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RESEARCH PAPER

Sex ratio rather than population size affects genetic diversity in *Antennaria dioica*

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ABSTRACT

- Habitat fragmentation and small population size can lead to genetic erosion in threatened plant populations. Classical theory implies that dioecy can counteract genetic erosion as it decreases the magnitude of inbreeding and genetic drift due to obligate outcrossing. However, in small populations, sex ratios may be strongly male- or female-biased, leading to substantial reductions in effective population size. This may theoretically result in a unimodal relationship between sex ratios and genetic diversity; yet, empirical studies on this relationship are scarce.
- Using AFLP markers, we studied genetic diversity, structure and differentiation in 14 highly fragmented *Antennaria dioica* populations from the Central European lowlands. Our analyses focused on the relationship between sex ratio, population size and genetic diversity.
- Although most populations were small (mean: 35.5 patches), genetic diversity was moderately high. We found evidence for isolation-by-distance, but overall differentiation of the populations was rather weak. Females dominated 11 populations, which overall resulted in a slightly female-biased sex ratio (61.5%). There was no significant relationship between population size and genetic diversity. The proportion of females was not unimodally but positively linearly related to genetic diversity.
- The high genetic diversity and low genetic differentiation suggest that *A. dioica* has been widely distributed in the Central European lowlands in the past, while fragmentation occurred only in the last decades. Sex ratio has more immediate consequences on genetic diversity than population size. An increasing proportion of females can increase genetic diversity in dioecious plants, probably due to a higher amount of sexual reproduction.

INTRODUCTION

Habitat fragmentation and small population size are known to reduce genetic diversity in plant populations and to increase genetic differentiation among populations (*i.e.* genetic erosion; Young *et al.* 1996). Consequently, many endangered species face genetic drift and increased levels of inbreeding (*e.g.* Heinicke *et al.* 2016). This is alarming, as a loss of genetic diversity is often accompanied by reduced reproductive fitness (reviewed in Leimu *et al.* 2006). However, genetic erosion can be considerably decelerated in plant species that exhibit prolonged clonal growth and/or increased longevity, particularly if the period of habitat fragmentation is relatively short compared to the species' life span (Mona *et al.* 2014). Furthermore, the mating system has a dominant impact on the strength of genetic erosion. More specifically, in outcrossing species, habitat fragmentation and decreasing population size may result in mate limitation (Thrall *et al.* 2014), and thus outcrossing species show higher susceptibility to genetic erosion than self-compatible species (Heinken & Weber 2013).

Dioecy (*i.e.* the occurrence of distinct male and female individuals) is a comparatively rare mating system, representing only 5–6% of all angiosperms (Renner 2014). The separation of sexes is a mechanism that ensures obligate outcrossing, which may promote high rates of gene flow and reduce the frequency of inbreeding due to the prevention of self-pollination (Allen & Hiscock 2008). As a consequence, theory predicts that dioecy should maintain high levels of genetic diversity and limit

genetic differentiation (Hamrick & Godt 1996; Barrett *et al.* 2010). However, among the few studies on genetic erosion in dioecious plant species, Lauterbach *et al.* (2012; for *Silene otites*) and Vandepitte *et al.* (2009; for *Mercurialis perennis*) revealed that dioecy does not inevitably avoid genetic erosion in fragmented populations. In fact, unbalanced sex ratios may critically decrease the effective size of populations, which may override the benefits of an obligate outcrossing mating system (Dubreuil *et al.* 2010).

In natural populations of dioecious plants, sex ratios often depart from the expected 1:1 ratio. Indeed, Barrett *et al.* (2010) found that out of 126 dioecious plant species, 70% were biased towards the male or female sex. The reasons for male- or female-biased sex ratios are likely related to different resource allocation patterns for sexual reproduction in males and females. While males produce only pollen, females produce ovaries, seeds and fruits. As such, females often spend more resources on successful reproduction than males (Obeso 2002). This can be related to the evolution of sex-specific life-history traits, and/or sex-specific stress susceptibility, resulting in distinct adaptation strategies of males and females to their natural habitat (Espírito-Santo *et al.* 2003; Iszkuło *et al.* 2008; Munné-Bosch 2015). Consequently, biased sex ratios may occur as a result of distinct growth rates and mortality of males and females in specific habitats (Varga & Kytöviita 2011). In addition, demographic stochasticity, which is particularly pronounced in small and isolated populations, may randomly alter sex ratios (Vandepitte *et al.* 2009). Thus, populations that consist of only a few individuals show a high probability that either males or females are overrepresented, which may result in reduced sexual reproduction due to mate limitation or, ultimately, in the total loss of sexual reproduction in populations comprising only one sex (Rosche *et al.* 2014).

Yet, to what extent sex ratios can influence genetic diversity in plant populations remains obscure. One might expect that species-specific optimal sex ratios should result in frequent sexual reproduction. As such, genetic diversity should show a unimodal relationship to sex ratio, with the highest genetic diversity in populations possessing optimal sex ratios (*i.e.* sex ratio resulting in highest reproductive success), and declining genetic diversity towards both extremes of female- and male-biased sex ratios. However, the only two empirical studies that related biased sex ratios to population size and genetic diversity in dioecious species revealed contrasting results. Hilfiker *et al.* (2004) found female-biased sex ratios in small, and genetically depleted populations of *Taxus baccata*. Vandepitte *et al.* (2009) detected a positive relationship between the proportion of females and genetic diversity in *M. perennis* and related this finding to environmental conditions favouring reproductive success. Overall, a thorough understanding of the interplay of fragmentation, small population size and sex ratio, on the one hand, and genetic diversity, on the other, is still lacking.

Here, we studied sex ratio and genetic diversity in 14 Central European lowland populations of *Antennaria dioica* (L.) P. Gaertn. (Asteraceae), a dioecious species that shows slightly female-biased sex ratios in natural populations (Eriksson 1997; Varga & Kytöviita 2011). In our study region, both size and connectivity of *A. dioica* populations have severely declined over recent decades, mainly due to land-use change and nutrient deposition (Rosche *et al.* 2014 and references therein). In dioecious plants, such small and fragmented populations may face an increased extinction risk, where demographic stochasticity results in deviations from the optimal sex ratio. Using this study system, we tested the following hypotheses: (i) genetic diversity is generally low and genetic differentiation is high in Central European populations of *A. dioica* due to effects of habitat fragmentation and decreasing population size; and (ii) genetic diversity shows a unimodal relationship with sex ratio, since both female- or male-biased sex ratios result in mate limitation.

MATERIAL AND METHODS

Study species

Antennaria dioica is a dioecious and diploid (2n = 2x = 28; Bayer 1984) chamaephyte. The species exhibits clonal growth through surface-creeping runners and occurs in dense patches that are often, but not inevitably, monoclonal (Rosche *et al.* 2014). Inflorescences are sexually dimorphic: female anthodia and involucral bracts are long and narrow, whereas male anthodia and involucral bracts are broad and short. Flowers are effectively pollinated by dipterans, coleopterans, lepidopterans and hymenopterans (Öster & Eriksson 2007). Wind-dispersed achenes are light (0.05 mg; Öster & Eriksson 2007) and build a transient seed bank (up to 3 years; Schütz 1989). To our knowledge, no information is available on how many years a distinct clone of *A. dioica* can live in natural habitats. However, in view of our first hypothesis, it is pertinent to note that increased longevity may substantially increase the resistance of species to the detrimental effects of fragmentation, such as demographic (Rosche *et al.* 2017) and environmental fluctuations (Rosche *et al.* 2018a).

The geographic distribution of *A. dioica* stretches over an extensive longitudinal gradient from Western Europe to Eastern Russia (Tutin *et al.* 1976). The species is mainly distributed in subalpine and mountainous zones, reaching altitudes of up to 3000 m a.s.l., but can be occasionally found in lowlands, *e.g.* in Central Europe. In the latter habitats, it occurs in a wide variety of semi-natural and natural sites, including dry calcareous or neutral-acidic grasslands, heathlands, and semi-open oak and pine woodlands, which have shallow and nutrient-poor soils (Ellenberg *et al.* 2001). Although such open habitats were widespread in Central Europe in recent history, their extent has declined considerably due to abandonment of traditional land use forms (Korneck *et al.* 1996).

Sampling scheme

For a previous study (Rosche *et al.* 2014), we sampled a total of 32 populations in central, northern and eastern Germany, as well as in the western Czech Republic, which covered a large proportion of all known stands in this region. In this study, we specifically focused on the clonal structure of the 32 *A. dioica* populations and found that 18 populations consisted of a very low number of genets (*i.e.* monoclonal up to five genets). Moreover, the majority of these very small populations had either exclusively male ($n = 4$) or exclusively female ($n = 9$) patches. For estimating genetic diversity and differentiation in the present study, we did not consider these 18 very small populations, because the small number of genets does not allow for any meaningful assessment of genetic diversity and among-

population genetic structure (Bonin *et al.* 2007). Here, we rather focus on the 14 largest populations, all of which contained both sexes and had at least nine genets (Table 1). These 14 populations were separated by at least 3 km (maximum 539 km). In each population, we collected fresh leaves from ten to 14 different *A. dioica* rosettes, which were equally distributed over the entire population (see Rosche *et al.* 2014 for further details). Leaf material was stored on silica gel until further use.

Population size was estimated through (1) counting the number of distinct patches (*i.e.* 'number of patches'), and (2) calculating the total area of patches in the entire population (*i.e.* 'cumulative patch size'). Patches were defined as a spatially coherent group of ramets separated by at least 20 cm from another group of ramets. We determined patch area by analysing photographs of individual patches based on a size standard in ArcMap 8.1 (ESRI, Redlands, CA, USA) and visually estimated the percentage coverage of *A. dioica* rosettes within each individual patch from the photographs. Subsequently, we calculated the size of each individual patch as patch area × percentage cover of *A. dioica* rosettes/100. Finally, we summed the sizes of all individual patches within each population to obtain an estimate for the cumulative patch size for the entire population. In populations that consisted of more than 20 patches, we only determined the patch size of 20 haphazardly chosen patches and multiplied the mean patch size with the total number of patches to infer the cumulative patch size of the considered population.

To estimate sex ratio, we counted the number of male, female and non-flowering patches in each population. Patches encompassing both sexes were scored for both genders. Based on the number of flowering patches, sex ratio was calculated as the proportion of females. We decided to use an estimate based on the number of patches rather than on the number of inflorescences per population because (1) most *A. dioica* patches are monoclonal (Rosche *et al.* 2014) and (2) their inflorescence numbers underlie strong annual fluctuations.

Analysis with AFLP

We applied the same DNA extraction and AFLP procedure as described in Stein *et al.* (2014), except for the selection of primers. For the selective amplification, ten different primer combinations were tested on 15 samples for their level of variability, resulting in four primer combinations chosen to fingerprint all samples: 5′-EcoRI+AAG*FAM-3′/5′-MseI+CTT-3′, 5′-EcoRI+AGC*HEX-3′/5′-MseI+CTT-3′, 5′-EcoRI+AAG*FAM-3′/5′-MseI+CAC-3′ and 5′-EcoRI+AGC*HEX-3′/5′-MseI+CAC-3′. Polymorphic DNA bands were scored as present (1) or absent (0) using the automatic peak scoring and selection function of the MegaBace Fragment Profiler software 1.2 (Amersham Biosciences, Freiburg, Germany) and corrected manually. Monomorphic peaks were deleted from the output table. Samples that did not produce electropherogram patterns of sufficient quality (*e.g.* smeared and weak bands) were omitted from further analyses. Out of all 135 samples that revealed clear electropherograms, 28 randomly chosen samples (21%) were run repeatedly under the conditions outlined above to identify non-reliable loci. In particular, out of 454 scored AFLP loci, 168 were polymorphic and showed reliable patterns when comparing among replicates. Our error rate of the 168 loci was 2.8%, which is in line with other AFLP studies (Hansen *et al.* 1999).

Table 1. Overview of studied *Antennaria dioica* populations. Geographic coordinates in latitude °N and longitude °E. No. patches: population size estimated as number of distinct patches, Cum. size (m^2): population size estimated as cumulative patch size in m^2, N: number of genets included in the analyses, $B_r(9)$: Band richness rarefied to the minimum sample size of nine genets per population, H_e: expected heterozygosity, PPB: proportion of polymorphic bands, ♀: number of female patches, ♂ number of male patches, ♂/♀: number of patches including both sexes, non-flowering: number of patches that did not flower.

Population	Locality	No. patches	Cum. size (m^2)	°E	°N	Sex ratio	♀	♂	♂/♀	Non-flowering	N	$B_r(9)$	H_e	PPB
BB1	Brandenburg, Mallnow	12	2.14	52°29′21	14°26′46	0.73	7	2	1	2	9	1.462	0.198	58.9
BB2	Brandenburg, Bühlow links	176	60.72	51°37′35	14°21′39	0.60	65	40	10	61	10	1.540	0.199	57.7
BB3	Brandenburg, Bühlow rechts	47	12.68	51°37′42	14°21′15	0.72	29	9	4	5	13	1.511	0.212	57.7
SA1	Saxony-Anhalt, Döblitz	48	10.60	51°33′4	11°50′36	0.74	27	9	1	11	10	1.525	0.212	61.3
SA2	Saxony-Anhalt, Lerchenhügel	8	2.06	51°32′53	11°51′57	0.71	4	1	1	1	10	1.546	0.217	60.7
SH1	Schleswig-Holstein, Löwenstedt	8	2.48	54°37′33	9°08′52	0.75	5	1	1	1	9	1.550	0.214	63.7
SH2	Schleswig-Holstein, Nordoer Heide	7	0.86	53°53′11	9°30′26	0.50	1	1	0	5	10	1.462	0.201	56.5
SY1	Saxony, Zöblitz	3	6.20	50°39′13	13°15′59	0.33	0	1	1	1	9	1.379	0.177	47.0
TH1	Thuringia, Rüdigsdorf	39	19.55	51°32′05	10°48′37	0.33	10	24	3	2	10	1.530	0.203	64.3
TH2	Thuringia, Pfaffenberg	30	18.08	51°31′30	10°50′57	0.67	18	8	2	2	10	1.579	0.218	67.3
TH3	Thuringia, Goldener Mann	36	5.08	51°25′22	11°04′21	0.57	12	9	0	15	9	1.509	0.193	57.7
TH4	Thuringia, Engerda	7	2.01	50°47′56	11°24′19	0.80	4	1	0	2	9	1.550	0.216	60.7
TH5	Thuringia, Martinsroda	64	34.36	50°47′47	11°29′29	0.53	22	18	20	4	11	1.480	0.181	59.5
UR1	Ústecký Region, Mědinêc	12	6.76	50°25′28	13°06′42	0.64	6	3	1	2	10	1.498	0.201	67.3

Clone identification

To identify clones, we conducted pair-wise comparisons of all samples using the AFLPdat package (Ehrich 2006) in R 3.2.3 (R Development Core Team 2015). To this end, the threshold of pair-wise band differences between two genetically differing individuals (*i.e.* two different genets) was set visually with the help of a histogram showing the pair-wise differences of bands (see Stein *et al.* 2014). In addition, we calculated the expected band difference as BD_{exp} = number of polymorphic loci × error ratio (see Douhovnikoff & Dodd 2003). Both approaches revealed a threshold of maximum five band differences between ramets of the same genet (*i.e.* clone). For further calculations in the present study, *i.e.* estimating genetic diversity and differentiation, we included only one sample per genet in our analyses.

Genetic diversity

The proportion of polymorphic bands (PPB) and Nei's expected heterozygosity (H_e) were calculated in AFLPsurv 1.0 (Vekemans *et al.* 2002) assuming Hardy-Weinberg equilibrium and applying a Bayesian method with non-uniform prior distributions of allele frequencies (Zhivotovsky 1999). Due to the dominant character of AFLPs, we further performed another band-based approach for estimating genetic diversity (Bonin *et al.* 2007): Band richness (B_r), *i.e.* the mean number of phenotypes expected per locus, as calculated with AFLPdiv 1.1 (Coart *et al.* 2005). We rarefied this estimate to a minimum sample size of nine individuals per population [$B_r(9)$], which allowed us to correct for different sample sizes (see also Al-Gharaibeh *et al.* 2016).

Genetic differentiation

To explore relationships among populations, we calculated pair-wise Nei's genetic distances in Arlequin 3.5.1.2 (Excoffier & Lischer 2010) and used them to generate a Neighbour-Net with the software SplitsTree 4.14.2 (Huson & Bryant 2006). Furthermore, we performed an analysis of molecular variance (AMOVA) in Arlequin to investigate the distribution of genetic variation within and among populations. We extracted Φ statistics (analogues of F statistics) and tested significance based on 999 permutations. In addition, we applied a Mantel test (Mantel 1967) using the vegan package 2.3-2 (Oksanen *et al.* 2015) to test for a correlation between genetic (pair-wise $F_{ST}/(1 - F_{ST})$ values) and geographic ($\log_e$-transformed) distances among populations.

Statistical analyses

To test for linear relationships between response variables describing genetic diversity (*i.e.* H_e, $B_r(9)$ and PBB) and the predictors sex ratio and population size, we fitted multiple linear regression models in R. Here, we fitted two different models for each response: one with cumulative patch size as a measure of population size, and one with number of patches as a measure of population size. To test for unimodal relationships between sex ratio and genetic diversity, we additionally fitted multiple linear regression models that included the genetic diversity estimates (H_e, $B_r(9)$ and PPB) as response variables and population size (number of patches; linear relationship) as well as both the linear and the quadratic terms for sex ratio as predictors. All multiple linear regression models were simplified by step-wise backward selection to obtain minimum adequate models. Here, we removed fixed effects terms with $P > 0.05$ based on F tests. Any transformation decisions were based on visual inspection in R (*i.e.* graphical assessment of normality of errors and homogeneity of variance; see Crawley 2014) and were as follows: cumulative patch size, number of patches, H_e, and $B_r(9)$ were $\log_e$-transformed, sex ratio was logit-transformed, and PPB remained untransformed. To test whether sex ratio is correlated with one of the two measures of population size, we applied Pearson correlation tests using the same transformations of variables as described above.

RESULTS

Genetic diversity and genetic differentiation

Genetic diversity within populations was moderately high: expected heterozygosity (H_e) varied between 0.18–0.22 (mean = 0.2; Table 1), percentage of polymorphic bands (PPB) between 47.0–67.3% (mean = 60.2%) and band richness [$B_r(9)$] between 1.38–1.58 (mean = 1.51). The Neighbor-Net network did not reveal any clear partitioning among the 14 populations (Fig. 1). A Bayesian STRUCTURE analysis and a principal coordinates analysis also showed no obvious genetic structure among the populations (data not shown). Accordingly, the AMOVA revealed that the molecular variance was mainly within (89%) and not among (11%) populations. As such, the overall Φ_{ST} value of 0.11 demonstrated moderate genetic differentiation. The pair-wise F_{ST} values varied between 0.008 (89 km; populations SA2 and TH4) and 0.143 (220 km; populations BB1 and SY1). We found a weak but significant correlation between genetic and geographic distance among populations (Mantel statistics: $r_M = 0.25$, $P < 0.05$; Fig. 2).

Population size and sex ratio

The number of patches varied between three and 176, where cumulative patch size varied between 0.9 and 60.7 m² (Table 1). Both variables were highly positively correlated ($r = 0.81$, $P < 0.001$). The percentage of flowering patches within each population varied between 29% and 100% (mean 77%). Females were dominant in 11 out of the 14 populations. Sex ratio varied between 32.5% and 80.0% females, with a mean of 61.5%. There was no correlation between sex ratio and population size, neither for the number of patches and sex ratio ($r = -0.01$, $P > 0.05$) nor for cumulative patch size and sex ratio ($r = -0.30$, $P > 0.05$).

Influence of population size on genetic diversity

There was no linear relationship between H_e and number of patches or cumulative patch size ($F_{2,11} = 0.01$, $P > 0.05$ and $F_{2,11} = 0.03$, $P > 0.05$), between PPB and number of patches or cumulative patch size ($F_{2,11} = 0.87$, $P > 0.05$ and $F_{1,12} = 0.78$, $P > 0.05$) and between $B_r(9)$ and number of patches or cumulative patch size ($F_{2,11} = 3.72$, $P > 0.05$ and $F_{2,11} = 2.36$, $P > 0.05$).

Influence of sex ratio on genetic diversity

The percentage of females was linearly positively related to H_e ($F_{1,12} = 11.36$, $P < 0.01$; Fig. 3a) and to $B_r(9)$ ($F_{1,12} = 5.41$,

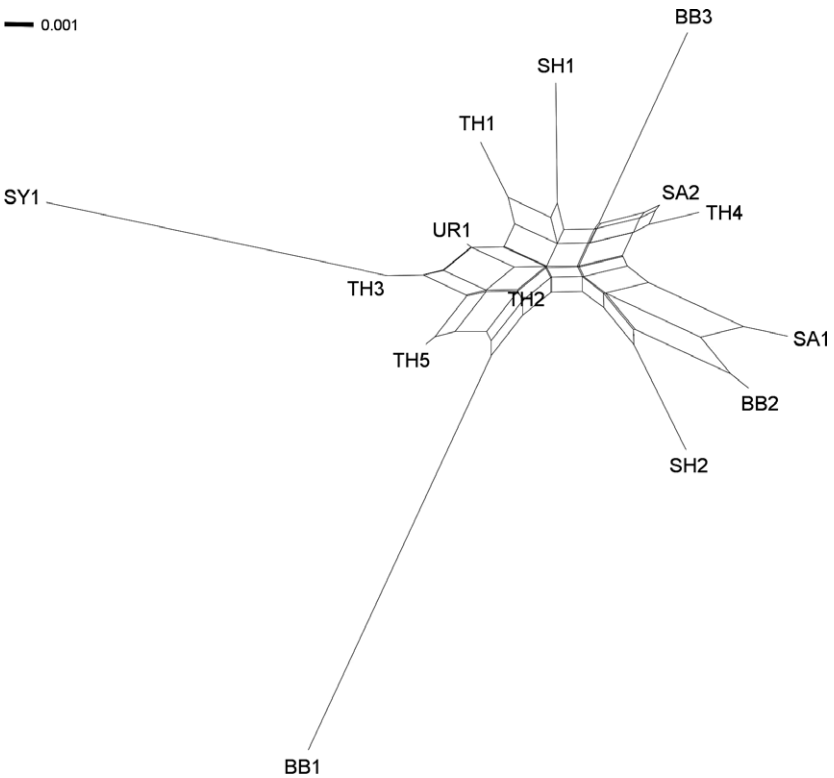

Fig. 1. Neighbour-net network based on Nei's pair-wise genetic distance among the 14 *Antennaria dioica* populations. Population abbreviations are given in Table 1.

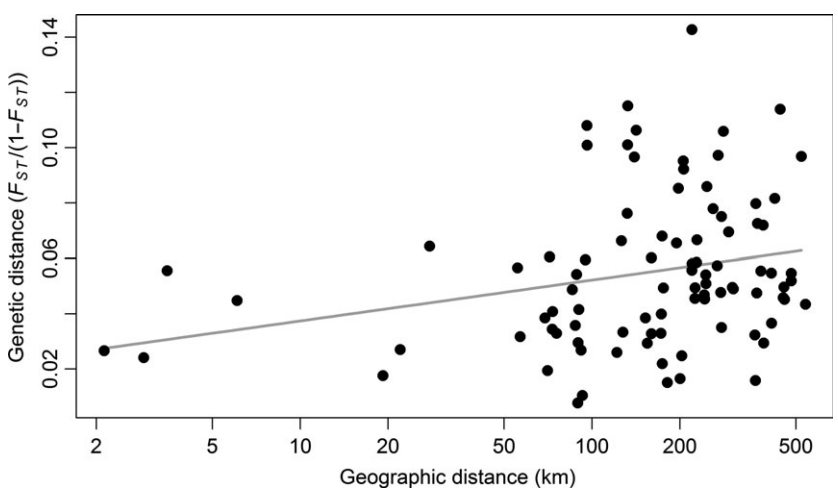

Fig. 2. Correlation between logarithmic geographic distances and genetic distances among the studied *Antennaria dioica* populations.

$P < 0.05$; Fig. 3b), but not to PPB ($F_{1,12} = 2.22$, $P > 0.05$). There was no unimodal relationship between sex ratio and any of the genetic diversity measures (H_e: $F_{2,11} = 0.65$, $P > 0.05$; $B_r(9)$: $F_{3,10} = 0.46$, $P > 0.05$; PPB: $F_{3,10} = 0.01$, $P > 0.05$).

DISCUSSION

High genetic diversity and low differentiation may result from population history

Despite overall small population size (mean: 35.5 patches), *A. dioica* showed surprisingly high within-population genetic diversity ($H_e = 0.18$–0.22, PPB = 47.0–67.3%), which was similar or even higher than reported from AFLP studies on other dioecious species, such as *S. otites* ($H_e = 0.16$–0.24, PPB = 45.6–66.0%; Lauterbach *et al.* 2012) and *M. perennis* (PPB = 0–41%; Vandepitte *et al.* 2009). In addition, genetic diversity exceeded values found in other obligate outcrossing species (mean of 21 AFLP-studies: $H_e = 0.19$, PPB = 50.4%; reviewed in Reisch & Bernhardt-Römermann 2014). Genetic differentiation was moderate ($\Phi_{ST} = 0.11$) and much lower than reported for the above two dioecious species, *M. perennis* ($\Phi_{ST} = 0.39$; Vandepitte *et al.* 2009) and *S. otites* ($\Phi_{ST} = 0.36$; Lauterbach *et al.* 2012), and the mean for other obligate outcrossers ($\Phi_{ST} = 0.2$; reviewed in Reisch & Bernhardt-Römermann 2014).

In general, the high genetic diversity and low differentiation are remarkable, given that *A. dioica* populations in the German

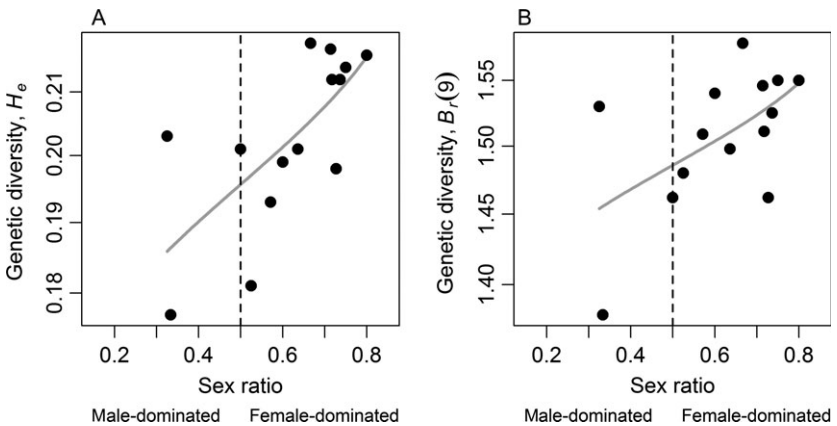

Fig. 3. Relationships between sex ratio and genetic diversity measured as (A) expected heterozygosity (H_e) and (B) band richness [$B_r(9)$]. Solid lines represent predictions of the minimum adequate linear models analysing 14 *Antennaria dioica* populations. Sex ratio reflects the proportion of female patches within populations. Dashed vertical lines refer to a sex ratio of 50% males and 50% females. Note that the majority of the populations are female-dominated.

lowlands are strongly declining and that the high level of fragmentation makes efficient gene flow among populations unlikely (Rosche *et al.* 2014). Therefore, our results may have arisen from larger population size and frequent gene flow in the past. Isolation occurred mainly throughout the last decades and was probably not long enough to result in substantial genetic erosion (*i.e.* very few generations since strong fragmentation; see also Rosche *et al.* 2018b). In fact, *A. dioica* is characterised by three attributes that are widely accepted to slow genetic erosion: pronounced perennial life span (Rosche *et al.* 2016), clonal propagation (Stein *et al.* 2014) and obligate outcrossing (Barrett *et al.* 2010).

Also, the weak but significant isolation-by-distance pattern and the lack of a clear genetic structure of *A. dioica* populations in the Central European lowlands suggest that populations could have formerly had a much more continuous distribution, or were functionally connected, *e.g.* by seed dispersal *via* domestic livestock (Rico *et al.* 2014). Remarkably, several studies on other outcrossing dry grassland species that are currently rare in our study area also revealed low genetic differentiation among populations (*e.g. Pulsatilla vulgaris*, Hensen *et al.* 2005; *Adonis vernalis*, Hirsch *et al.* 2015). Contrasting examples are *Globularia bisnagarica* (Φ_{ST} = 0.53; Honnay *et al.* 2006), *Muscari tenuiflorum* (Φ_{ST} = 0.21; Hornemann *et al.* 2012) and *Silene chlorantha* (Φ_{ST} = 0.36; Lauterbach *et al.* 2011), but these species were never reported as common in the dry Eurasian grasslands and have probably never formed large continuous populations.

In contrast to a large body of literature (see Leimu *et al.* 2006 for a review), genetic diversity was not related to population size in *A. dioica*. A positive relationship between population size and genetic diversity was also absent in other species that declined rapidly relative to their life span (*e.g.* Mona *et al.* 2014). Again, this supports that the studied *A. dioica* populations were larger in the past and have faced a sudden decline in size throughout the last decades. In accordance, Münzbergová *et al.* (2013) found that current allelic diversity in the long-lived grassland species *Succisa pratensis* is rather related to habitat connectivity in the past than to current population size. This implies that fragmentation-mediated genetic erosion is not generally absent in long-lived species but may occur with some delay. In addition, the lack of effects of population size on genetic diversity may also be related to the fact that our population size estimates do not inherently reflect effective population size, as single patches may consist of more than one individual (as an example see population SY1; Table 1). More specifically, in dioecious species, genetic diversity may not be solely related to classical estimates of population size but also to the frequency of females and males.

The proportion of females increases genetic diversity

Our study is among the first to reveal that the sex ratio in dioecious plants can significantly affect genetic diversity. Since sex ratio can have immediate consequences for generative reproduction, it seems to be more important for maintaining genetic diversity than population size in *A. dioica*. However, in contrast to our hypothesis, the proportion of females was not unimodally but positively linearly related to genetic diversity in *A. dioica*. The only previous study that found a significant relationship between sex ratio and genetic diversity also found that genetic diversity increases linearly with the proportion of females (*M. perennis*; Vandepitte *et al.* 2009). However, most of their populations were male-biased (mean 66.7% males in their study), and therefore an increasing number of females may simply represent more balanced sex ratios and the increase in number of mates (pollen receptors). For species with female-dominated sex ratios, such as *A. dioica*, an increasing number of females may be favoured due to increased seed production, which is especially important when reproductive success is strongly controlled by low seedling recruitment rather than pollen limitation. Indeed, Eriksson (1997) found very few established seedlings of *A. dioica* in the field, while Varga & Kytöviita (2011) suggested that *A. dioica* rarely suffers from pollen limitation, most likely due to effective pollinators (but see Öster & Eriksson 2007). In accordance with that study, we found that females in all of our 14 populations generated viable seeds, whereas established seedlings were largely absent (K. Schrieber unpublished data). Note that in extremely small populations, the probability of strongly unbalanced sex ratios is much more pronounced due to increasing demographic stochasticity (see also Vandepitte *et al.* 2009). Indeed, Rosche *et al.* (2014) found that 13 out of 32 *A. dioica* populations comprised only one sex (females: nine, males: four) and were consequently not able to generate seeds. However, as long as a few males are present, an increasing number of females does not necessarily result in pollen limitation, but may rather increase the success of generative reproduction.

Our 14 study populations of *A. dioica* were mostly female-biased (61.5% females in this study; 56.0% females in Eriksson 1997; 66;.0% females in Varga & Kytöviita 2011). This finding

contrasts with the observation that male-biased sex ratios are twice as frequent as female-biased sex ratios in dioecious plant species, which is usually explained by the increased need for resources in females to produce ovaries, seeds and fruits (Barrett *et al.* 2010; Munné-Bosch 2015). Yet, in *A. dioica*, there is no difference in adult mortality between the sexes and no spatial sex segregation (Varga & Kytöviita 2011). As such, early-acting genetic factors, or differing germination and seedling mortality may be responsible for genetically controlled female bias in natural populations (see also Che-Castaldo *et al.* 2015). This may indicate selection for female bias in *A. dioica*, which could result from its favourable effects on genetic diversity.

CONCLUSIONS

The unexpectedly high genetic diversity and low differentiation suggest that populations of *A. dioica* were much larger and formed a more continuous distribution in the past. While the effects of population size on genetic diversity may occur with some delay, our results revealed that sex ratio has more immediate consequences on genetic erosion. Specifically, a larger proportion of females could be crucial for preserving within-population genetic diversity of *A. dioica*, which has important implications for its conservation management: considering the low seedling recruitment in this species, populations could benefit from a female bias that can ensure sufficient seed output to counteract the random loss of genets and negative population growth. In the currently very small populations, the probability of unfavourable sex ratios is high and could negatively affect reproductive success.

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