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# Multiple paternity increases phenotypic diversity in *Tetrix subulata* pygmy grasshoppers

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## Abstract

Multiple paternity within clutches has been recorded among a variety of organisms. The degree of genetic similarity between parents may influence the number and viability of offspring. Females may therefore mate with several males as an insurance against sterile, low quality or genetically incompatible mates, but also to obtain half sibling offspring that are genetically and phenotypically more diverse. We examine the links between polyandry, multiple paternity and offspring phenotypic diversity in the color polymorphic pygmy grasshopper *Tetrix subulata*. By experimentally mating virgin females and genotyping the resulting offspring using microsatellite markers, we demonstrate that polyandrous females can produce offspring sired by different males. Analyses of microsatellite data and color patterns of captive reared families produced by wild caught females that were not mated in the laboratory, confirmed that multiple paternity occurs in the wild, and that it may increase color morph diversity among half-siblings. Polyandrous mating behavior may thus influence the evolutionary dynamics and maintenance of color polymorphism in this species.

## Key words

color polymorphism, grasshoppers, microsatellites, multiple paternity, offspring diversity, Orthoptera, polyandry, relatedness, *Tetrix subulata*

## Introduction

In sexually reproducing organisms, where the individual genotypes represent combinations of maternally and paternally inherited alleles, the degree of relatedness between parents may influence not only the quality of individual offspring, but also the diversity among different offspring (e.g., Marshall *et al.* 2003). Thus, a female should produce more diverse offspring if her eggs are fertilized by a male that is less closely related to her, because this results in offspring which are more heterozygous and genetically more distinct from each other. Offspring diversity may be increased also if the female mates with a male with a higher level of overall heterozygosity (Brown 1997; Weatherhead *et al.* 1999). Another way by which the diversity among offspring may be increased is if the female mates with more than one male and if more than one of the males contribute parentage, such that the offspring become half-siblings (Ochando *et al.* 1996; Foerster *et al.* 2003; Bretman & Tregenza 2005; Mäkinen *et al.* 2007; McLeod & Marshall 2009). Potential benefits of producing genetically and phenotypically more variable offspring include reduced probability that all offspring be ill-suited to future selective regimes (Yasui 1998; Forsman *et al.* 2007; Yue & Chang 2010), increased disease resistance (Baer & Schmid-Hempel 1999), and reduced competition among phenotypes that utilize different subsets of available resources (Forsman *et al.* 2008; Caesar *et al.*

2010). Despite the potential ecological importance, few studies have investigated whether and the degree to which multiple paternity influences genetic and phenotypic diversity among offspring in the wild.

Here we examine the roles of polyandry and multiple paternity for the ecological and evolutionary dynamics of color pattern polymorphism in the pygmy grasshopper *Tetrix subulata*, a small (up to 15-mm body length, average dry body mass *ca* 0.07 g), diurnal, ground dwelling insect species that inhabits biomes ranging from tropical rainforests to arctic regions of Europe, Asia and America (Rehn & Grant 1955; Holst 1986). Previous investigations have demonstrated that *T. subulata* females mate with more than one male, both in captivity and under natural conditions (Caesar *et al.* 2007; Caesar & Forsman 2009), and indicate that *T. subulata* males vary in genetic quality or fertilization ability (Caesar & Forsman 2009). Pygmy grasshoppers provide one of the classic examples of color polymorphism (Fig. 1) (Nabours 1929; Fisher 1939; Forsman *et al.* 2011). Morphs represent co-adapted gene complexes that differ in body size, reproductive life-history, thermoregulatory behaviors, physiology, predator avoidance behaviors, microhabitat choice and diet (Forsman 1999; Forsman & Appelqvist 1999; Forsman 2001; Forsman *et al.* 2002; Ahnesjö & Forsman 2003, 2006; Karpestam & Forsman 2011). That *T. subulata* females experimentally mated with several males produce more color morph diverse offspring suggests that polyandry may result in half-siblings (Forsman *et al.* 2007). However, shared paternity and its role in natural populations has not yet been confirmed using parentage analyses based on molecular genetic markers.

In this study, we experimentally mated captive reared virgin *T. subulata* females to four males each, in sequence, and then genotyped the resulting offspring using microsatellites to test whether polyandrous females produce offspring that are half-siblings sired by different males. In addition, we genotyped un-manipulated wild caught females and their captive reared mature families to examine whether multiple paternity occurs under natural conditions in the wild and whether it increases color morph diversity among the offspring.

## Materials and methods

*Study species.*—*Tetrix subulata* pygmy grasshopper adults survive over one reproductive season, and late instar nymphs and immature adults hibernate during winter and emerge in spring when the mating season begins (April in our study area in southeast Sweden). Females are polyandrous (Caesar *et al.* 2007; Caesar & Forsman 2009) and produce multiple egg pods, each of which may contain up to 35 eggs. Nymphs develop through five (males) or six (females) instars before they eclose into the adult form.

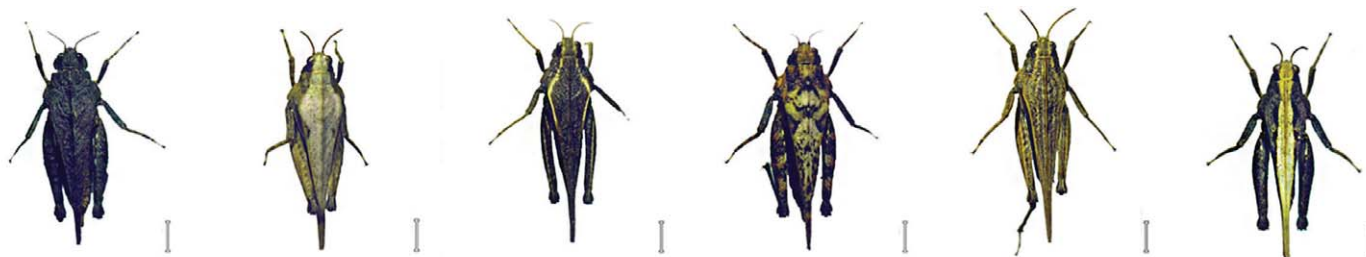


Fig. 1. Examples of *Tetrax subulata* color morphs. For color version, see Plate I.

Pygmy grasshoppers exhibit a multitude of color morphs within populations (Fig. 1). Ground colors of their pronotum are grey, brown, olive green or black of varying intensity. Some individuals are monochrome, while others are distinctly patterned. The pattern elements include several widths and colors of longitudinal stripes along the median pronotum, vertical bands on the femora of the jumping legs, or specks or spots about the middle or periphery of the pronotum or on the jumping legs.

*Experimental mating of captive reared virgin females: testing for multiple paternity.*—To investigate whether polyandrous *T. subulata* females produce offspring sired by different males, we used captive reared virgin females and males. These individuals were offspring to females collected as adults from 12 natural populations (Läckeby, Norra Mossen, Linneryd, Hällasjö, Långkärr, Vanserumbäck, Filmstället, Fliseryd, Studioglas, Jordtorp, Gölen and Knisa Mosse) in Småland, southeast Sweden, on 9<sup>th</sup> to 22<sup>nd</sup> of May, 2007. Females were brought to the laboratory for egg laying; hatched offspring were reared by family in 10L buckets, filled to one third with a mixture of soil and peat, maintained out of doors for growth and hibernation over winter (for details see Forsman *et al.* 2011).

At the onset of the mating experiment the following spring, 6<sup>th</sup> to 9<sup>th</sup> of May, 2008, the mature captive reared individuals were classified for sex. Only mature females ( $N = 33$ ) from rearing buckets without males were used for the experiment. Females were transferred to individual plastic cages ( $150 \times 85 \times 210$  mm) supplied with one small cup filled with soaked cotton for drinking, one cup with soil as oviposition media and food, a piece of potato as supplementary food, and housed in the laboratory at 25°C. Each female was offered four different males in sequence. Each male was present for 24 hours, and females were not allowed to rest between males. Pairs were not observed during the entire experiment and we therefore cannot be certain that the females mated with each of the four presented males. However, in a previous mating experiment similar to this one, Caesar and Forsman (2009) applied colored fluorescent powder on the genitalia at the tip of the abdomen of *T. subulata* males before they were introduced to the females and inspected the females' genitalia after the experiment, and found that of 60 females that were presented with four different males in sequence, no female mated with only one of the males, whereas eight females (13%) mated with two different males, 27 females (45%) mated with three different males, and 25 females (42%) mated with all four males. We also have evidence that females may mate with multiple males under natural conditions in the wild (Caesar *et al.* 2007).

We used males that originated from different populations to ensure genetic diversity and increase resolution in the paternity reconstructions. After use, males were put into 95% EtOH until DNA extraction. After removal of the last male, the female was left in the cage for four days to lay eggs, and then removed and stored in 95% EtOH until DNA extraction. Cages were searched for egg pods

that were placed on moist cotton inside a plastic Petri dish (80 mm in diameter) for incubation at  $25 \pm 1^\circ\text{C}$  and approximately 75% relative humidity. Only 42% (14 of 33) of the females oviposited. Clutches were small and contained on average 10.9 eggs (range 1 – 19,  $SD = 4.9$ ). Hatching success was 66% (range 10–100%,  $SD = 30$ ). Females produced on average only 7.9 (range 1 – 17,  $SD = 5.7$ ) nymphs (about half as many as produced by wild caught females, see below). Hatchlings were preserved in 95% EtOH for subsequent DNA extraction. We selected four females, their experimentally provided males and the resulting newly hatched offspring for subsequent genotyping and parentage analyses (see below), such that our data set included one large, two medium sized, and one small family.

*Un-manipulated wild caught females: testing if multiple paternity increases color morph diversity in the wild.*—To investigate whether multiple paternity occurs under natural conditions in the wild, and whether it increases color morph diversity among half-sibling offspring, adult females ( $N = 52$ ) were collected from a natural population in Bredsåtra ( $N56^\circ 50.923$ ,  $E16^\circ 47.441$ ), a pasture nearby a man-made pond on the island of Öland, on 19<sup>th</sup> of May, 2008. Females were brought to the laboratory and housed in separate cages for egg-laying (see above) without access to males: they were assumed to have mated in the wild prior to capture. Their eggs were incubated in the laboratory, as described above. Hatched nymphs were counted, transferred by family to separate 10 L buckets where they were maintained for growth during summer, and then counted and analysed for survival and color morph diversity in autumn (Caesar & Forsman 2009; Forsman *et al.* 2011). Of the 52 control females, 46 (88%) produced 732 hatchlings, whereof 260 (36%) were still alive at the autumn inventory. Average family size at the autumn inventory was 5.7 (range 1–24,  $SD = 5.5$ ,  $N = 46$ ). We selected the 14 largest families (average 12.1, range 7 – 24,  $SD = 4.1$  individuals) produced by un-manipulated females for genotyping.

*DNA extraction and microsatellite analysis.*— We extracted DNA from four virgin females in the first mating experiment, their experimentally provided mates and their resulting newly hatched offspring, and from 14 un-manipulated wild caught females and their captive reared mature offspring. Genomic DNA was extracted from the femur of adult individuals and from entire newly hatched nymphs. Prior to DNA extraction, each sample was centrifuged at 5000 rpm for 30s and 5mL supernatant was transferred to new-labelled Eppendorf tubes. REExtract-N-Amp Blood PCR Kit (Sigma, St Louis, MO) was used for DNA extraction. 30mL lysis buffer was added to each supernatant, incubated for 5min and 100mL neutralization buffer was added and the samples were stored at  $-20^\circ\text{C}$  in a freezer until microsatellite analysis. PCR amplifications were performed in 10 mL final reaction volumes using a PTC-200 DNA Engine Cycler (BioRad, Foster City, CA). Individual mixes consisted of 1 mL template DNA (c. 10ng), 0.5  $\mu\text{M}$  of each primer and 5  $\mu\text{L}$  REExtract-N-Amp PCR

reaction Mix (Sigma, St Louis, MO).

We used microsatellites that have previously been characterised for *T. subulata* (Johansson 2012; Johansson *et al.* unpubl.). The seven forward primers were 5'-end labelled with fluorescent dye; *Tsub78* and *Tsub86* with VIC (Applied Biosystems), *Tsub179*, *Tsub34*, *Tsub6* and *Tsub39* with 6-FAM (Applied Biosystems) and *Tsub74* with NED (Applied Biosystems). The PCR was set with an initial denaturation of 5 min at 94 °C, followed by 38 cycles of denaturation at 94 °C for 30s, annealing at 62 °C for 1.30 min, extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. PCR products were diluted 1:10, resolved in an automatic sequencer ABI 3730XL (Uppsala Genome Center), and results were scored in GeneMapper ver. 4.0 (Applied Biosystems, Foster City, CA). Microsatellite screening and amplification by PCR is described elsewhere (Johansson 2012).

*Analyses of multiple paternity.*—We used two methods for paternity inference of offspring in the four families produced by experimentally mated virgin females. The allele counting parental reconstruction method (Jones *et al.* 2010), based on data for three microsatellite loci (*Tsub78*, *Tsub86* and *Tsub34*) (Johansson 2012), was used to estimate the minimum number of fathers necessary to explain the variation in genotypes in the progeny arrays within each family. This approach requires highly polymorphic markers, and is particularly useful for species with large numbers of progeny, since the prospects of successful reconstruction diminishes rapidly if an unknown parent has fewer than 8-10 offspring in the family array (Jones *et al.* 2010). Consequently, this approach is likely to result in an underestimate of the number of fathers that have contributed parentage to half-sibling arrays. We therefore repeated the analyses with the maximum likelihood-based method in the program COLONY 2.0 (Wang 2004; Jones *et al.* 2010) based on seven loci (*Tsub78*, *Tsub86*, *Tsub179*, *Tsub34*, *Tsub6*, *Tsub39* and *Tsub74*). COLONY can be used to estimate full- and half-sib relationships and assign parentage using multi-locus genotypes. The program has no maximum limit of contributing fathers and estimates the most probable number of fathers instead of the minimum (Wang 2004; Wang & Santure 2009). Further, COLONY also allows for incomplete sampling of candidate parents, missing data and scoring error. We set the error rate of genotyping to 0.025 as suggested by Wang (2004), and one long run. Polygamy was specified as a mating parameter in accordance with information needed by the program (Jones & Wang 2010).

To estimate the number of sires for the families produced by un-manipulated wild caught females we used three different methods; 1) allele counting, 2) GERUD 2.0 and 3) COLONY 2.0. We first used allele counting to calculate the minimum number of fathers by the estimation of the minimum number of alleles in a family at two polymorphic loci, divided by two (Jones *et al.* 2010). Next, we estimated the minimum number of fathers per clutch using the computer based software GERUD 2.0 that takes into account multi-locus data and the entire progeny array at once (Jones 2005; Jones *et al.* 2010). When GERUD 2.0 returned multiple father solutions for progeny arrays, the solutions were ranked by likelihood-based probabilities (Jones 2005). GERUD can be used as a conventional estimate of the number of paternal contributions but does not handle missing data, and because of this we were only able to apply this method to 5 of the 14 families produced by wild caught females. For further estimation we therefore used COLONY 2.0 which allows for missing data, has no maximum limit of contributing fathers and estimates the most probable rather than the minimum number of sires (Jones & Wang 2010).

To test if polyandry results in increased color morph diversity among half-siblings in the wild, we used data for the 14 genotyped un-manipulated wild caught females and their captive reared offspring. Data were analysed using ANCOVA and comparisons of least squares means, where number of morphs was treated as the dependent variable, family size was included as covariate, and minimum number of sires as estimated by the conservative allele counting approach was treated as an explanatory variable.

## Results

Parentage analysis based on microsatellite loci for four captive reared females experimentally mated with four different males in sequence and their newly hatched offspring demonstrated that mating with more than one male can result in multiple paternity. The four females produced on average 11 (range 7 to 16, SD = 3.7) hatchlings. The minimum number of sires contributing parentage to these families as estimated based on the allele counting method was one for two of the families (consisting of 7 and 10 offspring), and two for the other two families (10 and 16 offspring). The most probable number of sires for the same families estimated using COLONY 2.0 averaged 3.75 (range 1 – 6, SD = 2.06) males per clutch (Fig. 2).

The results of our parentage analysis based on data for un-manipulated wild caught females and their captive reared offspring confirmed that multiple paternity occurs under natural conditions. The 14 females produced on average 12 (range 7 – 24, SD = 4.1) offspring which survived until maturity. The mean estimated minimum number of sires contributing parentage to these families as estimated by allele counting was 1.9 (range 1 – 4, SD = 0.6,  $N = 14$  families). The minimum number of sires estimated using GERUD 2.0 software for the 5 families without missing data ranged from one to three possible father solutions (mean = 1.8, SD = 0.84,  $N = 5$  families) (Fig. 3). The most probable number of sires per family as estimated using COLONY 2.0 averaged 3.2 (range 1 – 6, SD = 1.42,  $N = 14$  families) (Fig. 4). A comparison of the estimated number of sires generated by the three different methods for the 5 families without missing data is presented in Fig. 3.

Analysis of data for the captive reared families produced by un-manipulated females further uncovered that color morph diversity increased with increasing family size (ANCOVA,  $F_{1,11} = 6.53$ ,  $P = 0.026$ ) and with increasing minimum number of sires ( $F_{1,11} = 6.94$ ,  $P = 0.023$ ). Comparisons of least-squares means revealed that, for the average family size of 12 mature offspring, number of color morphs increased from 3.2 on average in families in which all offspring shared the same father, to 5.7 morphs in families consisting of half-sibling offspring sired by three or more different males (Fig. 5), demonstrating that multiple paternity may promote phenotypic diversity among individual offspring within sib-ships in the wild.

## Discussion

In this study, we experimentally mated captive reared virgin *T. subulata* pygmy grasshopper females to multiple males, genotyped their newly hatched offspring using microsatellites and were able to establish, for the first time, that polyandrous pygmy grasshopper females can produce progeny sired by different fathers. Importantly, we found signatures of multiple paternity not only under controlled conditions in the laboratory, but also in captive reared families produced by un-manipulated females that had mated under natural conditions in the wild. Our study further demonstrated that the number of color morphs in captive-reared families increased



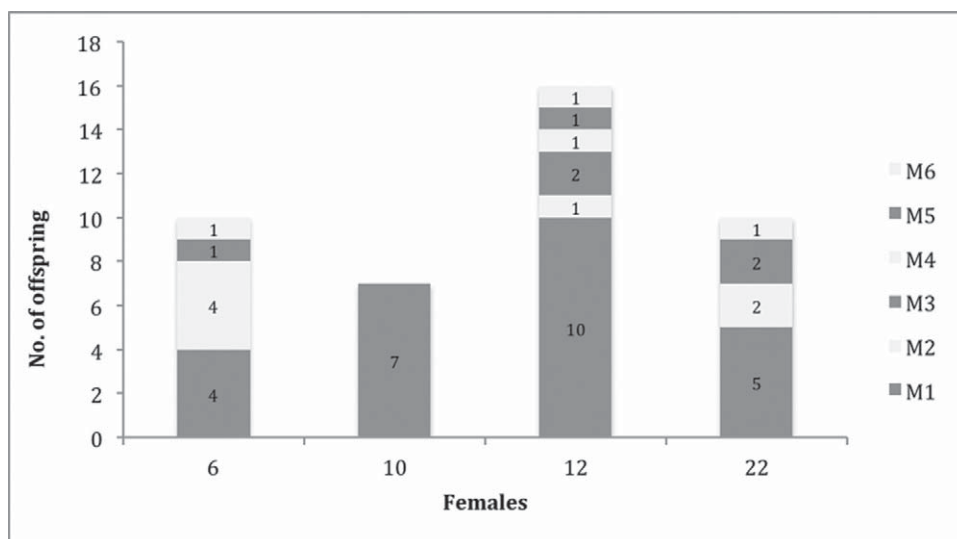


Fig. 2. The most probable division of offspring into full-sib *Tetrix subulata* families produced by experimentally mated captive reared virgin females, as estimated by COLONY. Each bar represents the offspring of one female. The pairs are divided and numbered according to the number of offspring attributed to each male.

with increasing minimum number of fathers, as estimated using parental reconstruction based on microsatellite loci. Taken together, our present findings provide evidence that polyandry can result in multiple paternity and increase the genetic and phenotypic diversity among half-siblings in *T. subulata*. Polyandrous mating behavior in free-ranging pygmy grasshoppers has been reported earlier (Caesar *et al.* 2007), but there has been no previous conclusive evidence (due to lack of co-dominant genetic markers for this species) that it translates into multiple paternity and increased diversity among the resulting offspring. Although our present analysis is based on limited sample sizes, the results corroborate those of Forsman *et al.* (2007) who found that *T. subulata* females mated to one male produced less diverse offspring than did females mated to two similar color morph males and females mated to two different color morph males.

Polyandry and multiple paternity was recorded almost a century ago also in the North American pygmy grasshoppers *Apotettix eurycephalus* and *Paratettix texanus* (Nabours 1929). In one experiment, eight virgin females of *P. texanus* were given opportunity to mate with several (four to eight) males of different dominant color patterns, and the normal recessive, such that there would be no doubt as to the identity of the paternity of the offspring. Based on the distribution of color patterns among the resulting offspring, it was concluded that in all eight cases, several (three to seven) males shared the parentage of the offspring produced by each female (Nabours

1929). Taken together, the above findings implicate the polyandrous mating system and multiple paternity in pygmy grasshoppers as promoting of the maintenance of genetic and phenotypic diversity within populations, as suggested for other organisms (*e.g.*, McLeod & Marshall 2009; Yue & Chang 2010).

Multiply-mated pygmy grasshopper females may obtain several fitness benefits by producing more color morph diverse offspring. If there are many color variants within a group of siblings, the probability increases that at least one morph will be well protected against predators (Bond 2007). The coexistence of two or more color variants may also reduce predator efficiency and increase survival in polymorphic prey populations (Glanville & Allen 1997; Wennersten & Forsman 2009). As stated above, pygmy grasshopper color variants differ in a suite of correlated and functionally important morphological, physiological, behavioral and life-history traits (Forsman 1999; Forsman & Appelqvist 1999; Forsman *et al.* 2002; Ahnesjö & Forsman 2006). The superior success of more variable groups is therefore expected in part due to greater generalism (summed across individuals) with regard to thermal requirements, dietary preferences, or life-history characteristics (Forsman *et al.* 2008; Forsman *et al.* 2012). Accordingly, there is experimental evidence that, at high density, survival of captive pygmy grasshoppers is enhanced by high phenotypic diversity, indicating that resource utilization is more efficient and competition less manifest in polymorphic groups (Caesar *et al.* 2010).

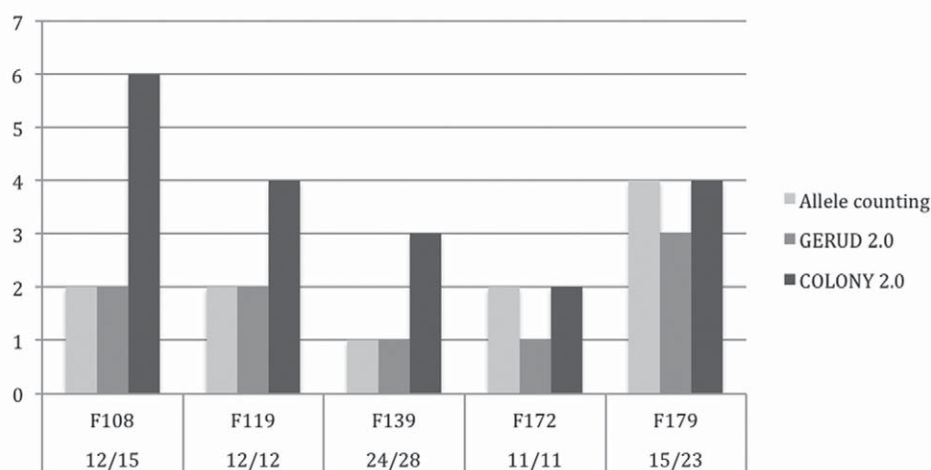
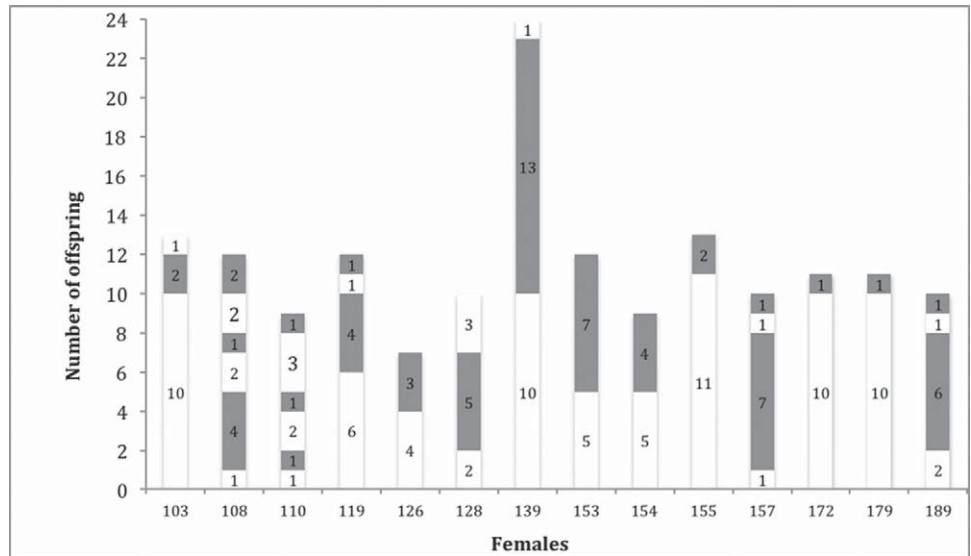


Fig. 3. The minimum number of sires in 5 half-sib families produced by unmanipulated wild caught *Tetrix subulata* mothers, as estimated by three different methods; allele counting: an estimation of minimum number of sires as the number of paternal alleles at two polymorphic locus (*Tsub86* and *Tsub74*), GERUD 2.0 software: estimation of minimum number of sires based on multi-locus genotypes, and COLONY 2.0 software: estimation of the most probable number of sires based on multi-locus genotypes. Below the female name is the number of genotyped offspring/total number of offspring per female.

Fig. 4. The most probable division of offspring into full-sib families produced by un-manipulated wild caught *Tetrix subulata* females, as estimated by COLONY. Each bar represents the offspring of one un-manipulated wild caught female. The pairs are divided and numbered according to the number of offspring attributed to each male.



Phenotypically and genetically more variable populations are also hypothesized to be better able to withstand and adapt to changing conditions (Bolnick *et al.* 2003; Kolbe *et al.* 2004; Forsman *et al.* 2008; Hughes *et al.* 2008; Bell & Gonzalez 2009; Chevin *et al.* 2010; Wennersten & Forsman 2012). In agreement with this notion, there is evidence that diversity may promote establishment in new environments. Forsman *et al.* (2012) introduced small groups of only six to seven *T. subulata* individuals to meadows, clear-cuts, pastures and coastal meadows in the wild, and found that establishment success increased with increasing color morph diversity in the founder group. Results were similar when the experiment was repeated under semi-natural conditions in outdoor enclosures (Wennersten *et al.* 2012). This suggests that the offspring of multiply mated females that disperse to new areas are more likely to successfully found new populations.

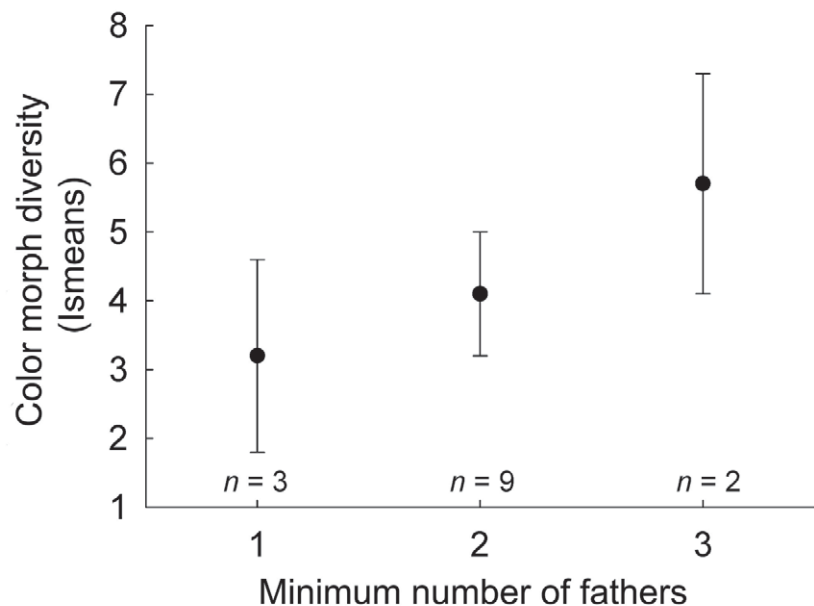
In summary, our present study demonstrates that *T. subulata* pygmy grasshopper females that have mated with multiple males may produce offspring that are half-siblings sired by different males, and confirm that such half-sibling families are more color morph diverse. These results are consistent with the notion that females

may mate with multiple males to avoid reproductive failure due to low quality, sterile or genetically incompatible males (see also Caesar & Forsman 2009), and to obtain fitness benefits mediated by the production of more diverse offspring (Forsman *et al.* 2007). Our present findings further indicate that the polyandrous mating behavior and multiple paternity may influence the maintenance of color polymorphism in pygmy grasshopper populations.

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Fig. 5. Effects of multiple paternities on colour morph diversity among *Tetrix subulata* families. Data for wild caught females mated in the wild, whose offspring were hatched and reared to maturity in captivity. Least squares means show the average number of color morphs for a family size of 12 mature offspring, as estimated from linear regression of number of colour morphs on number of individuals. Data for 14 families. Minimum number of fathers for each family was estimated using the conservative allele counting approach based on three microsatellite loci. Error bars indicate 95% confidence limits. Colour morph diversity within families increased significantly with increasing family size ( $F_{1,11} = 6.53, P = 0.026$ ) and with increasing minimum number of sires ( $F_{1,11} = 6.94, P = 0.023$ ).



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