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# Fine-scale spatial genetic structure suggests modest risk of inbreeding in natural populations of *Argiope bruennichi*

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## ABSTRACT

**Background:** One may expect inbreeding avoidance via mate choice to evolve if two circumstances arise: the risk of inbreeding is high but avoidable, and there is sufficiently severe inbreeding depression.

**Organism:** The European wasp spider (*Argiope bruennichi*) in which males are monogynous or conditionally bigynous and polyandrous females cannibalize up to 80% of their mates following copulation.

**Goal:** Assess the potential for inbreeding and inbreeding avoidance by determining the fine-scale spatial genetic structure of natural spider populations. Investigate pre-copulatory inbreeding avoidance mechanisms.

**Methods:** Search for patterns of fine-scale spatial genetic structure across three populations using 16 polymorphic microsatellite loci before and after mate search. Sample twice. In the first, estimate genetic distances of sub-adult males and juvenile females using spatial autocorrelation analysis. In the second, determine genetic distances of guarding males and guarded females. In addition, monitor mate acceptance and rejection in the field, genetically screen the mating partners, and assess the genetic distance between the male and female.

**Results:** We found no fine-scale genetic substructure and no evidence for clusters of related juveniles. The probability of encountering genetically similar mates following male mate search was around 10% on average and differed between populations. Furthermore, the data revealed no correlation between genetic similarity and male rejection against virgin females.

*Keywords*: dispersal, genetic population structure, inbreeding depression, mating strategy, microsatellite, polyandry.

## INTRODUCTION

Mate choice will evolve if the benefits of choice outweigh the costs (Andersson, 1994), which is especially likely if there is large variation in the quality of potential mating partners. In non-resource-based mating systems, mate quality is generally associated with either the

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additive effects of good genes or non-additive effects of compatibility (Jennions and Petrie, 2000; Neff and Pitcher, 2005; Simmons, 2005).

Non-additive benefits ensure that combinations of parental genes are beneficial for the offspring, such as optimizing immune defence (Penn and Potts, 1999; Penn, 2002; Milinski, 2006; Schwensow *et al.*, 2008) or avoiding inbreeding depression (Tregenza and Wedell, 2000; Zeh and Zeh, 2003). For instance, if inbred individuals suffer a marked reduction in fitness through the loss of heterozygosity and the increased impact of recessive deleterious alleles (Charlesworth and Charlesworth, 1987; Keller and Waller, 2002; Charlesworth and Willis, 2009), and if these costs do not outweigh kin-selected benefits (but see Kokko and Ots, 2006), the evolution of inbreeding avoidance mechanisms makes adaptive sense. However, selection on inbreeding avoidance adaptations can differ in strength between natural populations (Barrett and Charlesworth, 1991; Crnokrak and Barrett, 2002; Jennions *et al.*, 2004).

Clearly, selection on inbreeding avoidance might be relaxed in populations with a low probability of meeting a genetically different mating partner, and equally in populations where females rarely encounter male siblings. Conversely, inbreeding avoidance should be favoured in populations with a certain risk of experiencing inbreeding but also a high enough chance of encountering compatible partners. This will depend on factors such as the relationship between immigration and emigration and density. Furthermore, depending on a number of possible trade-offs, selection might favour inbreeding avoidance before or after copulation.

It is thus relevant to consider encounter rates of mating partners *per se*, which affect mating rates of both sexes and interact with the costs and benefits of mating (Bleu *et al.*, 2012; Kokko and Mappes, 2013). For example, a high risk of remaining unmated may select against pre-copulatory rejection of related mating partners despite high costs of inbreeding. High uncertainty of mate encounters may then favour alternative strategies such as multiple mating and trading up from poor-quality mates (Kempenaers *et al.*, 1992; Bateman *et al.*, 2001; Pitcher *et al.*, 2003; Laloi *et al.*, 2011). This mechanism is usually associated with females that secure fertilization of their eggs by initially accepting every male but then selectively forage for additional mating partners of higher quality. The degree to which post-copulatory mechanisms reduce the negative effects of inbreeding depends on the interplay of polyandry and paternity bias mechanisms towards unrelated mates. Females that mate multiply promote the availability of sperm from multiple males at the time of fertilization, which enables them to cryptically favour sperm of preferred high-quality males post-copulation (Eberhard, 1996).

Several studies have investigated whether animals mate multiply to avoid inbreeding (Birkhead and Moller, 1995; Olsson *et al.*, 1996; Zeh *et al.*, 1998; Kraaijeveld-Smit *et al.*, 2002; Mack *et al.*, 2002; Tregenza and Wedell, 2002; Garner and Schmidt, 2003; Bretman *et al.*, 2004, 2009; Simmons *et al.*, 2006; Bilde *et al.*, 2007; Firman and Simmons, 2008; Tuni *et al.*, 2013). All these studies used species in which males have a higher potential maximal mating rate than females. In such mating systems, the costs of indiscriminate mating are lower for males than for females if mating with a sister does not have a negative impact on reproductive success with other females. Females who provide the larger parental investment suffer the full fitness costs through inbreeding (Parker, 1979; Smith, 1979) and are hence under stronger selection to avoid these costs. This is fundamentally different in monogynous and/or bigynous mating systems in which males have similar or even lower maximal mating rates than females, so that both sexes share an interest in minimizing the costs of incompatibility (Fromhage *et al.*, 2005; Fromhage and Schneider, 2012). Under such conditions, selection should favour means to decrease the probability that genetically related individuals meet each other, such as through sex-specific dispersal (Schiegg *et al.*, 2006; Szulkin and Sheldon, 2008). Such mechanisms are, however, well known for vertebrates but far less so for invertebrates. Perhaps as a consequence, it is among invertebrates that post-copulatory avoidance or reduction of inbreeding costs has often been observed. Surprisingly, only a handful of studies have measured the individual risk of inbreeding in natural populations of invertebrates (Trontti *et al.*, 2005; Hardy *et al.*, 2008; Bretman *et al.*, 2011).

Here we attempt to assess the individual risk of inbreeding in natural populations of the wasp spider *Argiope bruennichi* (Scopoli, 1772). This species has a monogynous mating system with a conditional option of a bigynous strategy for males (Fromhage and Schneider, 2012; Welke *et al.*, 2012), while females mate multiply, although rarely more than twice (Zimmer *et al.*, 2012). Females are not choosy about their first mate, but if they encounter a second male that is not related, they will re-mate and then may store relatively more of this second male's sperm (Schneider and Lesmono, 2009; Welke and Schneider, 2009). Females possess paired genital openings that lead to two separate sperm-storage organs (spermathecae) (Eberhard, 2004; Foelix, 2011; Herberstein *et al.*, 2011). Each spermatheca can be filled independently with sperm from a different male during separate copulation bouts, which facilitates cryptic female choice for genetically compatible sperm and a paternity bias towards non-siblings. However, for a selection pressure on inbreeding avoidance to be relevant, the potential for inbreeding and the opportunity to avoid it must be present.

*Argiope bruennichi* males invest maximally in securing full paternity with a single female (monogynous strategy) or they divide their investment between two females (bigynous mating strategy). Males always die after two copulations but may be killed by the female after their first copulation (Schneider *et al.*, 2006). Due to the high investment in mating, males are choosy and more likely to be monogynous with more fecund (fat and old) virgin females (Schulte *et al.*, 2010; Welke *et al.*, 2012). Furthermore, males have been observed to reject virgin females for reasons other than size and age (Schulte *et al.*, 2010; Zimmer *et al.*, 2012). In a comprehensive field study, male mate rejection could not be related to any measure of female phenotype (Schulte *et al.*, 2010). These observations led to the prediction that males reject virgin females on the basis of their genetic compatibility. If a male is experimentally provided with a sibling as his only mate, he will actively maximize his chances to escape sexual cannibalism in order to continue searching for another mate for his terminal investment (bigynous strategy) (Welke and Schneider, 2010). These laboratory findings suggest that males can distinguish between related and unrelated females and make adaptive decisions. However, do these findings also apply in the field, and are the above adaptations a result of the risk of inbreeding in nature? If both sexes of these spiders use the option of mating with one or two partners as a trading-up mechanism under natural conditions, we would expect no pre-mating rejection of related females. In light of the costs of inbreeding, we predict only a modest risk of encountering a related mating partner.

Alternatively, field conditions might differ from laboratory conditions and males might reject related females as mating partners, but whether or not they apply pre-copulatory mate choice may depend on the local situation, such as the availability of females.

In order to quantify the probability of individual inbreeding and hence the potential of post-copulatory inbreeding avoidance, we sampled *A. bruennichi* of both sexes from transects during two different developmental stages, namely before and after males left their webs and searched for females. We replicated the samplings in three geographically separated populations. Furthermore, we conducted a field experiment in another population of *A. bruennichi* to investigate the existence of male pre-copulatory inbreeding avoidance mechanisms.

We expected to find a population structure characterized by the risk of encountering genetically similar mates during a mating season (e.g. through patches of related spiders) but one that also provides the possibility to locate more compatible mating partners within reach of an individual. These prospects might vary among populations, promoting flexible strategies that accommodate the trade-off between choosiness and reduced mating success. Furthermore, the risk of individual inbreeding and consequently the potential for inbreeding avoidance might vary temporally depending on male mobility. Males mature earlier than females and leave their webs after maturation to actively search for females. At that time, they accrue in the vicinity of sub-adult females (Zimmer *et al.*, 2012). Males can then decide to remain close to their natal website and guard the nearest female as shown for the spider *Stegodyphus lineatus* (Bilde *et al.*, 2005), or conduct a risky mate search to find females further afield.

We investigated the presence of sibling clustering through spatially structured sampling of immature spiders from three *A. bruennichi* populations. We determined their genetic similarity using established microsatellite markers known to detect siblings. We tested whether neighbouring individuals were genetically more similar than distant individuals. Furthermore, by collecting and genotyping sub-adult females and the adult males in their immediate vicinity within the same three populations, we assessed the probability that mature males guard genetically similar sub-adult females. Finally, to test whether males avoid inbreeding pre-copulation, we closely observed virgin females in a natural population at the beginning of the mating season. We noted male and female behaviour prior to mating and collected the visiting males after they had decided whether to leave the female or to mate with her. Visiting males and the corresponding females were genetically screened and compared with one another.

## MATERIALS AND METHODS

### Study species

The orb-web spider *Argiope bruennichi*, which spread from Southern Europe and Asia to Northern Europe around 1930 (Guttmann, 1979; Kumschick *et al.*, 2011), has colonized sunny, open meadows with long grass and low vegetation (Bellmann, 2006). They build large orb-webs, often a little above the ground and decorated with a stabilimentum (Bruce, 2006). The mating season begins in mid-July and lasts only 3–4 weeks. Males and females of the species mature in July, with most males maturing a few days earlier than the females. Females start to produce their egg sacs a month after maturation. The spiderlings hatch out of the eggs after about 4 weeks but overwinter inside the egg sacs. They emerge in spring and initially use a communal hunting web. After one or two moults, they disperse by ballooning (long distance) or bridging (short and flexible distance) to build their own webs at suitable sites and grow to maturity by July. Males build webs until they mature and upon maturation they can be found roving the meadows in search of potential mates. Females, in contrast, are relatively sedentary. Occasional changes of web location generally occur within a radius of ~1 m (Zimmer *et al.*, 2012).

Population genetic studies on *A. bruennichi* show a high genetic diversity even in recently colonized habitats (Krehenwinkel and Tautz, 2013) and in meadows that have been populated for less than two generations (Zimmer *et al.*, 2014). These patterns suggest that this species colonizes new habitats with individuals from several origins (Krehenwinkel and Tautz, 2013).

*Argiope bruennichi* exhibits a strong sexual size dimorphism with males much smaller than females. They are sexually cannibalistic spiders and females cannibalize up to 80% of their mates during mating (Schneider *et al.*, 2006). Females show highly stereotypic attack behaviour soon after genital contact. Males can influence their chance of surviving their first copulation by timing when copulation terminates. Long copulations invariably end in male death (Schneider, 2014).

Male *A. bruennichi* have two secondary copulatory organs, the pedipalps, which they use to transfer sperm (Foelix, 2011). They damage their pedipalps during copulation and the pieces that break off remain in the female's genital opening, acting as a mating plug to prevent the female from mating again (Nessler *et al.*, 2007). However, males can use only one of their pedipalps at a time, thus the two sperm storage organs of females can only be filled through two separate copulation bouts. Since males damage their pedipalps during copulation, they are limited to a maximum of two copulations in total.

## Sampling

Sampling was used to quantify the spatial genetic population structure at a spatial scale of walking distance of male spiders. We collected individuals from three geographically separated populations in Northern Germany: Hamburg-Billwerder (HH-Billwerder, 53°52′53.79″N, 10°10′95.91″E), Pevestorf (53°06′46.77″N, 11°41′20.58″E), and Tostedt (53°28′97.09″N, 9°73′51.68″E). All three sites constitute typical habitat occupied by *A. bruennichi*, dry grasslands with patchy vegetation. Each of the sites is isolated, with a road on one side and a forest or industrial area on the other. High densities of grasshoppers were available as prey in all three meadows.

We conducted sampling on two occasions. The first was conducted on three consecutive days at the end of June 2013, one day for each population. At this time of the year, shortly before the start of the reproductive season, both sexes are yet to mature and sub-adult females and sub-adult males sit in their own webs. For this sampling, we used ropes to mark a longitudinal transect 1.5 m by at least 24 m, allowing us to capture a representative sample of the population. We established a grid by connecting the two ropes that formed the long sides of each transect with ropes every 2 m along their length. We sampled all individuals located within each transect. If the transect did not contain at last 50 spiders, it was extended beyond 24 m until a sample of at least 50 spiders was achieved. We collected 66 individuals within a transect 24 m long from the meadow in HH-Billwerder, 59 individuals within a transect 24 m long from the meadow in Pevestorf (Lower Saxony), and 53 individuals within a transect 26 m long from the meadow in Tostedt (Lower Saxony). We GPS-mapped each web within each transect individually in each population, and also noted the web location on a map based on the rope-grid. On the basis of GPS-mapping, we mapped and visualized distributions of individuals and computed the distances using Geographic Distance Matrix Generator (Ersts, 2013). Subsequently, all collected individuals were genetically screened (see below).

The same three populations were revisited at the beginning of July 2013 when the protandrous males had matured but most females were still sub-adults. At that time, adult virgin males had left their own web and had begun searching for potential mating partners and besieging the webs of sub-adult females. This time, collections were conducted in sections of the meadows outside the previously used transects and thus were unaffected by the first sampling. We had marked the boundaries of transects with bamboo poles so that each previously sampled area was clearly identifiable. During the second sampling, we collected 20–21 sub-adult females with their male visitors from each of the three populations. Any difference in genetic distances between males and females of the first and second samplings would provide information about how male mate search might affect the probability of sibling encounters.

## Microsatellite analysis

To determine the genetic similarity between pairs of individuals within the three sampling populations of *A. bruennichi* and the relatedness between potential mating pairs of the fourth population, we used microsatellite markers. Genetic divergence between individuals ('genetic distance') was measured as the individual proportion of shared alleles (POSA). The genetic distance estimate ranges from 0 to 1. Smaller genetic distances (close to 0) indicate that individuals are genetically similar, whereas larger genetic distances (close to 1) reveal less similarity between individuals. Previous experiments revealed that siblings (hatched from the same egg sac) could be confidently detected with the markers and had a genetic distance of ≈0.3, while randomly sampled pairs of spiders from the same population had values of ≈0.5 or larger (Zimmer *et al.*, 2014). Note that our intention was to identify sibling clusters that likely occur as a consequence of synchronous hatching if at least a proportion of hatchlings remain in the vicinity of their birthplace. Our goal was not to draw conclusions about the overall genetic substructure of populations.

For microsatellite analyses, we extracted DNA with the 5 PRIME ArchivePure DNA kit according to the manufacturer's protocol (5 PRIME, Hamburg, Germany). Our study species was genotyped for a set of 16 previously developed microsatellite loci for *A. bruennichi* (Krehenwinkel and Tautz, 2013). PCR amplification was undertaken using the Qiagen Multiplex PCR Kit Protocol (Qiagen, Hilden, Germany). We used ABI ROX as size standard. Genotyping was performed on an Applied Biosystems 3730 DNA Analyzer. Subsequently, microsatellite alleles were called using GeneMapper v.4.0 (Applied Biosystems) and genetic distances between individuals, as well as pair-wise $F_{ST}$-values among populations, were calculated using Microsatellite Analyser (MSA) v.4.05 (Dieringer and Schlotterer, 2003). The TreeMaker program (provided by S. Piry) was used to construct a neighbour-joining tree based on the genetic distance matrix. The tree was further edited with FigTree v.1.4.2 (Rambaut, 2014). Tests for Hardy-Weinberg equilibrium were performed using Markov chain methods in Arlequin v.3.5.1.3 (Excoffier and Lischer, 2010); it was revealed that some microsatellite loci deviated from Hardy-Weinberg equilibrium. Using the software Microchecker v.2.2.3 (Van Oosterhout *et al.*, 2004), we tested each locus and confirmed the presence of null alleles (when one or more alleles fail to amplify during PCR) in some loci. This corroborates the notion that null alleles are relatively widespread in spiders (Rutten *et al.*, 2001; Bilde *et al.*, 2009), including *A. bruennichi* (Zimmer *et al.*, 2014). However, analyses using the same microsatellite loci with null alleles were redone using a 1200 bp fragment of the mitochondrial COI gene and showed no difference in the results of a previous study (Zimmer *et al.*, 2014). This indicates that the present microsatellite loci are suitable genetic markers despite the occurrence of null alleles.

### Spatial autocorrelation analysis

The occurrence of genetic population structure on a small spatial scale was investigated using spatial autocorrelation analysis. This method tests whether genetic similarity between all possible pairs of individuals at one sample site depends on the geographic distance between them. If so, a pattern of isolation by distance exists that assumes a higher genetic similarity between nearby individuals (Barbujani, 2000). Calculations were conducted using Spatial Genetic Software v.1.0c (SGS) (Degen *et al.*, 2001) by considering the mean number of alleles in common (NSC), over all 16 previously developed microsatellite loci, between pairs of samples in a given spatial distance class. We assessed the best performance of distance classes by repeating the spatial autocorrelation analysis using different classes and comparing the results. The results remained the same regardless of the classes used, but were best illustrated with five distance classes: 0–5, 5–10, 10–15, 15–20, and 20–25 m. Significant deviation of all values from a spatially random distribution of alleles was tested using a Monte Carlo permutation applied in SGS. The mean number of alleles in common over all pairs of individuals constitutes a reference value. Isolation by distance exists when individuals at short distances are significantly more similar than the reference value, whereas individuals at greater distances show insignificant values (Barbujani, 2000).

### Pre-copulatory inbreeding avoidance by males

We conducted this field experiment in a meadow in the landscape conservation area of Buxtehude (53°45′39.76″N, 9°67′43.68″E) in Lower Saxony (permission was provided verbally by the owner) from 12 to 26 July 2013. The study site is dry grassland with patchy vegetation including a few low bushes surrounded by a forest and a small forest track. Shortly before the mating season, we marked the location of around 150 sub-adult females in the population with bamboo poles. Each day we monitored the developmental status of each sub-adult female to forecast her date of final moult due to the swelling of her external genital structure (epigyne) (see Zimmer *et al.*, 2012). In addition, we noted the number of males staying close to sub-adult females' webs or in their web each day. As soon as females moulted to maturity, we gave them an individual number and observed them continuously until the first male visited them. We monitored if he rejected the female or mated with her, and noted any other behavioural interactions. Rejection was defined as follows: the male touched the web or the female and then left the web and walked away without any attempt to copulate. Thereafter, we collected the male and the female and froze them at −80°C to determine their relatedness through microsatellite typing (see above). We assessed the genetic distance of 40 mating pairs (15 rejected, 25 accepted pairs). To analyse whether male decisions depend on genetic relatedness, we used a logistic regression conducted in JMP v.7.0.

## RESULTS

### Spatial distance and relatedness

Spatial autocorrelation analysis of genetic structure in the three *A. bruennichi* populations comprising juvenile and sub-adult individuals (first sampling) showed no dependency between individual genetic distances (number of alleles in common, NSC) and spatial distances (Table 1) of individuals. Individuals that were close together did not share more

**Table 1.** Spatial autocorrelation analysis of fine-scale genetic structure in three geographically separated *A. bruennichi* populations (Pevestorf, HH-Billwerder, and Tostedt)

| Population | Mean NSC over all pairs | Distance class limits (m) | | | | |
|---|---|---|---|---|---|---|
| | | 0–5 | 5–10 | 10–15 | 15–20 | 20–25 |
| Pevestorf | 0.8690 | 0.8773$^{\text{N.S.}}$ | 0.8734$^{\text{N.S.}}$ | 0.8375$^{\text{N.S.}}$ | 0.8077$^{\text{N.S.}}$ | |
| HH-Billwerder | 0.7890 | 0.7973$^{\text{N.S.}}$ | 0.8089$^{+*}$ | 0.7825$^{\text{N.S.}}$ | 0.7524$^{\text{N.S.}}$ | 0.7038$^{-*}$ |
| Tostedt | 0.7420 | 0.7384$^{\text{N.S.}}$ | 0.7378$^{\text{N.S.}}$ | 0.7360$^{\text{N.S.}}$ | 0.7536$^{\text{N.S.}}$ | 0.7733$^{\text{N.S.}}$ |

*Note*: Values for number of alleles in common (NSC) indicate a mean differentiation of samples within each distance class. – , number of alleles in common significantly lower; + , number of alleles in common significantly greater than a mean differentiation over all distance classes. $*P < 0.05$; N.S. = not significant.

**Table 2.** Pair-wise $F_{\text{ST}}$-values (below diagonal) and $P$-values (determined by permutation; above diagonal), together with averaged proportion of shared alleles (POSA) for three geographically separated *A. bruennichi* populations (Pevestorf, HH-Billwerder, and Tostedt)

| | Pevestorf | HH-Billwerder | Tostedt | POSA |
|---|---|---|---|---|
| Pevestorf | | 0.0001 | 0.0001 | 0.53 ± 0.002 |
| HH-Billwerder | 0.039671 | | 0.0081 | 0.57 ± 0.002 |
| Tostedt | 0.039662 | 0.007386 | | 0.59 ± 0.003 |

alleles than those that were far apart. Our data provide no evidence for clustering of siblings, either before or after mate search. The three populations were not inbred, but genetically different from each other (see Table 2). A neighbour-joining tree (Fig. 1) shows that all sites were genetically mixed. Bootstrap values that connect more than two individuals are mostly 0 with only four values higher than 10 (median = 0, interquartiles = 0.4), supporting the absence of any small-scale spatial substructure.

During the second sampling, when adult virgin males were accumulating in the vicinity of sub-adult females that were close to their final moult, the mean genetic distance between females and visiting males was 0.54 ± 0.03 ($N = 23$) for the Pevestorf population, 0.56 ± 0.03 ($N = 20$) for the HH-Billwerder population, and 0.58 ± 0.02 ($N = 22$) for the Tostedt population. Hence, the majority of males visited females that were genetically different. The probability of arriving at the web of a genetically similar female (defined as a genetic distance less than 0.36) varied between 4.55% and 17.39%, suggesting that there is a slight probability that males visit females that are as genetically similar as siblings, leading to the potential for inbreeding (Table 3).

## Pre-copulatory inbreeding avoidance

Overall, we marked and observed 107 females that moulted to maturity during the field experiments. Of these 107 females, 28 (26.17%) died, disappeared or had no visitors during the observation period and were not included in the data set. Sixty-two females (57.94%) had at least one male visitor who mated with them. Of these 62 females, 35 (56.45%) had at

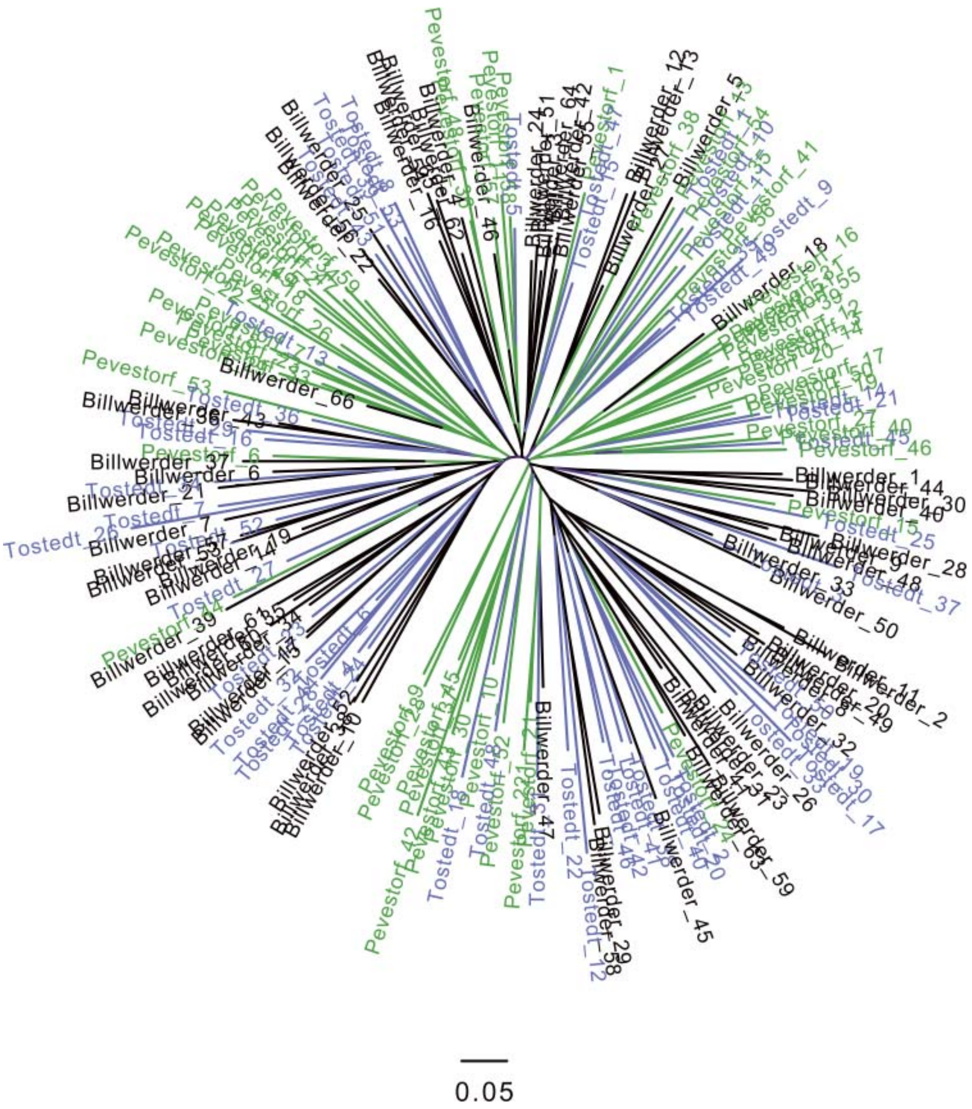

**Fig. 1.** Neighbour-joining tree generated from POSA (proportion of shared alleles) distance matrix. Data are derived from three geographically separated populations of *A. bruennichi* individuals: Pevestorf (green branches in the online version), HH-Billwerder (black branches in the online version), and Tostedt (blue branches in the online version).

least one male on her web during her final moult, resulting in an opportunistic mating, meaning the male copulated with the defenceless female during her final moult (Foellmer and Fairbairn, 2003; Uhl *et al.*, 2015). It took males on average 26.49 ± 7.28 min ($N = 33$) from first contact to mate with the virgin female.

Seventeen of the 107 females (15.89%) were rejected by at least one male visitor. Three of these 17 males (17.65%) rejected a female after touching her web; the other 14 (82.35%) rejected the female after walking to the centre of the hub and touching her body.

**Table 3.** Summary of results for first and second sampling from three *A. bruennichi* populations (Pevestorf, HH-Billwerder, and Tostedt)

| Population | *N* (pairs of males and nearest female at sampling 1) | Possibility that nearest female is genetically similar at sampling 1 | *N* (pairs at sampling 2) | Possibility to have a genetically similar male mate in the vicinity/web |
|---|---|---|---|---|
| Pevestorf | 24 | 12.5% | 23 | 17.39% |
| HH-Billwerder | 26 | 7.69% | 20 | 5% |
| Tostedt | 26 | 0% | 22 | 4.55% |

On average, males rejected virgin females 17.42 ± 10.06 min ($N = 12$) after web contact. We excluded four males as outliers from the data due to the long time it took them to make a decision (373 ± 31.37 min).

Contrary to expectations, males did not reject virgin females on the basis of their relatedness. Males that rejected virgin females were no more genetically similar to the females than males that accepted virgin females and mated with them (logistic regression: $\chi^2_1 = 0.006$, $P = 0.94$, $N = 40$). The average genetic distance of rejected pairings was 0.61 ± 0.02 ($N = 15$) and 0.61 ± 0.01 ($N = 25$) for accepted pairings.

## DISCUSSION

Spatial autocorrelation revealed no evidence for a fine-scale genetic substructure in three replicated *A. bruennichi* populations: neighbouring spiders were no more genetically alike than on average, so there were no isolation-by-distance patterns. Nevertheless, we were able to identify a modest individual inbreeding risk within populations. The webs of genetically similar individuals (with a genetic distance of less than 0.36) were occasionally in direct proximity to one another. If these individuals were not of the same sex, they could encounter one another during mate choice. However, the probability of encountering genetically similar mates did not explain pre-copulatory mate rejection. When we genotyped pairs upon encountering one another in the field, we found that females rejected by males were no more genetically similar than the population average.

Being typical web-building spiders, *A. bruennichi* spiderlings disperse by ballooning, with the attendant high risk of ending up in unsuitable habitat. However, there is no consensus as to whether ballooning is obligate after hatching out of the egg sac (Follner and Klarenberg, 1995) or not (Walter *et al.*, 2005). Intuitively, some hatchlings should be short-distance dispersers that remain at their natal site of known quality (Walter *et al.*, 2005). Since spiderlings hatch out of the egg sac simultaneously (which in *A. bruennichi* is several hundred individuals), short-distance dispersers would create a population substructure with patches of closely related individuals. This is found in the sub-social spiders *Stegodyphus lineatus* (Bilde *et al.*, 2005) and *S. tentoriicola* (Ruch *et al.*, 2009), in which newly established nests are found accumulated around maternal sites, thus promoting inbreeding.

Population genetic studies on *A. bruennichi* suggest that a relatively large number of individuals from several origins colonize new habitats (Krehenwinkel and Tautz, 2013), and that diversity is high even in recently founded populations (Zimmer *et al.*, 2014). Population genetic estimates do not necessarily consider the probability that individuals could still mate with relatives due to a fine-scale substructure. We observed high genetic diversity but still identified a modest risk of inbreeding within natural populations of *A. bruennichi*, suggesting that early juvenile dispersal ensures sufficient genetic mixture even in recently colonized populations. These results lend further support to the conclusion that range expansion in *A. bruennichi* is not constrained by inbreeding (Zimmer *et al.*, 2014).

Settlement of juveniles in space may not be relevant for mating if sex-specific dispersal at a later date reduces the proximity of siblings. In the presence of a clustering of related individuals, male mate search may extend a sufficient distance so that copulation is with more distantly related females. Female web-building spiders are relatively sedentary, while males are mobile after maturation and actively search for receptive females (Foelix, 2011). Using our data set, we were able to determine whether the spatial arrangement of mating partners is different from that of juveniles. We did not find a clustering of relatives among juveniles, and subsequently no difference in the spatial arrangement of the mating pairs. Males leave their webs once mature and probably stay with the first female they encounter regardless of whether she is a sibling or not. The estimated probability that males encounter genetically similar females varied between our populations: it was around 5% in two populations (HH-Billwerder and Tostedt) but rose to 17% in Pevestorf. Interestingly, among the sites in Northern Germany that we investigated in this and in a previous study (eight populations in total), Pevestorf was the oldest population. Given the high costs of inbreeding, the observed inbreeding risk of 5–17% may be sufficiently high to select for mechanisms that reduce the costs of inbreeding while trade-offs prevent the evolution of mate rejection to avoid inbreeding. This interpretation is in line with results from a wild population of crickets in which there was also a fairly low risk of inbreeding (Bretman *et al.*, 2011) combined with a high probability of encountering another, more compatible mating partner. As another parallel to our system, these crickets also evolved post-copulatory inbreeding avoidance mechanisms to reduce the costs of inbreeding (Bretman *et al.*, 2004, 2009). Clearly, further field studies on different systems are required to determine whether the probability of inbreeding affecting one to two of 10 mating pairs could be sufficiently high to justify selection on inbreeding avoidance mechanisms and how high the costs of inbreeding need to be. In *A. bruennichi*, despite a modest risk the benefits are high, particularly as sib-mating significantly reduces hatching success (Zimmer *et al.*, 2014) while the effects on offspring fitness are small. A second generation of inbreeding, however, leads to a drastic reduction in fitness by lowering adult lifespan in both sexes and causing smaller size in males in the congener *A. australis* (Welke, 2012). It is adaptive that both males and females reduce the costs of an incompatible first mating by actively inviting re-mating. Laboratory studies of several *Argiope* species have shown that polyandry facilitates cryptic female choice (Elgar *et al.*, 2000; Schneider and Lesmono, 2009) and can reduce the costs of inbreeding for females (Welke and Schneider, 2009). Females may achieve this by controlling the number of sperm that they store from a second mate. Females of *A. lobata* have been shown to store similar numbers of sperm during first copulations with related or unrelated males, but sperm numbers in storage are significantly lower for second, related males (Welke and Schneider, 2009). Males of *A. bruennichi* reduce the costs of inbreeding by opting to mate with two different females (bigynous strategy) if mated to a sister first; males copulate very briefly with sisters and thereby lower the risk of sexual cannibalism (Welke and Schneider, 2010). Short copulations increase the chances for males to survive copulation and survivors search for a better second mating opportunity (Fromhage and Schneider, 2012). Monogynous males copulate once or twice with the same female and thereby maximize paternity success with this female (Fromhage *et al.*, 2005; Fromhage and Schneider, 2012). Hence, in *Argiope*, both sexes do not reject closely related sexual partners when virgin and are only choosy when further mating opportunities arise.

From the male perspective, pre-mating rejection of genetically similar females seems to be more parsimonious than copulating and opting for a bigynous strategy. Even brief copulations entail a risk of sexual cannibalism and males may be better off trying to find a compatible partner from the outset. One explanation for the absence of rejection of genetically similar females may be that some reproductive success, even with a reduced fitness, is better than no mating at all. A risk of remaining unmated is often used as an explanation for trading-up strategies (Bleu *et al.*, 2012). However, high densities and the genetic diversity in *A. bruennichi* do speak against this. Furthermore, mate rejection does occur in the field as reported here and in a previous field study (Schulte *et al.*, 2010), arguing against complete random mating. No phenotypic variables could be found to explain mate rejection (Schulte *et al.*, 2010), nor did we find relatedness to be responsible for mate rejection. This leaves us to speculate that rejections might be due to genetic incompatibilities that were not detected using microsatellites. Apart from in the laboratory, where we can control for genealogy, genetic comparisons only reveal genetic similarity that may or may not arise because individual spiderlings hatch out of the same egg sacs. Hence, it is possible that these spiders recognize siblings but not genetic similarity *per* se. An alternative explanation is that rejected females were unsuitable mates because they were infected with parasites (Foelix, 2011) or endosymbionts, which are quite common in spiders (Gunnarsson *et al.*, 2009; Goodacre, 2011), although unknown for *Argiope*. It is unclear how males collect information about female compatibility or parasite infection but chemical profiles are likely candidates. We know from previous studies that *Argiope* males can distinguish between females of different reproductive status using silk-based and airborne sex pheromones (Herberstein *et al.*, 2002; Gaskett *et al.*, 2004; Schulte *et al.*, 2010). Sex pheromones may indeed code more information than just mating status, as shown in other spiders and insects (Johansson and Jones, 2007). However, our current understanding of *Argiope* pheromones is limited. Nevertheless, both sexes accept a risk of inbreeding as virgins and probably opt for multiple mating to reduce the negative impacts of inbreeding. However, the costs of inbreeding are not high enough to select for pre-copulatory mate rejection of unmated individuals.

To our knowledge, this is the first detailed microsatellite-based study of fine-scale (<30 m) spatial genetic structure in an orb-web spider. Although we used highly polymorphic genetic markers and autocorrelation analysis, we failed to detect genetic substructure in *A. bruennichi* spiders. The reliability of our findings may be impaired by possible mistakes in measurements through an inappropriate sampling scale or small sample sizes. Obtaining accurate results depends on the sampling scheme and on the selected distance intervals. Sampling along transects is a good strategy, because such small to large scales are considered to represent adequate pairs of individuals that are easy to map within transects (Vekemans and Hardy, 2004). Sampling within a single transect in two dimensions might not represent precisely the distribution of individuals within populations and could miss or underestimate potential genetic structure (Vekemans and Hardy, 2004). As *A. bruennichi* spiders seem to disperse or walk without direction, using transects in multiple dimensions may be better than single scales to assess spatial genetic structure in this species. Furthermore, to estimate genetic structure, the sample sizes must be large enough to ensure sufficient numbers of pair-wise comparisons in each selected distance class. Degen (2000) recommended a minimum of 30 pairs per distance class for analysis of spatial genetic population structure. As our analysis represents at least 30 pairs in all distance classes except for the 25-m distance in one of the three populations (Pevestorf), we assume that our sample size was adequate to detect potential genetic structure in the three *A. bruennichi* populations. However, the use of suitable genetic markers is critical. Although the information content of microsatellites is expected to be sufficient for reliable estimates of spatial genetic structure, the number of genetic markers might not have been adequate: it is easier to detect weak spatial genetic structure with more markers (Vekemans and Hardy, 2004). However, our markers helped distinguish siblings from non-siblings (Zimmer *et al.*, 2014) and since we were targeting the spatial distribution of individuals that share similar numbers of alleles, as siblings are known to, the use of 16 genetic markers should have been sufficient. Finally, the sampling within 3–4 populations may not be representative and other populations may well possess more substructure. Although possible, our confidence in the pattern is strengthened by the very similar findings for our populations. Furthermore, the rapid range expansion and the overall high genetic diversity across the entire range, including very recently colonized populations, and the absence of bottlenecks emphasize that the species has a potent dispersal strategy. Even if many spiders were to remain at their birth site, immigration of individuals from other sources would likely dilute any fine-scale genetic substructure.

In conclusion, we did not detect fine-scale genetic substructure in natural *A. bruennichi* populations but observed a modest probability of encountering a genetically similar individual. Hence, inbreeding is possible and is already known to reduce hatching success and offspring fitness. The probability of encountering genetically similar mating partners differed slightly among the populations and might overall be large enough to favour the evolution of inbreeding avoidance in nature but not large enough to select against indiscriminate mating of virgin males and females. The presence and spatially random distribution of related individual spiders suggests that the dispersal mode does not lead to complete emigration. Rather, an unknown proportion of spiders remain in their original habitat and intermingle with immigrating individuals leading to a high local mix of diverse genotypes (Zimmer *et al.*, 2014).

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