental instability (Lens et al., 2002). Often used as a fitness proxy (Lens et al., 2002), this phenomenon has been found to reflect negative fitness effects such as from either inbreeding or outbreeding depression (Demontis et al., 2010; Dongen, 2006; Lacy & Alaks, 2013). In our study, possible negative correlations – expected under inbreeding depression – or positive correlations – expected under outbreeding depression – between fluctuating asymmetry and variation in microsatellite loci may uncover any such effect.
- vi. *Does habitat fragmentation influence gyne size?* Gyne size polymorphism is known from various ant species (Buschinger & Heinze, 1992; Seifert, 2018: 22 of 178 Central European ant species). It even relates to alternative reproductive tactics with smaller gynes founding colonies by help of others, such as by readoption in the maternal colony or another colony close by and larger gynes performing long-distance dispersal flights before founding alone (Rüppell & Heinze, 1999). Gyne body size has been suggested to negatively correlate with habitat patchiness and dispersal risk (Schlick-Steiner et al., 2007 and references therein); alternatively, a positive correlation could result from larger gynes better dispersing to more distant habitat islands (cf. Merckx et al., 2018). We here test habitat fragmentation for correlation with gyne body size.

## MATERIALS AND METHODS

### Study area, sampling and DNA extraction

Worker ants and alate reproductives were collected near Retz between April and October 2004. In the approximately 200 km<sup>2</sup> study area (Figure 1c, Figure S1, Supporting Information), all islands of potentially suitable habitat, that is, continuous natural xerothermic grassland not partitioned by structures uninhabitable by *Messor structor* (e.g., paved roads, buildings, farmland, dense scrubland, forests),

were searched for nests of the ant. The search aimed at an exhaustive screening of each habitat island, facilitated by the very easy detectability of these ants' activity above soil surface under favourable weather conditions, resulting in the finding of 54 inhabited islands in total. Habitat islands were GPS mapped (Crux II, EMTAC Technology Corp., Taiwan) and digitised; some islands were digitally reshaped by analysing orthophotos of 2011 (source: [www.geoimage.at](http://www.geoimage.at), retrieved 20 February 2015). For each island, surface area, centroid coordinates, and distance to the closest habitat island were calculated in ArcGIS v10.2 (Esri, USA). For all *M. structor* nests found, GPS coordinates were recorded, and from inside all nests, ants were sampled. In more detail, ca. 50 randomly selected workers were sampled as were all alate gynes encountered while sampling these workers, without focused searching for gynes. To avoid multiple sampling of nests, a minimum distance between nest entrances of five metres was maintained; in most cases, the distance was more than 10 metres. DNA was extracted from degasted workers using the GenElute Mammalian DNA Extraction Kit (Sigma, USA) following the manufacturer's instructions and eluted in 50 µL elution solution.

### Mitochondrial DNA

A part of the mitochondrial cytochrome oxidase subunit I gene (COI) was amplified as described by Schlick-Steiner et al. (2006) and Sanger-sequenced by a commercial provider using the amplification primers. All sequences were checked for signatures of nuclear-mitochondrial pseudogenes (Song et al., 2008), edited manually, and aligned with Clustal X v2.0 (Larkin et al., 2007). Comparison with haplotypes published by Schlick-Steiner et al. (2006) and Steiner et al. (2018) allowed linking results to previous work in a Europe-wide context. Tamura-Nei distances between and within lineages were calculated with Mega 7.0 (Kumar et al., 2016).

### Microsatellites

Eight microsatellite loci were used, namely Ms1A, Ms1B, Ms1E, Ms2A, Ms2C, Ms2D (Arthofer et al., 2005), MsMic13j and MsMic24b (Steiner et al., 2011). PCR conditions and fragment analysis were as described in Arthofer et al. (2005) and Steiner et al. (2011). Allele scoring was done manually in PeakScanner v1.0 (Applied Biosystems, USA). Data were checked for scoring errors in MicroChecker v2.2.3 (van Oosterhout et al., 2004). Null allele estimation was calculated with FreeNA (Chapuis & Estoup, 2007) in two ways, that is, using the overall dataset as a single population as well as using each habitat island as a separate population. Allele number, frequency and observed heterozygosity over all loci were calculated in GenAlEx v6.501 (Peakall & Smouse, 2012).

For assessing the number of refugia, the microsatellite allele-size distributions were analysed. The distribution of allele frequencies of neutral loci at mutation-drift equilibrium depends on the model of evolution. The allele size of microsatellites likely changes with the

gain or loss of single repeat units (Slatkin, 1995; ant example: Schlick-Steiner et al., 2015), and frequencies would thus, in an idealised population, be expected to follow a normal or Poisson distribution (Kimura & Ohta, 1978). Independent of the applicable model, low frequencies are expected to dominate (Luikart et al., 1998; Nei et al., 1976). Because some microsatellite loci in our dataset had multiple alleles at high frequencies, deviations from normal distribution were tested for at  $p = 0.05$  based on the mean and standard deviation of allele frequencies of each locus. Alleles with significantly higher frequencies were marked and bp lengths of gaps between them calculated.

Because individuals within an ant nest are likely to be related to each other and relatedness influences tests for (a) departures from Hardy Weinberg Equilibrium (HWE) and (b) Linkage Disequilibrium (LD), a single worker per nest was used in testing each locus for (a) and (b). In more detail, two approaches were applied in testing for (a) and (b), that is, using all habitat islands together as a single population and using only habitat islands harbouring at least five nests and treating these islands as separate populations; the number of five nests represented a compromise between including just habitat islands with higher numbers of nests and the number of habitat islands meeting the criterion. The Markov chain method implemented in Genepop on the web v4.2 (Rousset, 2008) was applied with 1000 dememorizations, 1000 batches and 1000 iterations per batch. Bonferroni-Holm correction (Rice, 1989) for multiple comparisons was applied to (a) and (b). Allelic richness (AR) was calculated using rarefaction to account for different nest numbers across habitat islands, implemented in HP-Rare v1.0 (Kalinowski, 2005) by averaging across all loci and assuming two genes (based on a minimum of two individuals per nest).

Nest-level mean locus heterozygosity (MLH) and the nest-level squared distance between two alleles at a locus as mean over all loci ( $d^2$ , Coulson et al., 1999) were calculated by averaging across the individual workers of each nest and used as proxies of genetic variation in the asymmetry analyses (see *Materials and methods, Morphometrics*). The coefficient  $F_{IS}$  (Wright, 1949) was not calculated because of low numbers of nests per island (see *Results*). Habitat-island-mean AR, MLH and  $d^2$  (as well as the morphometric index MW/CW, for details, see *Materials and methods, Morphometrics*) were calculated and used as dependent variables in linear regression analyses (type III sum of squares) performed with the natural logarithms of the habitat-island parameters surface area, perimeter, distance to the closest habitat island, as well as with the natural logarithm of the number of nests found per habitat island (which likely is influenced by habitat-island size and quality) as independent variables using SAS 9.4 (SAS Institute, USA). For significant results of the regressions with habitat-island-mean AR, MLH and  $d^2$  as dependent variables, to test for a potential influence of null alleles (estimated for each habitat island), the frequency of these alleles was used as an additional covariable. Logarithmic transformation was used to reduce skewness of data within variables and to linearize the relationship among variables; throughout, outliers were identified and excluded via the nonparametric method of Tukey's fences (targeting values outside the 1.5-fold

interquartile range), and before regression analyses, collinearity of independent variables was validated via the condition index, interpreting index values of  $<30$  as non-problematic. Additionally, habitat-island-mean AR, MLH and  $d^2$  (as well as MW/CW) were used as dependent variables in simple linear regression analyses (type III sum of squares) using the natural logarithm of Hanski's connectivity index  $i_4$  as an independent variable. The index  $i_4$  was calculated using the formula in Hanski et al. (1994); given the absence of knowledge of migration distances in these ants, the constant that can be used in the exponent to give more or less weight to long distances was set to 1.  $i_4$  is a measure for connectivity of individual habitat islands but is calculated based on the distances among and the surface areas of all islands considered. Thus, in terms of fragmentation,  $i_4$  decreases for a habitat island with its increasing distance from all other habitat islands and/or with decreasing surface area of all other islands (but not of itself). Habitat-mean MLH and  $d^2$  were also used as dependent variables in simple linear regression analyses with AR as independent variable. The significance level  $\alpha$  was set to 0.05 throughout.

Analysis of molecular variance (AMOVA) was done to analyse variation in the nuclear data according to habitat islands and mtDNA lineages using GenAEx. Isolation by distance (IBD) was tested assessing the correlation between pairwise relatedness (calculated following Lynch and Ritland (1999) and converted into a distance matrix following Legendre and Legendre (1998)) and log-transformed geographic distance in a paired Mantel test with 999 permutations in GenAEx. Only one, randomly selected worker per nest was included to avoid inflated correlation due to high relatedness of nestmates resulting from one or a few sexuals producing the entire worker population in a colony.

Population structure was estimated using FLOCK v3.1 (Duchesne & Turgeon, 2009; Duchesne & Turgeon, 2012) and STRUCTURE v2.3.4 (Pritchard et al., 2000). FLOCK, employing a non-Bayesian algorithm, is considered resistant to deviations from HWE and LD (Duchesne & Turgeon, 2012); settings were  $K = 2$  to  $K = 25$  with 50 runs per  $K$ . The Bayesian method implemented in STRUCTURE was used to test for  $K = 1$  to  $K = 25$  with 10 replicates for each  $K$ , a burn-in of 50,000 iterations, 250,000 iterations for data generation, and habitat islands as locprior information (Hubisz et al., 2009). STRUCTURE HARVESTER v0.6.94 (Earl & Vonholdt, 2012) was used to identify the optimal  $K$  (Evanno et al., 2005); the results were visualised in DISTRUCT v1.1 (Rosenberg, 2004). Additionally, population structure was visualised using discriminant analysis of principal components via the R package ADEGENET, which does not make assumptions regarding HWE and LD (Jombart, 2008).

## Wolbachia

The primers *wsp81F* and *wsp691R* (Braig et al., 1998), frequently used for *Wolbachia* diagnostics, resulted in unsatisfactory amplification success. For *wsp691R*, this was due to two mutations in the primer-binding site; sequencing of the binding site of *wsp81F* failed (see

Appendix S1). Instead, the primers *wsp151F* and *wsp599R* (Ruangareerate & Kittayapong, 2006), targeting the *Wolbachia* surface protein (*wsp*) gene, were used. PCR reactions contained 1 × MyTaq buffer (Bioline, UK), 0.2 μM of each primer, 0.125 U MyTaq DNA polymerase (Bioline) and 0.5 μL of template DNA in a reaction volume of 5 μL. All reactions were carried out on a UnoCycler 732-1200 (VWR, USA) with 2 min at 94°C; 35 cycles of 30 s at 94°C, 45 s at 50°C, 60 s at 72°C; 10 min at 72°C. Amplified products were visualised on 2% agarose gels stained with GelRed (Biotium, USA). Samples with positive reactions were re-amplified under identical conditions except that the reaction volume was 10 μL and sequenced by a commercial provider using the forward PCR primer. Samples showing multiple peaks after sequencing were cloned using the InsTAclone PCR cloning kit (ThermoFisher Scientific, USA) following the manufacturer's protocol and transformed into DH5α cells. Plasmid DNA was purified by alkaline lysis and sequenced using M13 primers by a commercial provider.

*Wolbachia* multi locus strain typing (MLST) was performed following Baldo et al. (2006) utilising the primers *fbpA* F1/R1, *coxA* F1/R1, *gatB* F1/R1, *hcpA* F1/R1 and *ftsZ* F1/R1. Annealing temperatures ( $T_a$ ) were changed to 55°C for *fbpA*, 50°C for *gatB* and *hcpA* and 48°C for *ftsZ*. PCR was done using 1 × MyTaq buffer, 0.2 μM of each primer, 0.25 U MyTaq DNA polymerase and 0.8 μL of template DNA in a 10 μL reaction volume. Cycler settings were 2 min at 94°C; 35 cycles of 30 s at 94°C, 45 s at  $T_a$ , 60 s at 72°C; 10 min at 72°C. Products were sequenced by a commercial provider using the forward primers. The sequences of MLST loci and of a fragment of the *wsp* gene were searched against GenBank and the *Wolbachia* MLST Database (<http://pubmlst.org/wolbachia/>).

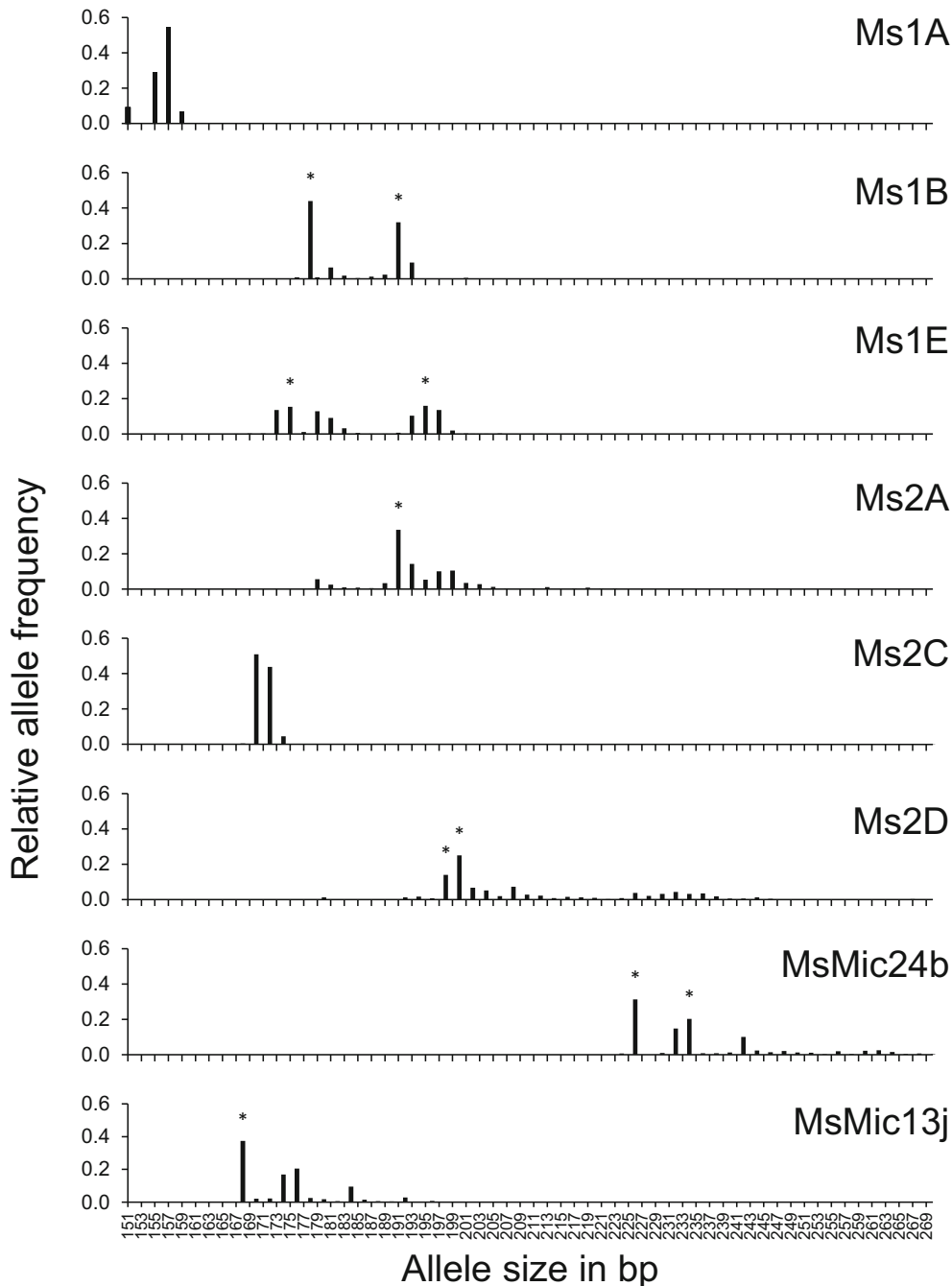
*Wolbachia* copy number per ant cell was assessed in a modified qPCR assay following McGarry et al. (2004). Fragments of *wsp* as used for diagnostics and fragments of the microsatellite MsMic24b were cloned as described above, and extracted plasmid DNA was sequenced to ensure correct insertion into the vector. Plasmid extracts were adjusted to a DNA concentration of 1 μM using a spectrophotometer (Nanodrop ND-1000; ThermoFisher Scientific), and dilution series ranging from 10<sup>-4</sup> to 10<sup>-9</sup> μM were used as standards for titre determination. Of 90 samples tested positive for *Wolbachia*, 24, all belonging to strain *wMes4*, were chosen for titre determination. The subset included, when available, groups of four randomly chosen samples (Research Randomizer v4.0; Urbaniak & Plous 2013) showing the same amplification success concerning *wsp* and MLST loci. Analysis was done in a RotorGene Q cycler (QIAGEN, Germany). Each reaction contained 1 × RotorGene SYBR Green PCR master mix, 0.2 μM forward and reverse primer, and 1 μL template DNA in a total of 10 μL. All samples ran in triplicate, and negative controls contained 1 μL water instead of a template. Plasmid standards for *wsp* and MsMic24b were included in each run. PCR conditions were 5 min at 50°C; 40 cycles of 30 s at 94°C, 45 s at 55°C, 60 s at 72°C; a final melting step from 60°C to 95°C in 0.5°C increments every 2 s was applied to assess specificity. The results were exported from the RotorGene Q Series software v1.7.94, and concentrations were calculated in SPSS v23 (IBM, USA).

## Morphometrics

Workers from nests representing the range of heterozygosity detected as characterised by MLH and  $d^2$  were measured, that is, 185 workers from 51 nests (2 to 6 per nest, on average 4), using a Leica M165 C stereomicroscope at magnifications 48 to 360×. Based on their high reproducibility (B. Seifert, unpubl.) and sufficient variation in a small-scale pilot study on the colonies sampled (not shown), five morphometric characters were selected and measured each on the left and the right body side: EL = eye length (maximum large diameter of the elliptic eye; all structurally defined ommatidia, pigmented or not, were included), Fe2L = length of midfemur (measured on extensor side, from the distalmost point of femur to borderpoint between femur and trochantellus, equivalent measurements possible with view on plane or edge of femur and intermediate viewing positions), PnHL = pronotum hair length (length of hair at frontolateral corner of pronotum), SLd = scape length (maximum straight line scape length in dorsal view, excluding the articular condyle; the scape was tilted to a position perpendicular to the swivelling plane of the funiculus segment), SPPLd = distance between the centre of the propodeal stigma and the posterior margin of the propodeal lobe (measured in dorso-caudo-lateral view; the specimen was carefully tilted to the position resulting in the true maximum of the distance).

As an asymmetry proxy, the composite fluctuating asymmetry (CFA) was calculated following Garrido and Perez-Mellado (2014). Thus, for each of the five morphometric characters, (a) the value for the left side was subtracted from that for the right side, (b) the absolute value of the difference was divided by the absolute value of the difference averaged across all individuals measured, and (c) the results for all five characters were summed up. Additionally, asymmetry corrected for directional asymmetry (DA) following Lacy and Alaks (2013) was used to calculate CFAcorrDA; DA may or may not stem from developmental instability (Lens et al., 2002) and would thus, if inherent to the data, inflate the CFA and hamper its usefulness as a fitness proxy. Thus, for each character, the value for the left side was subtracted from that for the right side, and from the value of the difference (a positive or negative value), the real number of the difference averaged across all individuals was subtracted, followed by steps (a) and (b) resulting in one CFAcorrDA value per individual. To test for any relation between genetic diversity and the asymmetry proxies, using SAS 9.4, nest means of CFA and CFAcorrDA were subjected to linear regression analyses (type III sum of squares) with MLH and  $d^2$  as independent variables, and habitat-island means of CFA and CFAcorrDA were subjected to linear regression analyses (type III sum of squares) with AR. A significance level  $\alpha$  0.05 was used.

All alate gynes sampled were measured, that is, 929 gynes from 118 nests (1 to 85 per nest, on average 8.47), using a Leica M125 stereomicroscope at 37.5× magnification. Two characters were measured: CW = cephalic width (maximum width of head capsule including compound eyes), MW = mesosoma width (largest width of pronotum in dorsal view). Using CW as a measure for



**FIGURE 2** Relative frequencies of all alleles of the eight microsatellite loci genotyped in 660 workers of *Messor structor*, sorted according to size in base pairs (bp). The numbers of alleles per locus were four in MS1A, 15 in MS1B, 24 in MS1E, 24 in MS2A, six in MS2C, 31 in MS2D, 23 in MsMic24b, and 16 in MsMic13j. \* indicates alleles with higher frequencies than expected under normal distribution (see Material and methods).

overall body size, mesosoma size was standardised by calculating the ratio MW/CW. Habitat-island-mean MW/CW was used as dependent variable (together with the genetic parameters AR, MLH and  $d^2$ ) in regression analyses (type III sum of squares) performed with the habitat-island parameters surface area, perimeter and distance to the closest habitat island, as well as the number of nests per island, and the connectivity metric  $i4$  (for details, see *Materials and methods, Microsatellites*).

## RESULTS

### Habitat islands and ant nests sampled

The 54 habitat islands populated by *Messor structor* had surface areas of 0.05 to 48.25 ha (mean  $\pm$  standard deviation, SD = 5.86  $\pm$  8.72 ha; Table S1, Supporting Information), and perimeters from 0.18 to 4.83 km (mean  $\pm$  SD = 1.06  $\pm$  0.88 km). The distances to the closest

habitat island were 0.002–2.2325 km (mean  $\pm$  SD = 0.49  $\pm$  0.44 km). The maximum distance between two islands was 20.7 km. In total, 226 *Messor structor* nests were found, from one to 18 per island (mean  $\pm$  SD = 4.2  $\pm$  3.9).

## mtDNA

The COI sequences of 645 workers were successfully amplified (habitat-island means 2.0 to 3.0 workers per nest; overall average 2.9 workers per nest; Table S1); sequencing the remaining 33 workers failed despite multiple attempts. The two lineages expected based on a comprehensive taxonomic analysis that also demonstrated their conspecificity (Steiner et al., 2018), Lineage 3 and 4, were identified in 51 and 593 individuals, respectively, and no additional lineage was found. The Tamura-Nei distances were 3.33% between the lineages and 0.02% (Lineage 3) and 0.19% (Lineage 4) within them.

## Microsatellites

Overall, genotyping was successful in 660 workers (habitat-island means 2.0 to 3.0 workers per nest; overall average 2.9 workers per nest; Table S1), with 11.2–12.1 workers per locus and habitat island on average (Table S2, with descriptive statistics at the habitat-island level for each microsatellite locus used). Four to 31 alleles per locus (mean  $\pm$  SD = 17.9  $\pm$  8.8; Figure 2) were found. In three loci, namely Ms1B, Ms1E and MsMic24b (Figure 2), allele-size distribution significantly deviated from normal distribution, with gaps of eight to 20 bp between the most frequent allele sizes. The observed heterozygosity over all loci ranged from 0.25 to 0.75 (mean  $\pm$  SD = 0.55  $\pm$  0.13). When using all habitat islands together as a single population, the estimated null allele frequencies per locus ranged from 0.063 to 0.188 (mean  $\pm$  SD = 0.125  $\pm$  0.032). When using the 54 habitat islands as separate populations, the number of habitat islands without null alleles differed across loci and ranged from 12 to 31 (mean  $\pm$  SD = 21.8  $\pm$  5.6; corresponding to 40.3  $\pm$  10.3%). Because of the low average frequencies of null alleles across the 54 habitat islands and the inconsistency in their occurrence across habitat islands, all loci were retained for further analyses, as done by Ozerov et al. (2016). All loci significantly deviated from HWE when using all habitat islands together as a single population (data not shown). When only habitat islands harbouring at least five nests were analysed and treated as separate populations ( $n = 18$ ), eight islands deviated from HWE in four loci, namely Ms2D, Ms1E, MsMic24b and MsMic13j (Table S3). In total, less than 7% of 144 comparisons deviated from HWE, and we retained all loci for further analyses. When using all habitat islands together as a single population, Ms1E and MsMic13j remained significant for LD after Bonferroni-Holm correction. When using the 18 habitat islands as separate populations, no linkage was observable, but sample size was too low for some islands for performing exhaustive comparisons. In sum, we kept all loci for further analyses.

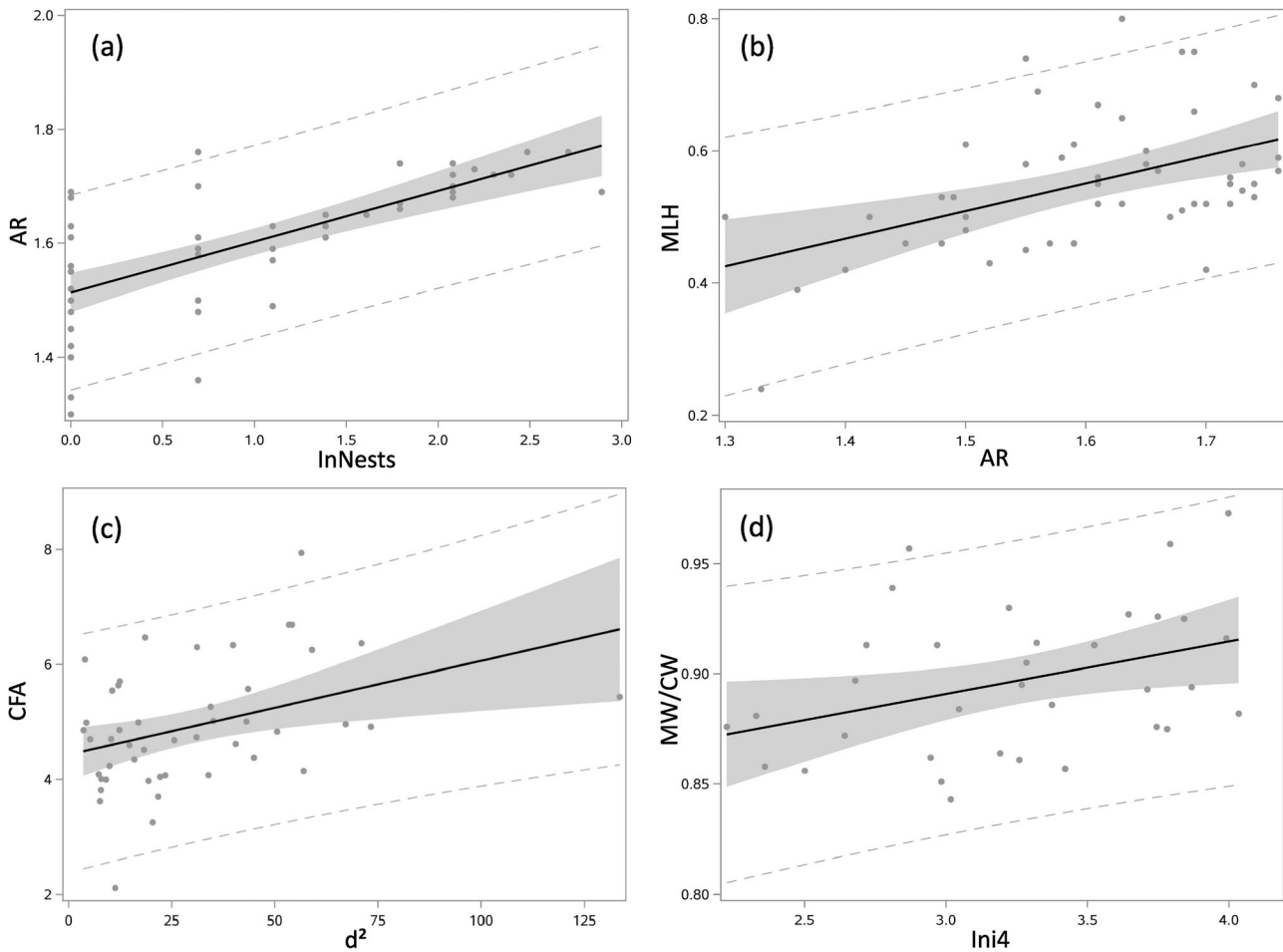
AR averaged over all loci ranged from 1.3 to 1.8 (mean  $\pm$  SD = 1.6  $\pm$  0.1;  $n = 54$  habitat islands). AR was explained by the number of nests per habitat island ( $n = 52$ ,  $p < 0.0001$ ,  $R^2 = 0.498$ , estimate = 0.0892, Figure 3a, after exclusion of the islands M80 and M86 that were identified as outliers in the regression of gyne mesosoma size given  $i_4$  (see *Morphometrics*); including M80 and M86:  $n = 54$ ,  $p < 0.0001$ ,  $R^2 = 0.495$ , estimate = 0.0894, see Figure S2 for all regressions using AR as dependent variable). An additional regression to test whether the increase of AR with increasing number of nests per island could, in fact, be linked to the frequency of null alleles and thus be an artefact rejected this ( $n = 54$ ,  $p_{\text{InNests}} < 0.0001$ ,  $p_{\text{null alleles}} = 0.8771$ ,  $R^2 = 0.495$ , estimate<sub>InNests</sub> = 0.0878, estimate<sub>null alleles</sub> = 0.0583). Contrasting the results for the number of nests per habitat island, AR was not explained by the habitat islands' surface area nor perimeter nor distance to the closest habitat island. MLH ranged, at the nest level, from 0.2 to 0.9 (mean  $\pm$  SD = 0.6  $\pm$  0.2;  $n = 226$ ) and, at the habitat-island level, from 0.2 to 0.8 (mean  $\pm$  SD = 0.6  $\pm$  0.1;  $n = 54$ ); nest-mean squared distance between two alleles at a locus as mean over all loci ( $d^2$ ) ranged from 2.4 to 133.6 (mean  $\pm$  SD = 27.5  $\pm$  19.4;  $n = 226$ ) and habitat-island  $d^2$  from 2.4 to 62.7 (mean  $\pm$  SD = 27.1  $\pm$  12.8;  $n = 54$ ). Habitat-island-level MLH was returned as dependent on AR ( $n = 52$ ,  $p = 0.0004$ ,  $R^2 = 0.227$ , estimate = 0.4184, Figure 3b, after exclusion of the islands M80 and M86; including M80 and M86:  $n = 54$ ,  $p = 0.0004$ ,  $R^2 = 0.219$ , estimate = 0.5405, see Figure S3 for all regressions using MLH as dependent variable). However,  $d^2$  was not explained by AR (Figure S4: all regressions using MLH as dependent variable). The habitat-island-mean values of neither MLH nor  $d^2$  were explained by any of the habitat-island parameters. In the AMOVA, 10% and 2% of nuclear variation were explained by habitat islands and mtDNA lineages, respectively (Table S4). IBD was weak ( $r_{xy} = 0.047$ ,  $p_{\text{rxy-rand}\geq\text{rxy-data}} = 0.001$ ).

In the FLOCK analysis, no plateau was found for any  $K$  (testing up to  $K = 25$ ), meaning that the program was inconclusive about the correct number of  $K$ . Calculating delta  $K$  following Evanno et al. (2005) from the STRUCTURE results suggested one or two genetic clusters, the two solutions being indistinguishable in this approach as  $K = 1$  is not evaluated using this method (Figures 4 and S5). The microsatellite-based STRUCTURE clusters and the mtDNA lineages did not correlate ( $r_{xy} = 0.047$ ,  $p = 0.239$ ). In the ADEGENET analysis, no structuring in the population became visible (Figure S6).

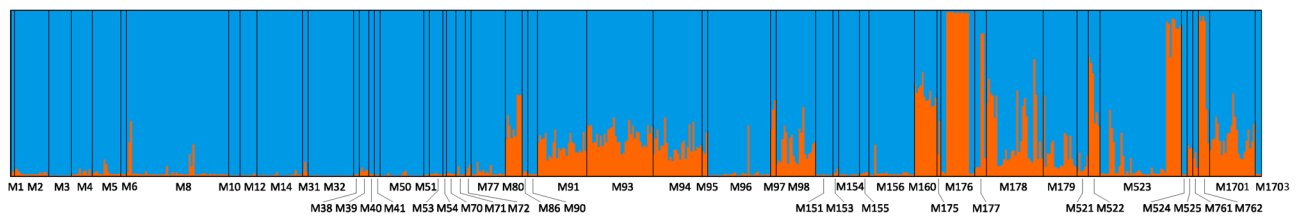
## Wolbachia

Analysis of 94 workers from 34 habitat islands yielded 51 positive reactions (54%). After sequencing, 11 samples were suspected to contain traces of more than one genotype and were therefore cloned; no multiple infections were confirmed in this step.

The general multi locus strain typing (MLST) primers of Baldo et al. (2006) amplified successfully in some samples, but success differed among the six genes. Amplification success of the 94 samples ranged from 4 to 81% (Table S5), and 95.7% amplified for at least one



**FIGURE 3** Linear regression of (a) allelic richness (AR) of habitat islands given the natural logarithm of the number of nests (lnNests) found on the same habitat islands ( $n = 52$ ,  $p < 0.0001$ ,  $R^2 = 0.498$ , estimate = 0.0892); (b) mean locus heterozygosity (MLH) of islands given AR found on the same islands ( $n = 52$ ,  $p = 0.0004$ ,  $R^2 = 0.227$ , estimate = 0.4184); (c) worker fluctuating asymmetry (calculated as composite fluctuating asymmetry, CFA) of nests given the nest-level squared distance between two alleles at a locus as mean over all loci ( $d^2$ ) for the same nests ( $n = 47$ ,  $p = 0.0074$ ,  $R^2 = 0.149$ , estimate = 0.0163); and (d) gyne mesosoma size (calculated as mesosoma width divided by head width, MW/CW) of islands given the natural logarithm of the isolation index  $i_4$  (lnI4) found on the same islands ( $n = 35$ ,  $p = 0.0279$ ,  $R^2 = 0.138$ , estimate = 0.0237). In (a)–(d), the habitat islands M80 and M86 were excluded; for details, see Methods, section Microsatellites. Grey areas indicate 95% confidence intervals.



**FIGURE 4** Results of STRUCTURE for  $K = 2$ , sorted left to right by geographic position of habitat island from NE to SW. Each coloured bar represents a single worker of *Messor structor*, and black vertical lines separate habitat islands.

gene and were therefore treated as *Wolbachia* positive. All *wsp* and MLST sequences were identical to those of either wMes3 ( $n = 27$ ) or wMes4 ( $n = 67$ ; Table S6) reported by Steiner et al. (2018; see <https://doi.org/10.5061/dryad.mj43d20> for GenBank associations). No novel strain was found. For 24 samples, qPCR was successful; *Wolbachia* copy number per host cell ranged from 0.06 to 3.83 (mean  $\pm$  SD =  $1.31 \pm 0.89$ ; Table S6).

### Morphometrics

As measured by CFA, fluctuating asymmetry ranged from 2.1 to 7.9 in nests (mean  $\pm$  SD =  $4.9 \pm 1.1$ ;  $n = 47$  nests) and from 3.7 to 6.2 in habitat-island means ( $n = 14$  habitat islands); as measured by CFA-corrDA, it ranged from 1.8 to 7.8 in individual workers (mean  $\pm$  SD =  $4.9 \pm 1.1$ ;  $n = 47$  nests) and from 3.7 to 6.3 in habitat-island

means ( $n = 14$  habitat islands). Fluctuating asymmetry increased with increasing heterozygosity as estimated by  $d^2$  both when not correcting for DA (CFA; nest level,  $n = 47$ ,  $p = 0.0074$ ,  $R^2 = 0.149$ , estimate = 0.0163, Figure 3c; see Figure S7 for all regressions using CFA as dependent variable) and when correcting for it (CFAcorrDA; nest level,  $n = 47$ ,  $p = 0.0115$ ,  $R^2 = 0.134$ , estimate = 0.0162; Figure S7: all regressions using CFAcorrDA as dependent variable). No relation was found with AR nor MLH.

Mesosoma size (MW/CW) ranged from 0.8 to 1.1 in individual gynes (mean  $\pm$  SD =  $0.9 \pm 0.0$ ;  $n = 929$  gynes) and from 0.8 to 1.0 in habitat-island means ( $n = 37$  habitat islands). It increased with increasing habitat connectivity as measured by  $i_4$  after exclusion of the habitat islands M80 and M86 that were identified as outliers ( $n = 35$ ,  $p = 0.0279$ ,  $R^2 = 0.138$ , estimate = 0.0237, Figure 3d; see Figure S8 for all regressions using MW/CW as dependent variable); no relation was found when M80 and M86 were included. No relation was found with any of the habitat-island parameters nor with the number of nests per habitat island.

## DISCUSSION

A combined dataset of mitochondrial DNA (mtDNA), microsatellites, *Wolbachia*, and worker and gyne morphometrics was generated for the harvester ant *Messor structor*, a grassland habitat specialist, in a 200 km<sup>2</sup> area in Austria (Central Europe). Over the past millennia, this area underwent substantial changes, and in the past century, rural engineering has resulted in both strongly decreased area and a strongly increased isolation of grassland. In the following, we discuss our findings in the light of our six research questions on the population history and conservation biology of *M. structor* in the area.

### i. From how many refugia did the ants originate that colonised the study area after the LGM?

For evaluating the consequences of habitat fragmentation, knowledge of population history and thus of postglacial recolonisation can be important. The study area belonged to the area of continuous permafrost during the LGM and was thus not inhabitable by *M. structor* (Lindgren et al., 2016). Rather, we consider colonisation of the study area from two different glacial refugia as the most plausible explanation for our mtDNA and microsatellite results. Concerning mtDNA, this is because two divergent lineages are present in our study site, termed Lineage 3 and 4 in Steiner et al. (2018), who had included 18 of the 226 nests analysed here. The high divergence in mtDNA between lineages suggests colonisation from different source populations, which is in line with the overall pattern of mtDNA-lineage distribution provided by Steiner et al. (2018), with Lineage 3 occurring west of Austria and Lineage 4 occurring in eastern Austria.

The two-refugia hypothesis is supported by the microsatellite data, in that the distribution of allele sizes is discontinuous in the loci Ms1B, Ms1E and MsMic24B (Figure 2). Further, in a mutation-drift equilibrium, low allele frequencies would be expected to dominate

(Luikart et al., 1998; Nei et al., 1976). In Ms1B, Ms1E and MsMic24B, we rather observe two frequency maxima in size distribution, separated by a large gap. We infer the gap to be a product of separated population history, with drift having acted as a diverging force on the allele-size distributions of the populations during isolation, followed by new contact. Divergence and, in the extreme, incipient speciation have been unveiled by such allele-size distributions, for example, in the kelp *Lessonia nigrescens* (Tellier et al., 2011) and in ants of the *Camponotus ephippium* complex (Macaranas et al., 2001).

### ii. Do reproductive barriers separate populations in the study area?

In inferring potential gene-flow reductions by habitat fragmentation, knowledge of reproductive barriers that existed before habitats became fragmented can be useful. We infer that no reproductive barrier exists for *M. structor* in the study area: admixture of microsatellite alleles in a contact zone between the two mitochondrial lineages is so strong that in the microsatellite variation, association of individuals with the mtDNA lineages is practically not detectable (AMOVA, Table S4). When assuming one genetically admixed population, the lack of aggression between different nests of *M. structor* across the study area found by Schlick-Steiner et al. (2005) is indirectly supported. As is the case in many of the ant species investigated (Hakala et al., 2019; Helms IV, 2018), we may also here see slight indication of males being responsible for gene flow to a higher degree than females: the rarer mtDNA lineage 3 is clumped across habitat islands (Figure 1d), which could hint at philopatry of gynes given that mtDNA is inherited exclusively via females.

### iii. Has *Wolbachia* influenced genetic diversity?

*Wolbachia* can influence the distribution and frequency of genotypes, and knowledge about such effects is needed for sound inference of the conservation-genetic status of host populations. We found two closely related strains of *Wolbachia* to infect this *Messor structor* population. Of the six genes analysed, just one single-nucleotide polymorphism (SNP) in *coxA* separates wMes3 and wMes4. As the SNP is consistent in all individuals successfully sequenced and has been seen earlier (Steiner et al., 2018), we exclude PCR errors as a possible explanation. No sign of additional strains was found. Mitochondrial Lineages 3 and 4 were always associated with wMes3 and wMes4, respectively, confirming maternal coinheritance.

The infection rate was almost 100%, although bacterial density in worker ants was frequently low. Degastering the workers prior to extraction may have contributed to this low titre, as it is known that different tissues harbour different quantities of this intracellular symbiont (Clark & Karr, 2002). Age might also play a role as ant workers, which are evolutionary dead ends for the maternally inherited *Wolbachia*, probably lose their infection with age (Keller et al., 2001).

We did not detect reduced gene flow between individuals infected with the two *Wolbachia* strains – the strains perfectly coincide with the mtDNA lineages, between which there is no gene-flow reduction (see Research Question ii) – indicating that no cytoplasmic

incompatibility between the differently infected lines is present here. The negligible rate of uninfected ants in the study site supports the assumption that CI is expressed between infected and uninfected individuals.

iv. *Did habitat fragmentation change genetic diversity?*

To reconstruct the conservation-genetic consequences of habitat fragmentation, knowing the pre-fragmentation extent of genetic diversity is necessary. We have twofold evidence that a lot of genetic diversity arrived in the area postglacially: Two well-diverged lineages met, and each represented a considerable fraction of the within-lineage variation known from the respective distribution area (mtDNA data: this study; nuclear DNA as captured by amplified fragment length polymorphism for a subsample of the colonies included in this study: Steiner et al., 2018).

What is the conservation-genetic consequence of population size per habitat island? While we did not detect a direct effect of habitat-island area on any population-genetic parameter, we did find genetic diversity as measured by AR (which is corrected for sample size) to be lower on habitat islands with smaller populations (Figure 3), an indirect measure of island size and quality; we also found heterozygosity as measured by MLH to be lower with lower AR (Figure 4) – and both findings represent clear negative effects of small population size on genetic diversity of habitat islands. The lack of an effect of AR on the heterozygosity measure “squared distance between two alleles at a locus as mean over all loci” ( $d^2$ ) likely can be explained by allele size being used in calculating  $d^2$  (but not MLH). Fewer alleles available can be well expected to reduce  $d^2$  (as shown for MLH), but in the Retz population, this effect seems to be counterbalanced by the large differences between allele sizes of the same locus (Figure 2) that partly co-occur in individuals, probably due to the mixing of the two lineages.

What is the conservation-genetic consequence of island isolation? The extent of nuclear differentiation at the level of habitat islands of 10% (AMOVA, Table S4; compared with just 2% of nuclear variation in accord with the mtDNA lineages) suggests that gene flow among islands is reduced relative to that within islands. At the same time, the weak IBD suggests that this reduction of gene flow is not very strong. This is also reflected in the lack of any effect of distance on any of the measures of genetic diversity used – the distances to the closest island in our sample, that is, 320 m on average and 2325 m at the most, seem not to influence negatively genetic diversity (or cannot be demonstrated using our data, possibly because of colony longevity). Finally, the at least partial lack of nuptial flight observed in 2004 should be mentioned in this context, as mating in or close to the nest might promote inbreeding. However, in polygynous colonies, mating in or close to the nest does not necessarily mean mating of relatives and thus does not necessarily lead to inbreeding (Ingram, 2002).

v. *Does fluctuating asymmetry in workers correlate with genetic architecture?*

Genetic architecture matters to fitness – too much homozygosity or heterozygosity can result in inbreeding and outbreeding depression, respectively, and mating strategies have evolved to avoid both (Wei & Zhang, 2018 and references therein). However, these strategies are counteracted by, among others, extrinsic influences, such as when inbreeding results from habitat fragmentation (Mäki-Petäys & Breen, 2007; Vitikainen et al., 2011; Vitikainen et al., 2015) or outbreeding from the admixture of lineages diverged in allopatry (Frankham et al., 2011). We infer that both might apply to our data, inbreeding, as possibly indicated by MLH, and outbreeding, as deducible from the gaps in allele-size distribution, which is why  $d^2$  likely does not indicate inbreeding here (see previous section).

Before interpreting our fitness proxy (asymmetry in workers) with regard to genetic architecture, we note that in our data, asymmetry is not due to a directional effect. This is because the same results were returned when using an index without correction for DA (CFA) and an index correcting for it (CFAcorrDA). This is relevant in that DA may be due to other effects than developmental instability (Lens et al., 2002) and may thus, if inherent to the data, hamper the usefulness of CFA as a fitness proxy.

Our results are twofold. The first result is the lack of a relationship of lowered heterozygosity represented by the MLH data and asymmetry, that is, of inbreeding depression. This might possibly be due to there being no inbreeding depression because of (a) a reduced vulnerability of haplodiploid organisms to inbreeding depression because of the successful purging of bad alleles (but see, e.g., a *Formica exsecta* case study, Vitikainen et al., 2015); and/or (b) the longevity of ant colonies and their queens which enables them to stay longer undamaged by suboptimal conditions than shorter-lived organisms (Braschler & Baur, 2016). In any case, these effects would provide just a delay of inbreeding depression and, once started, inbreeding depression is expected to be more harmful in haplodiploid organisms than in diploids in the long run due to the production of inviable or sterile diploid males that can result in an extinction vortex (Zayed & Packer, 2005). While original models may have overestimated the frequency of occurrence of this extinction vortex, it has been demonstrated for several species and might become a more severe effect, especially if inbreeding is combined with other factors like suboptimal habitat quality (Leung & van der Meulen, 2022). Alternatively, the lack of demonstrated inbreeding depression might well be due to a low sensitivity of our fitness proxy, worker asymmetry, which was the only practical approach in the frame of this project. Possibly, use of life-history traits such as fecundity and/or longevity would have revealed inbreeding depression because they seem to be more sensitive than morphology (DeRose & Roff, 1999; ant example: Haag-Liautard et al., 2009).

The second result is the positive relationship of increased heterozygosity as represented by  $d^2$  with asymmetry (Figure 3c), possibly suggesting outbreeding depression. This would be in line with the demonstration of outbreeding depression using asymmetry measures by others (Demontis et al., 2010; Kurbalija et al., 2010). This result is not a consequence of habitat fragmentation, and we suggest that it rather indicates that this ant population might be in the delicate

situation of suffering from divergent alleles within individuals, combined with the additional negative effects of lowered genetic variation. The situation of both out- and inbreeding has been suggested for other haplodiploid organisms (wasp example: Greeff et al., 2009) and may even advance the extinction vortex (Zayed & Packer, 2005).

vi. *Does habitat fragmentation influence gyne size?*

The size of ant gynes can relate to dispersal capability (Helms IV, 2018). We found no relation between gynes' relative mesosoma size (corrected for absolute body size by calculating MW/CW) and surface area nor perimeter of habitat islands nor distance to the closest habitat island; however, the less connected (as measured by the connectivity measure  $i_4$ ) a habitat island was, the smaller was the mesosoma (Figure 3d). A smaller mesosoma houses less flight muscle, and flight ability can be inferred to be thus reduced. Reduced flight ability may in turn result in local copulation, that is, copulation on the habitat island of the maternal colony, followed by readoption of daughters into the colony after copulation (Heinze & Buschinger, 1989; Heinze & Keller, 2000). While we do not have at hand data on the colony founding behaviour of this *M. structor* population, for at least some of the colonies analysed here, a "lack of nuptial flight" was observed in 2004 as was asynchronous development across colonies (Schlick-Steiner et al., 2005), both in line with such a mode of colony foundation.

This scenario for our population would be in accord with the general suggestions that habitat patchiness may lead to (a) reduction in gyne size (Schlick-Steiner et al., 2007 and references therein) due to increased dispersal risk (but see, e.g., Haatanen & Sorvari, 2013 who found no environmental influence on gyne size) and (b) colony foundation via readoption of daughters. From the conservation-genetics point of view, this scenario would mean that increased habitat patchiness reduces genetic exchange both between and within islands, which would then worsen inbreeding that results from reduced AR due to reduced numbers of nests per habitat island. Body size and proxies thereof are considered to determine flight ability in ant gynes (Helms IV, 2018), but including also wing muscle mass and its relationship with body size (reviewed by Hakala et al., 2019) as well as wing surface area and its relationship with body size (wing loading, Betts & Wootton, 1988) may be worthy topics for future studies on this system.

## CONCLUSION

Here, we assessed the impact of habitat fragmentation – one of the major drivers of biodiversity loss worldwide (Haddad et al., 2015) – on the grassland-specialist ant *Messor structor* in a 200 km<sup>2</sup> area in Central Europe and reconstructed its population history as background for the conservation-biological interpretation. Two mtDNA lineages recolonised the area after the LGM, likely from two different refugia, which we also infer to be the reason for allele-size distribution gaps in three out of eight microsatellites used. Based on mtDNA data for the

ant's entire distribution range, provenience of the population studied here from two refugia resulted in high genetic diversity, which intermixes without gene-flow barriers, neither due to mtDNA lineage identity nor to infection with two different *Wolbachia* strains. Notwithstanding the lack of impact on the species' mating system, the *Wolbachia* data provided here represent a case study useful for assessing the conservation-genetic relevance of these endosymbionts more generally (cf. Hamm et al., 2014).

From relating population size per habitat island to AR, we infer that through reduced population sizes, habitat fragmentation has negatively impacted genetic diversity per island. This in turn is inferred to have resulted in reduced heterozygosity as measured by MLH, possibly reflecting inbreeding. The other measure of heterozygosity we used,  $d^2$ , did not show this effect, likely because it accounts for the difference between allele sizes within individuals and because these differences are large due to the large size differences of alleles from the two refugia. The fitness proxy we used, the asymmetry of workers, is not influenced by a DA effect and does, firstly, not reveal inbreeding depression (regression using MLH) but might secondly rather indicate potential outbreeding depression (regression using  $d^2$ ). The first may be due to the good mobility of the alates, particularly the males; the second may be seen as supporting the relevance of interpreting potential effects of habitat fragmentation in a historical context.

We found no effect of any habitat-island parameter (surface area, perimeter, distance to the closest habitat island) on either of the two heterozygosity measures, but the size of the mesosoma of gynes and thus of their flight muscles decreased with decreasing connectivity of habitat islands (connectivity measure  $i_4$ ). In ants, gyne size is known to influence colony foundation mode, and habitat fragmentation, in terms of both area and isolation, may thus influence the social biology of this ant.

What measure for quantifying heterozygosity may be best to use has been hotly discussed and depends on the particular research question (reviewed by Hansson, 2010). In this work, it turned out to be important to use both MLH and  $d^2$ , in that the first was interpreted to reflect potential inbreeding, the second, potential outbreeding.

Future analyses on the *Messor structor* population should include (1) comparing the data from this study area with data from a distribution area with continuous habitat; (2) assessing agrochemicals as stressors potentially contributing to developmental problems and thus asymmetry; (3) testing life-history traits as a potentially more sensitive proxy of potential inbreeding depression than asymmetry; (4) assessing larger minimum distances between islands than analysed here to identify potential effects of isolation on genetic diversity; (5) assessing the quality of habitat on each island such as the level of shrub encroachment and the availability of resources relevant to these ants for inclusion as additional parameters; (6) testing the hypothesis we have raised here that the connectivity of islands influences colony foundation mode; (7) assessing the role of male dispersal in maintaining gene flow and thus genetic diversity; (8) genome-wide sequencing to quantify the genome-wide extent of potential inbreeding (Kardos et al., 2016) relative to that of outbreeding in the same individuals;

and (9) revisiting the islands presented here to monitor ongoing fragmentation and to evaluate a potential lag phase of additional consequences in becoming apparent (e.g., Haddad et al., 2015).

From the conservation point of view, our finding of habitat islands with fewer nests harbouring less AR and of potential inbreeding (less MLH) coming with less AR highlights the impact of further population-size reductions on unmanaged patches in agricultural land, in line with studies on other species, including ants (Pacheco et al., 2013; Urrutia-Escobar & Armbrrecht, 2013). The analysed *M. structor* population may be particularly vulnerable, in that their fitness has been potentially reduced due to outbreeding because two diverged lineages met and admixed postglacially, topped by reduced heterozygosity due to habitat fragmentation. When these nests were sampled (2004), the genetic and demographic viability of the population may have been decreased – assessment of the population's status two decades later will be needed to evaluate this.

#### AUTHOR CONTRIBUTIONS

**Raphael C. Strohmaier:** Investigation; formal analysis; writing – original draft; writing – review and editing; visualization. **Wolfgang Arthofer:** Conceptualization; investigation; formal analysis; writing – original draft; writing – review and editing. **Karl Moder:** Formal analysis; writing – review and editing; visualization. **Heino Konrad:** Investigation; writing – review and editing. **Christian Stauffer:** Conceptualization; writing – review and editing. **Alfred Buschinger:** Conceptualization; writing – review and editing. **Nils Struck:** Investigation; formal analysis; writing – review and editing. **Herbert C. Wagner:** Investigation; formal analysis; writing – review and editing. **Bernhard Seifert:** Conceptualization; formal analysis; writing – review and editing. **Ross H. Crozier:** Conceptualization. **Florian M. Steiner:** Conceptualization; supervision; project administration; investigation; data curation; formal analysis; writing – original draft; writing – review and editing. **Birgit C. Schlick-Steiner:** Conceptualization; supervision; project administration; investigation; data curation; formal analysis; writing – original draft; writing – review and editing.

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#### CONFLICT OF INTEREST STATEMENT

The authors declare that no conflict exists for any of the authors.

#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Dryad at <https://doi.org/10.5061/dryad.n2z34tn8s>.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**Data S1:** Supporting Information

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