Restricted hybridisation in the secondary contact zone of closely related haplodiploid social spider mites

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Abstract

How frequently hybridisation and gene flow occur in the contact zones of diverging taxa is important for understanding the speciation process.*Stigmaeopsis sabelisi* and *Stigmaeopsis miscanthi* HG form are haplodiploid, social spider mites that infest the Chinese silver grass, *Miscanthus sinensis*. These two species are closely related and parapatrically distributed in Japan. In mountainous areas, *S. sabelisi* and *S. miscanthi* HG form are often found in the highlands and lowlands, respectively, suggesting that they are in contact at intermediate altitudes. It is estimated that they diverged from their common ancestors distributed in subtropical regions (south of Japan) during the last glacial period, expanded their distribution into the Japanese Archipelago, and came to have such a parapatric distribution (secondary contact). As their reproductive isolation is strong but incomplete, hybridisation and genetic introgression are expected at their distributional boundaries. In this study, we investigated their spatial distribution patterns along the elevation on Mt. Amagi using male morphological differences and investigated their hybridisation status using single-nucleotide polymorphisms by MIG-seq. We found their contact zone at altitudes of 150–430 m, suggesting that their contact zone is prevalent in the parapatric area, which is in line with a previous study. Interspecific mating was predicted based on the sex ratio in the contact zone. However, no obvious hybrids were found, and genetic introgression was estimated to be extremely low. Here, we discuss why gene flow is extremely restricted to the contact zone.

Introduction

Closely related species often overlap in their geographical distribution (Mayr, 1963). Contact frequencies and reproductive, ecological, and genetic relationships at their distribution boundaries are important for their coexistence and evolutionary consequences (Coyne and Orr, 2004; Johannesson *et al*., 2020). In particular, hybridisation is likely to occur if reproductive barriers are not completely established. In this case, they may fuse into a single species (Coyne and Orr, 2004) or diversify further by character displacement and/or reinforcement of reproductive barriers (Dobzhansky, 1959; Hoskin *et al*., 2005; Smadja and Butlin, 2006; Pfennig and Rice, 2014). If the fitness of the hybrids is high, a new species can be generated from hybridisation through developing reproductive isolation from its parental species (Rieseberg, 1997; Seehausen, 2004; Mallet, 2008; Abbott *et al*., 2013).Even though this does not result in new species, genetic introgression by hybridisation may bring genetic diversity to the parent species and drive their subsequent evolution (Edelman *et al*., 2019). Therefore, to understand the evolutionary relationships and speciation of closely related species with overlapping distributions, it is important to determine the frequencies of contact, reproductive isolation, and genetic introgression at their distribution boundaries.

The*Stigmaeopsis miscanthi* species group (Acari: Tetranychidae) is a haplodiploid spider mite that infests Chinese silver grass, *Miscanthus sinensis*, in East Asia (Saito *et al*., 2018, 2019). The mites construct woven nests on the undersurface of the host plant leaves and live in groups within the nests. They are called social spider mites because there are two to three generations of overlap among nest members, and they show cooperative nest building, nest sanitation, and brood care (Saito, 2009; Schausberger *et al*., 2021). Woven nests are protective against their natural enemies (predatory mites, predatory gall midges, ants, etc.); however, some predators, such as the phytoseiid mite *Typhlodromus bambusae*, can intrude into the woven nests. To protect nestmates and their offspring against predatory intruders, adult males and females counterattack the intruders cooperatively and sometimes kill the intruders if they are immature (Saitō, 1986a, 1986b; Yano *et al*., 2011; Saito *et al*., 2011). Males are aggressive not only against predatory intruders but also against conspecific males. They kill each other to establish their own harem (Saitō, 1990). The frequency of male killing varies among populations (Saito, 1995; Saito and Sahara, 1999; Sato, Egas, *et al*., 2013; Sato *et al*., 2019) and is associated with differences in male aggression and also with their reproductive, phylogenetic, and geographic relationships (Sato *et al*., 2000a, 2000b, 2015, 2015, 2018; Sato, Egas, *et al*., 2013). Five species and two forms have been described in this species group so far (Saito *et al*., 2018, 2019).

In Japan, *Stigmaeopsis sabelisi* with lower male aggression, *S. miscanthi* high-aggression form (hereafter, *S. miscanthi* HG form) with higher male aggression, and *S. miscanthi* mild-aggression form (hereafter, *S. miscanthi* ML form) with intermediate male aggression are distributed (Saito, 1995; Saito and Sahara, 1999; Sato, Egas, *et al*., 2013; Sato *et al*., 2019). *S. miscanthi* ML form is distributed in subtropical regions and is geographically isolated from the two other species (Sato, Egas, *et al*., 2013; Sato *et al*., 2019) (Fig. 1). On the other hand, *S. sabelisi* and *S. miscanthi* HG form show overlap in their geographic distribution: *S. sabelisi* is distributed in colder regions (from Aomori Prefecture to Kyushu Islands), whereas *S. miscanthi* HG form is distributed in

warmer regions (from Shizuoka Prefecture to the main island of Okinawa) (Fig. 1). Japan is mountainous, and in areas where both species are distributed, *S. sabelisi* and *S. miscanthi* HG form are found in the highlands and lowlands, respectively (parapatric distribution). A previous study inferred the population history of the species group using mtDNA (cytochrome c oxidase subunit I; COI) and estimated that *S. sabelisi* and *S. miscanthi* HG form were derived from an ancestral group with mild male aggression in the subtropical region during the last glacial period (20,000–40,000 years BP for *S. sabelisi* and 5,494–10,988 years BP for *S. miscanthi* HG form) (Sato *et al*., 2019). Considering their inferred history together with their ecological and reproductive relationships and the migration history of their host plant (Clark *et al*., 2014), it is predicted that (1) *S. sabelisi* was derived south of Japan and migrated into the Japanese archipelago just after the host plant expanded its distribution into the Japanese archipelago; (2) as temperature increased more, the ancestral groupexpanded its distribution northward and migrated into the Ryukyus Islands; (3) *S. miscanthi* HG form was derived from the ancestral group in and around the Japanese archipelago; and (4) *S. miscanthi* HG form expanded its distribution in the Japanese archipelago and drove *S. sabelisi* to the colder region through competition and reproductive interference (Saito *et al*., 2013; Sato, Sabelis, *et al*., 2013; Sato *et al*., 2015), resulting in their present geographic distributions (Sato *et al*., 2019).

A previous field study at Mt. Unzen, one of the mountains on the Kyushu Islands at the southern end of the parapatric area (Nagasaki Prefecture; Fig. 1), found that the distributions of these two species broadly overlapped at intermediate altitudes (100–400 m), and both species were collected from the same host plant colonies in the contact zone (Sato *et al*., 2008). It is known that their reproductive isolation is strong but incomplete; there is a strong post-mating and pre-zygotic reproductive barrier, but a few hybrids are produced from interspecific crosses (proportion of hybrids: 0– 30%) (Sato *et al*., 2000a, 2000b, 2015, 2018), and their hybrids are fertile (Sato, 2004). Therefore, hybridisation and gene flow are likely to occur in the contact zones formed on each mountain in the parapatric area. In particular, males of *S. miscanthi* HG form actively approach the females of *S. sabelisi* for mating, as they do for conspecific females (Sato *et al*., 2015). In addition, interspecific male fights occur easily between the two species, and males in the *S. miscanthi* HG form tend to win interspecific male fights (Sato, Sabelis, *et al*., 2013). This suggests that *S. sabelisi* females are at a higher risk of interspecific mating than *S. miscanthi* HG form females, indicating that genetic introgression is likely asymmetric. However, it has not been confirmed whether hybridisation occurs in the contact zones. Furthermore, their contact zone has been reported only on Mt. Unzen, and it is unclear whether their contact zones are widespread in their parapatric areas.

In this study, to address whether the contact zone of *S. sabelisi* and *S. miscanthi* HG form is widespread in their parapatric areas, we investigated their distribution patterns along the elevation on and around Mt. Amagi in Shizuoka Prefecture, Japan. Mt. Amagi was selected as the study site because it is located at the northern end of the parapatric area (Fig. 1). The presence of contact zones at both the southern and northern ends of the parapatric areas (Mt. Unzen and Mt. Amagi) supports the hypothesis that contact zones are prevalent in parapatric areas. To determine whether interspecific mating occurred in the contact zone, we analysed the sex ratio of mite colonies in the contact zone. The mites are haplodiploid, in which females develop from fertilised eggs and males develop from unfertilised eggs. Virgin females lay unfertilised eggs, and the number of eggs is significantly lower than that of mated females (Sato *et al*., 2000a, 2000b, 2018). Females mated with males of different species lay unfertilised eggs because of the strong post-mating pre-zygotic barrier (reproductive barrier in the egg fertilisation stage); however, the number of eggs was similar to that of females mated with conspecific males, possibly because females control the number of eggs by copulation stimuli (Sato *et al*., 2000a, 2000b, 2018). As females that mate with males of different species produce an overabundance of sons (Sato *et al*., 2000a, 2000b, 2018), the sex ratio would become relatively male-biased in mite colonies where interspecific mating occurs (Sato *et al*., 2008); although, the spider mite species originally showed an extremely female-biased sex ratio (male ratio: 0.10–0.20) (Sato and Saito, 2007). To address whether hybridisation and genetic introgression occurred between the two mite species, we analysed the genetic population structure of the mite colonies in their contact zones. Spider mites are too small to obtain sufficient DNA from a single mite for molecular analysis (body length is less than 0.5 mm). Therefore, in the genetic analyses, we used multiplexed Inter-Simple Sequence Repeat (ISSR) genotyping by sequencing (MIG-seq), which is useful for small amounts of low-quality DNA (Suyama and Matsuki, 2015). Single-nucleotide polymorphisms (SNPs) were detected using MIG-seq for genetic analysis. To confirm whether *S. sabelisi* and *S. miscanthi* HG form collected from Mt. Amagi produced hybrids, we performed cross-experiments. Previous cross-experiments have found hybridisation; however, these studies used different populations, so there is a possibility that the two species from Mt. Amagi do

not produce hybrids that are different from those reported in previous studies. Finally, based on these findings, we discuss the prevalence of their contact zones, interspecific mating, gene flow, and the effect of the contact zone on their evolution.

Materials and Methods

Study site and mite collection

We collected spider mites of *S. miscanthi* species group from the eastern area of Mt. Amagi (Shizuoka Prefecture, Japan) at altitudes of 20–680 m in July 2020 (Table 1, Fig. 1). This area is dominated by *S. sabelisi*;therefore, to ensure the collection of *S. miscanthi* HG form, we collected mites from the area northeast of Mt. Amagi at an altitude of 20 m, additionally (Shiofuki in Table 1). We brought host plant leaves with mite nests to the laboratory and recorded the number of males, females, and immatures in each nest using stereoscopic microscopes. Slide specimens of the collected males were prepared and used for species identification based on the male morphology. We stored the collected females in microtubes containing 99% acetone for genetic analysis. We reared the collected immatures on detached *M. sinensis* leaves under controlled conditions (25℃, 75–100% relative humidity, and 15:9 h light:dark). Since spider mites are haplodiploid, male genes are derived only from their mothers. Therefore, we prepared slide specimens of males developed from reared immatures within several days after mite collection and used them for species identification of females based on male morphology. We established laboratory mite cultures for each population using the remaining collected mites and used them for the cross-experiment.

Species identification by male morphology

As the relative length of leg I to leg III and body width were greater in males of the *S. miscanthi* HG form than in those of *S. sabelisi*, we used morphological differences to identify the species of the collected mites (Saito, 1995; Sato *et al*., 2000a, 2019; Sato, Egas, *et al*., 2013). One to five males were placed on a drop of Hoyer's solution on a slide glass and covered with cover glass. We placed a 10-g weight on the cover glass to flatten the mite body evenly for accurate measurement and dried it at 45℃ for more than 3 days. We took photos of the slide specimen with a microscope (Axioskop, Zeiss, Germany) and an eyepiece camera (Dino-Eye AM4023X, Anmo Electronics Corporation, Taiwan), after which we measured the lengths of the first and third legs (the four leg segments of the tarsus, tibia, genu, and femur), body length, and body width of the slide specimen using image-processing software (Image J ver. 1.53a; National Institute of Health, Bethesda, MD, USA) (Schneider *et al*., 2012). We calculated the relative length of the first to third legs (leg I to leg III) from the total of the four leg segment lengths and used the log-transformed values in the analysis. The specimens used to construct the linear discriminant function were measured three times, and the average values were used. The specimens used for species identification were measured once, and the value was used for species identification.

In a previous study conducted on Mt. Unzen (Nagasaki Prefecture, Japan), a contact zone was found at altitudes of 100–400 m (Sato *et al*., 2008). Therefore, we assumed that the mite colonies at altitudes below 100 m and above 500 m were *S. miscanthi* HG form and *S. sabelisi*, respectively. We constructed a linear discriminant function using body width (*BW*), body length (*BL*), and log-transformed values of the relative length of the first to third legs (*BLL*) of males from the colonies (*S. miscanthi* HG form: 31 males and 41 males developed from immatures collected from Shiofuki and Amagi 1 and 2; *S. sabelisi*: 28 males and 41 males developed from immatures collected from Amagi 17–20; Table 1). We used the discriminant function for species identification of males and males developed from immatures collected from colonies at an altitude of 100–500 m (Amagi 3–16; Table 1). For discriminant analysis, we used statistic software R ver. 4.0.2 (R Core Team, 2022) and the *MASS* package (Venables and Ripley, 2002).

Interspecific mating inferred from the sex ratio in the field

The sex ratio is expected to be relatively male-biased in mite colonies where interspecific mating occurs (Sato *et al*., 2008), because females that mate with different species produce an overabundance of sons (Sato *et al*., 2000a, 2000b, 2018). We estimated interspecific mating in the field by analysing the calculated sex ratio. Based on the results of species identification using male morphology, we categorised the mite colonies into three types: *S. miscanthi* HG, *S. sabelisi* alone, and both species. The male ratio was analysed using a generalised linear model (GLM) with a binomial error distribution and likelihood ratio test

(LRT). Multiple comparisons were performed using Tukey's post-hoc test. For the analysis, we used statistic software R ver. 4.0.2 (R Core Team, 2022) and the *multcomp* package (Hothorn *et al*., 2008).

Genetic analysis

In the genetic analysis, we used 237 females from 11 colonies at altitudes of 100–500 m (contact zone), two colonies below 100 m altitude (pure *S. miscanthi* HG form), and two colonies above 500 m altitude (pure *S. sabelisi*). DNA was extracted from a single female mite, which was stored in 99% acetone in a microtube, using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA).

To construct the MIG-seq library, we followed the protocol described by Suyama et al. (2022), which is modified from Suyama and Matsuki (2015). Briefly, an MIG-seq library was prepared using a two-step PCR method. In the first PCR, ISSR regions were amplified using MIG-seq primer set 1 (Suyama and Matsuki 2015). In the second PCR, indices and Illumina adapter sequences were added to the first PCR products. The Illumina MiSeq platform and MiSeq Reagent Kit v3 (150 cycles; Illumina) were used for sequencing. We skipped the sequencing of the first 17 bases of reads 1 and 2 (SSR primer region and anchors) using "DarkCycle". We detected genome-wide SNPs from the sequenced raw reads and filtered SNPs and females, as described in Suyama *et al.* (2022). After the removal of extremely short reads and low-quality reads using trimmomatic 0.39 (Bolger *et al*., 2014), 42,183,313 reads (177,989 ± 4,186 reads per sample) remained from 43,713,012 raw reads (184,443 ± 4,323 reads per sample). De novo SNP genotyping was performed using Stacks 2.55 (Rochette *et al*., 2019), utilizing the following parameters: minimum depth of coverage required to create a stack (*m*) of 3, maximum distance allowed between stacks (*M*) of 2, and number of mismatches allowed between sample loci while building the catalogue (*n*) of 2. Using the 'populations' program in Stacks, we removed SNP sites exhibiting high heterozygosity ($Ho \geq 0.6$) and filtered out SNP sites with fewer than three minor alleles. SNPs that were retained by 50% or more samples were included in the SNP dataset. To avoid linked SNPs, only the first SNP from each locus was considered. Observed (H_O) and expected (H_E) heterozygosities as well as nucleotide diversities at SNP sites were calculated using the 'populations' program in Stacks. To determine the presence of hybrids and genetic introgression between the species, the selected SNPs were used to analyse the population structure using STRUCTURE Software ver. 2.3.4 (Pritchard *et al*., 2000) and Structure Harvester (Earl and von Holdt, 2012). We conducted 30 independent runs with a burn-in of 100,000 steps, followed by an additional 100,000 steps using an admixture model. We estimated the log-likelihood of each cluster ($K = 1–10$). A Neighbour-Net network was constructed using SplitsTree4 4.14 (Huson and Bryant, 2006), utilising the uncorrelated P distance matrix and ignoring ambiguous sites.

Cross-experiment

We used laboratory cultures collected from Amagi 17 and Amagi 1 in the cross-experiment as *S. sabelisi* and *S. miscanthi* HG form, respectively (Table 1). We placed a detached *M. sinensis* leaf (1.0 × 3.0 cm) on water-soaked cotton wool in a Petri dish (5.0 cm diameter, 1.5 cm high; SPL Life Sciences, Gyeonggi-do, Korea). We collected a teleiochrysalis female from the mite culture and placed it onto the prepared leaf arena. One day after mite introduction, we checked the emergence and construction of a woven nest and then introduced a male collected from the mite culture onto the leaf arena. We allowed them to mate and oviposit for 10 days, after which we removed the females and males from the leaf arena and recorded the number of eggs. We checked the development of their offspring daily until they reached the adult phase and recorded their survival and sex. We performed cross-experiments between the populations: Amagi 17 (female) × Amagi 1 (male) and Amagi 1 (female) × Amagi 17 (male), and within species as controls: Amagi 17 (female) × Amagi 17 (male) and Amagi 1 (female) × Amagi 1 (male). As another control, we observed virgin oviposition because spider mite females produce sons without mating, and the number of eggs is significantly fewer in the virgin oviposition than in the mated oviposition in the species group (Sato *et al*., 2000a, 2000b, 2018). We carried out the cross-experiments under controlled conditions (25°C, 75–100% relative humidity, and 15:9 h light:dark).

To determine the pre-mating barrier, we compared the number of eggs among the cross combinations (interspecific crosses, intrapopulation crosses, and virgin oviposition) of each female species. For comparison, we used a GLM with a negative binomial error distribution because we detected overdispersion in the Poisson GLM. To determine post-mating and pre-zygotic barriers, we compared the offspring sex ratios among cross combinations (interspecific cross, intraspecific cross, and virgin

oviposition) using a GLM with a binomial error distribution or quasibinomial error distribution when overdispersion was detected in the binomial GLM. To determine the zygotic barrier, we compared the offspring survival ratios among the cross combinations (interspecific cross, intraspecific cross, and virgin oviposition) using a GLM with a quasibinomial error distribution, as we detected overdispersion in the binomial GLM. For the analysis, we used statistic software R ver. 4.0.2 (R Core Team, 2022).

Results

Contact zone inferred by species identification by male morphology

The discriminant function was obtained as follows:

$$Discriminant\ score = -13.160 - 0.0152DW + 0.022DL - 62.874RLL$$

where *DW*, *DL*, and *RLL* are the body width, body length, and relative lengths of the first to third legs, respectively. When the male individuals used for formulating the function were randomly sampled, the function provided 100% correct answers. Using the discriminant function, we identified 137 males for species identification in males and 175 males developed from the immatures for species identification in females, both of which were collected from colonies at altitudes of 100–500 m. For species identification in males, 32 and 105 individuals were identified as *S. miscanthi* HG form and *S. sabelisi*, respectively. Both species were found together in six colonies at altitudes of 160–420 m, although *S. sabelisi* dominated in seven colonies and *S. miscanthi* HG form in one colony (Fig. 2). In the species identification of females, 34 and 141 individuals were identified as *S. miscanthi* HG form and *S. sabelisi*, respectively. Both species were found together in five colonies at altitudes of 150–430 m, although *S. sabelisi* was dominant in nine colonies (Fig. 2). These results indicate that their contact zone exists at least in the area at altitudes of 150–430 m on Mt. Amagi.

We examined the morphology of 312 males in total and found that a male that developed from the immatures collected at an altitude of 350 m (Amagi 10; Table 1; Fig. 2) had a deformity (Fig. 3). The legs usually consist of four segments (tarsus, tibia, genu, and femur); however, in the deformed male, only three segments were present on one of the third legs (Fig. 3).

Interspecific mating inferred from the sex ratio in the field

The male ratio varied among the mite colonies (male ratio: 0.077–0.355; Fig. 4). The effect of colony type on the male ratio was significant (binomial GLM, $df = 2$, LRT = 15.123, $P < 0.001$). The male ratio in the colonies in which both species were found was significantly higher than that in the colonies of pure *S. sabelisi* or *S. miscanthi* HG form, although the male ratio was not significantly different between the colonies of pure *S. miscanthi* HG form and *S. sabelisi* (Tukey's test, both species vs. *S. miscanthi* HG form: $z = 3.756$, $P = 0.001$; both species vs. *S. sabelisi*: $z = 2.563$, P = 0.027; *S. miscanthi* HG form vs. *S. sabelisi*: $z = 1.389$, P = 0.344). These results indicate that the overproduction of sons by interspecific mating occurred in colonies where both species were found.

Hybridization and genetic introgression

A total of 111 SNPs from 189 loci in 237 females were used for the genetic analysis. All raw reads of MIG-seq data were submitted to the DDBJ Sequence Read Archive under the BioProject ID PRJDB17427. The *S. miscanthi* HG form populations had lower heterozygosity and nucleotide diversity than the populations of *S. sabelisi* (Table S1). Neighbour-Net analysis demonstrated the distinction between *S. sabelisi* and *S. miscanthi* HG form as two separate genetic clusters (Fig. 5). None of the samples occupied an intermediate position between *S. sabelisi* and *S. miscanthi* HG form. Under the delta *K* method, the best STRUCTURE model assigned individuals to two genetic clusters (Fig. S1), corresponding to *S. sabelisi* and *S. miscanthi* HG form, respectively (Fig. 6). A total of 232 individuals were assigned as pure *S. sabelisi* or pure *S. miscanthi* HG form, and five individuals collected from their contact zones were assigned as *S. sabelisi* with very small fragments of *S. miscanthi* HG form. These results showed that there were no hybrids and that genetic introgression was very low.

Reproductive isolation in laboratory cross-experiment

In the cross experiments with *S. sabelisi* females, the number of eggs in interspecific crosses was significantly higher than that in virgin oviposition (negative-binomial GLM, $z = 3.418$, $P < 0.001$; Table 2) and similar to that in intrapopulation crosses (negative-binomial GLM, $z = 0.793$, $P = 0.428$; Table 2), indicating that mating occurred in the interspecific cross. A few hybrids were produced in two of the 24 pairs of interspecific crosses; however, the female ratio in the offspring of interspecific crosses was significantly lower than that in intrapopulation crosses (binomial GLM, $z = 7.919$, $P < 0.001$; Table 2) and similar to that in virgin oviposition (binomial GLM, $z = 0.006$, $P = 0.995$; Table 2), indicating the presence of a strong pre-zygotic barrier. The survival rate of offspring from egg to adult in interspecific crosses was significantly lower than that in intrapopulation crosses (quasibinomial GLM, $t = 2.993$, $P < 0.01$; Table 2) but not significantly different from that in virgin oviposition (quasibinomial GLM, $t = 1.230$, $P = 0.223$; Table 2).

The results of the cross-experiments with *S. miscanthi* HG form females were similar to those of the cross-experiments with *S. sabelisi* females. The number of eggs in the interspecific cross was significantly higher than that in the virgin oviposition (negative-binomial GLM, $z = 4.870$, $P < 0.001$; Table 2) and was similar to that in the intrapopulation cross (negative-binomial GLM, $z = 0.513$, $P = 0.608$; Table 2), indicating that mating occurred in the interspecific cross. A few hybrids were produced in one of the 25 pairs of interspecific crosses; however, the female ratio in the offspring of interspecific crosses was significantly lower than that in intrapopulation crosses (quasibinomial GLM, $t = 6.311$, $P < 0.001$; Table 2) and similar to that in virgin oviposition crosses (quasibinomial GLM, $t = 0.004$, $P = 0.997$; Table 2), indicating the presence of a strong pre-zygotic barrier. The survival rate of offspring from egg to adult in interspecific crosses was significantly lower than that in intrapopulation crosses (quasibinomial GLM, $t = 2.692$, $P < 0.01$; Table 2) but not significantly different from that in virgin oviposition (quasibinomial GLM, $t = 1.565$, $P = 0.122$; Table 2).

Discussion

We found that the geographic distributions of *S. sabelisi* and *S. miscanthi* HG form overlapped widely at intermediate altitudes (150–430 m) on Mt. Amagi. We conducted a field survey on Mt. Amagi at the northern end of their parapatric area because a previous study detected a contact zone on Mt. Unzen at the southern end of their parapatric area (Sato *et al*., 2008; Fig. 1). As their contact zone was confirmed at both the southern and northern ends of their parapatric area, we concluded that their contact zone would be prevalent in their parapatric area and that their contact zone may have a significant impact on their evolution and ecological relationships.

In several host-plant fields in their contact zones, both species were found together for both sexes. This suggests that interspecific mating has occurred in these fields. In fact, the sex ratio of spider mites in the colonies of both species was male-biased compared to that of pure *S. sabelisi* or *S. miscanthi* HG form colonies. As for their reproductive barrier, the post-mating pre-zygotic barrier contributes the most (Sato *et al*., 2000a, 2000b, 2015, 2018), which causes the overproduction of males owing to their haplodiploid genetic system and regulation of the number of eggs by copulation stimuli. The male-biased sex ratio strongly supports interspecific mating in the contact zone. A previous study conducted on Mt. Unzen detected a male-biased sex ratio at intermediate altitudes (Sato *et al*., 2008). Therefore, we conclude that interspecific mating is widespread in their parapatric area.

A previous study reported the presence of a pre-mating barrier, but it was very weak and asymmetric (Sato *et al*., 2015). The post-mating pre-zygotic barrier contributes most to the reproductive barrier; however, it is also incomplete, and a few hybrids were produced in their cross-experiments in the laboratory (proportion of hybrids: 0–30%) (Sato *et al*., 2000a, 2000b, 2015, 2018). In this study, we carried out cross experiments using the populations collected from Mt. Amagi and confirmed that they produce hybrids, although the proportion of hybrids was slightly lower compared to those in previous studies (3–13%). Therefore, we expected that hybrids would exist in the contact zone and that gene flow would occur between them. However, our genetic analysis did not identify obvious hybrids, and it was estimated that genetic introgression was extremely low (Figs. 5 and 6). This suggests that gene flow is strongly restricted by mechanisms that act later than post-mating pre-zygotic isolation. Because their hybrids are fertile in the laboratory (Sato, 2004), they may have problems surviving in nature. For example, adult female spider mites enter diapause and overwinter. Diapause attributes differ between the two species: *S. sabelisi* takes a much longer time to emerge from diapause (high-intensity diapause), whereas the *S. miscanthi* HG form emerges from diapause very quickly (low-

intensity diapause) (Saito *et al*., 2002). The difference in diapause is considered the result of adaptation to colder and warmer regions in each species, and this difference contributes to maintaining their parapatric distribution. The diapause attributes of their hybrids have not yet been investigated; however, if they are not able to enter diapause or emerge from diapause at a suitable time in the local season, they will be selected against from the field, even though they are able to survive in the laboratory. It is also likely that hybrids have reproductive problems in nature. As described before, males of this species kill each other to establish their own harems, and only the victorious male can reproduce (Saitō, 1990). In this study, we identified a deformed male in the contact zone. We do not know if the deformity was caused by hybridisation, but it is worth investigating the influence of hybridisation on the morphology and fighting ability of males. Therefore, to understand why gene flow is extremely restricted in the contact zone, it is necessary to investigate hybrid traits, such as diapause attributes, morphology, and behaviour.

This field survey was conducted at the northern end of *S. miscanthi* HG form distribution. It is common for populations at the edge of the distribution to have extremely low genetic diversity owing to bottlenecks and genetic drift. Because the genetic diversity of *S. miscanthi* HG form populations has not been investigated in other regions, it is not possible to confirm whether the genetic diversity is lower in this area than in other areas. However, *S. miscanthi* HG form populations showed lower heterozygosity and nucleotide diversity than *S. sabelisi* (Table S1). This may explain the low genetic diversity of *S. miscanthi* HG form populations in this study. The low genetic diversity and harshness of the environment for *S. miscanthi* HG form likely make it difficult to produce hybrids. Therefore, before concluding that gene flow is extremely restricted between them in their parapatric area, it is worth conducting this study on other mountains where both species are abundant.

Although not as much as expected, some negligible genetic introgression was detected between *S. sabelisi* and *S. miscanthi* HG form (Fig. 6). As described previously, these two species have different distribution areas and male aggression levels: *S. sabelisi* is distributed in colder regions and shows lower male aggression, whereas *S. miscanthi* HG form is distributed in warmer regions and shows higher male aggression (Saito, 1995; Sato, Egas, *et al*., 2013). Male aggression varies among populations in each species, and the same relationship between winter coldness and male aggression was found in each species. *S. miscanthi* HG form populations distributed in relatively colder regions showed lower male aggression compared to populations distributed in warmer regions, and the same clinal trend was found in *S. sabelisi* populations (Saito and Sahara, 1999). Male aggression is genetically determined, as laboratory mite populations maintain similar aggression regardless of rearing temperature. To date, attempts have been made to explain the clinical trends of male aggression, both intra- and interspecies, through the application of kin selection theory (Saito, 1995; Saito and Sahara, 1999; Saito and Mori, 2005; Sato, Egas, *et al*., 2013). Severe winter colds can induce inbreeding in the spider mite species. Overwintered virgin females establish their spring nests by mating with the surrounding males; however, if there are no males, they produce haploid sons and mate with them for spring nest establishment. Whether males are around is affected by winter coldness because non-diapause males overwinter by chance if the winter coldness is mild (Saito, 1995; Sato, Egas, *et al*., 2013). As predicted by kin selection theory, male aggression is higher in populations where the average kinship in a colony is expected to be low from the spring nest establishment event, and vice versa. However, if genetic introgression affects male aggression, then a clinical trend in each species may be generated by genetic introgression. For example, *S. miscanthi* HG form populations distributed in relatively colder regions are expected to encounter *S. sabelisi* much more frequently than those in warmer regions. Milder male aggression was likely caused by genetic introgression from *S. sabelisi*. To test this hypothesis, it is necessary to investigate the inheritance of male aggression and genetic introgression status in other contact zones.

In this study, we revealed that *S. sabelisi* and *S. miscanthi* HG form have broad contact zones throughout their parapatric area, and interspecific mating occurs in their contact zones; however, gene flow is strongly restricted between them. The contact zones of closely related species influence their evolution in various ways; they can cause species breakdown, promote diversification by character displacement and/or reinforcement of reproductive barriers, and generate new species by hybridisation (Coyne and Orr, 2004; Johannesson *et al*., 2020). In spider mites, contact zones are likely to contribute to diversification. In particular, reinforcement of reproductive isolation can occur when the fitness of a hybrid is low. The traits and fitness of their hybrids have not been well investigated; however, it is quite likely that hybrids have problems surviving and reproducing in nature, considering that we did not find obvious hybrids in their contact zones, despite incomplete reproductive isolation. Reinforcement of reproductive isolation in spider mites was suggested by a previous study, which found that post-mating, a pre-zygotic barrier

evolved faster in *S. sabelisi* females collected from parapatric areas than in allopatric areas (Sato *et al*., 2018). This tendency was not found in *S. miscanthi* HG form females; however, it fits their behavioural differences (Sato *et al*., 2018). Specifically, males readily fight with different species of males for nests, *S. miscanthi* HG form males tend to win interspecific male fights (Sato, Sabelis, *et al*., 2013), and *S. miscanthi* HG form males are active to mate regardless of female species much more than *S. sabelisi* males (Sato *et al*., 2015). These behavioural differences suggest that *S. sabelisi* females are likely exposed to the risk of reproductive interference by other male species, whereas *S. miscanthi* HG form females are guarded by the same male species against such risk. We expect genetic analyses to provide insights into this hypothesis. However, in this study, we could not analyse the direction and details of genetic introgression because the gene flow was extremely low. As discussed before, their gene flow may be restricted by mechanisms acting later than post-mating pre-zygotic isolation. However, the amount of gene flow likely changes depending on the power relationships between *S. sabelisi* and *S. miscanthi* HG form. In this study, genetic analyses were performed on Mt. Amagi, where *S. sabelisi* was found to be superior. From this perspective, it would be worthwhile to conduct this study on other mountains.

Declarations

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Author contribution statement

Y. Sato conceived the study. Y. Sato, S. Konaka, Y. Tsumura, and Y. Suyama designed the study. S. Konaka, Y. Sato, and N. Matsumoto conducted the field surveys. S. Konaka performed the measurements, Y. Sato and S. Konaka analysed of male morphology. S. Hirota, Y. Suyama, S. Konaka, Y. Sato, and Y. Tsumura performed molecular analyses. Y. Sato conducted the cross-experiment and analysed reproductive isolation. Y. Sato and S. Hirota wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Data availability

All raw reads of the MIG-seq data are available from the DDBJ Sequence Read Archive under BioProject ID PRJDB17427. The morphology, sex ratio, and cross-experimental data are shown in the Supplementary Materials.

Research Ethics Statement

Not applicable, as the study was conducted on spider mites.

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Tables

Table 1 Location where spider mites of *Stigmaeopsis miscanthi* species group were collected and the numbers of nests, females, males immatures, eggs and the male ratio collected from each location.

Location	Latitude	Longitude	Altitude (m)	No. of					Male ratio
				Nest	Female	Male	Immature	Egg	
Shiofuki	34.96863	139.12685	20	8	48	4	23	133	0.08
Amagi 1	34.89088	139.13757	20	19	70	13	143	267	0.16
Amagi 2	34.84611	139.07361	50	18	74	17	157	331	0.19
Amagi 3	34.84833	139.06611	150	14	35	12	59	53	0.26
Amagi 4	34.88358	139.09616	160	10	13	6	26	79	0.32
Amagi 5	34.85333	139.06028	210	22	20	11	39	52	0.35
Amagi 6	34.88972	139.08833	250	7	15	3	25	61	0.17
Amagi 7	34.85750	139.05889	280	10	18	8	31	81	0.31
Amagi 8	34.88667	139.08917	290	4	6	2	22	20	0.25
Amagi 9	34.90222	139.10972	300	8	17	4	28	66	0.19
Amagi 10	34.88806	139.08306	350	6	7	2	10	25	0.22
Amagi 11	34.85582	139.06557	390	17	42	14	122	127	0.25
Amagi 12	34.90583	139.07472	400	28	80	33	139	293	0.29
Amagi 13	34.88917	139.07889	420	11	26	9	85	112	0.26
Amagi 14	34.85806	139.06444	430	21	40	4	50	172	0.09
Amagi 15	34.88583	139.07694	450	20	27	9	69	132	0.25
Amagi 16	34.90639	139.06500	460	35	103	29	164	228	0.22
Amagi 17	34.88361	139.07139	500	10	25	5	41	92	0.17
Amagi 18	34.86194	139.05944	530	11	20	6	47	45	0.23
Amagi 19	34.88139	139.06639	560	4	7	2	7	28	0.22
Amagi 20	34.87944	139.05972	680	10	19	5	30	94	0.21

Table 2 The number of eggs laid in 10 days after female emergence, the survival rate from egg to adult, and the female ratio in interspecific crosses, intra-population crosses and virgin oviposition in the cross experiments using *S. sabelisi* females (a) and *S. miscanthi* HG form females (b).

(a) Cross experiment of *S. sabelisi* female

Cross type	Cross combination		No. of pairs	No. of eggs			Survival rate from egg to adult			Female ratio in offspring		
	Female	Male		(Mean	±	SE)	(Mean	±	SE)	(Mean	±	SE)
Interspecific	Amagi 17 (*S. sabelisi*)	Amagi 1 (*S. miscanthi* HG form)	24	7.708	±	0.850	0.797	±	0.060	0.013	±	0.009
Intra-population	Amagi 17 (*S. sabelisi*)	Amagi 17 (*S. sabelisi*)	27	8.444	±	0.590	0.951	±	0.017	0.806	±	0.014
Virgin oviposition	Amagi 17 (*S. sabelisi*)	-	23	4.870	±	0.352	0.891	±	0.037	0.000	±	0.000

(b) Cross experiment of *S. miscanthi* HG form female

Cross type	Cross combination		No. of pairs	No. of eggs			Survival rate from egg to adult			Female ratio in offspring		
	Female	Male		(Mean	±	SE)	(Mean	±	SE)	(Mean	±	SE)
Interspecies	Amagi 1 (*S. miscanthi* HG form)	Amagi 17 (*S. sabelisi*)	25	8.320	±	0.725	0.773	±	0.038	0.030	±	0.030
Intra-population	Amagi 1 (*S. miscanthi* HG form)	Amagi 1 (*S. miscanthi* HG form)	22	7.818	±	0.737	0.932	±	0.032	0.820	±	0.023
Virgin oviposition	Amagi 1 (*S. miscanthi* HG form)	-	21	4.143	±	0.416	0.900	±	0.044	0.000	±	0.000

Figures

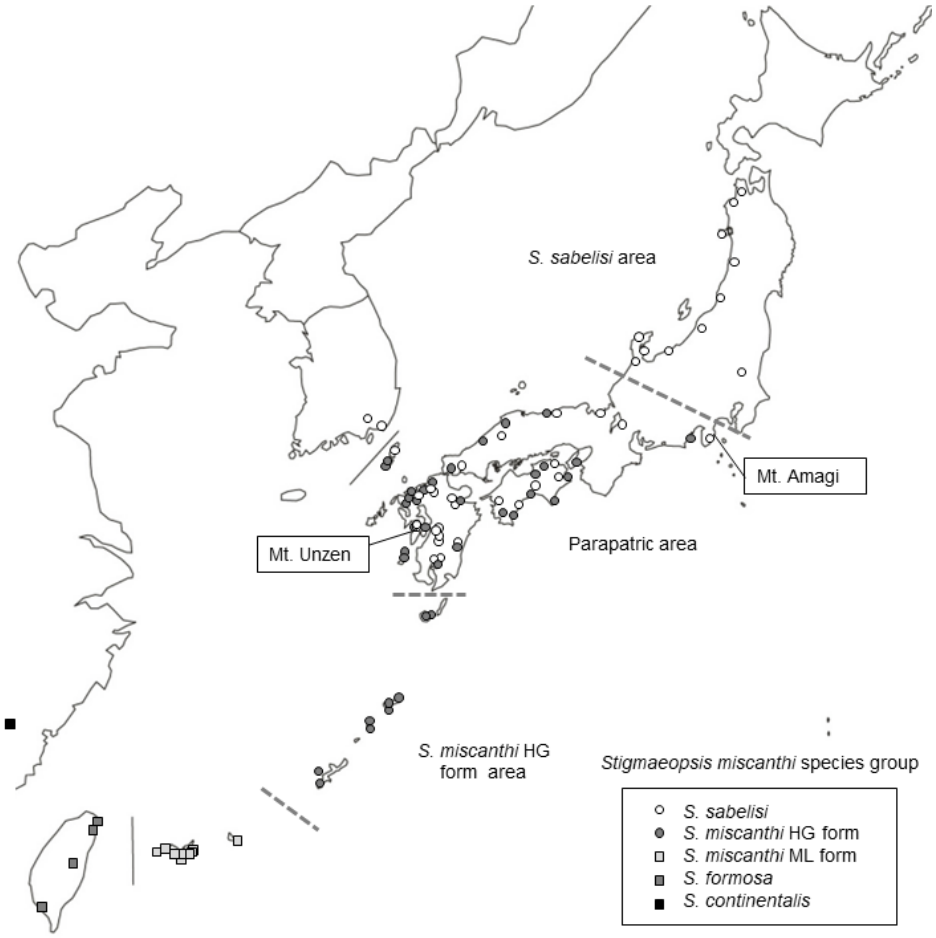

Figure 1

The geographic distribution of the social spider mite *Stigmaeopsis miscanthi* species group in and around the Japanese Archipelago (Saito, 1995; Saito & Sahara, 1999; Sato et al., 2013), and the locations of Mt. Unzen and Mt. Amagi, where the distribution of the two species at different altitudes was studied in a previous study (Mt. Unzen; Sato et al., 2008) and in this study (Mt. Amagi). Three species are distributed in Japan, and the geographic distributions of two species, *Stigmaeopsis sabelisi* and *S. miscanthi* HG form, overlap widely. In the overlapping area (parapatric region), *S. sabelisi* and*S. miscanthi* HG form are distributed in the highlands and lowlands, respectively.

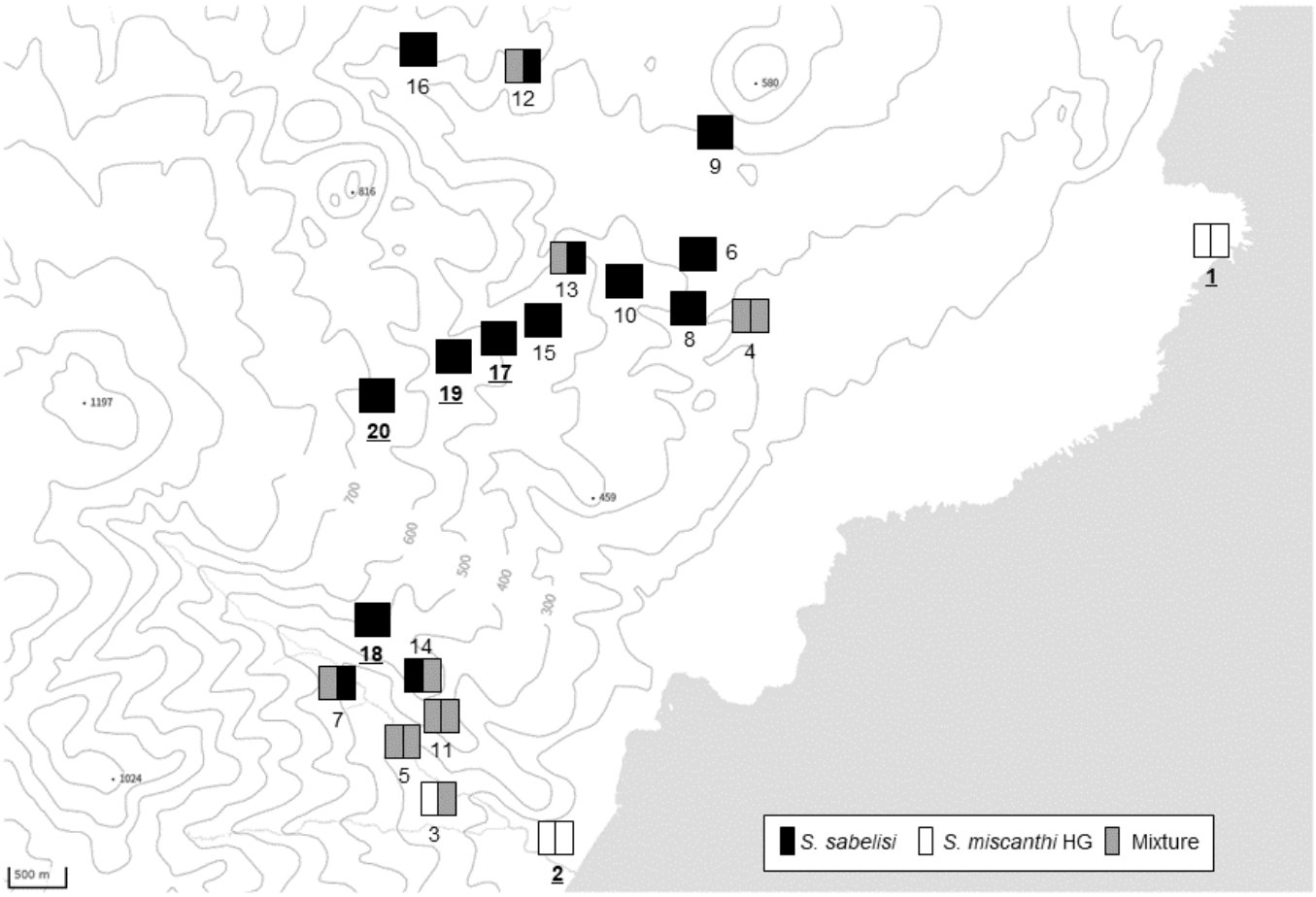

Figure 2

Distributional patterns of *S. sabelisi* and *S. miscanthi* HG form along altitude in both sexes based on the discriminant scores in the species identification by male morphology. Squares show the field points, and the numbers near the squares correspond to Table 1. The numbers with underlines are the colonies used to construct the discriminant function, and others are the colonies identified by the discriminant function. The colour of the square shows the species composition, and black, white, and grey indicate *S. sabelisi*only, *S. miscanthi* HG form only, and the mixture, respectively. The right part of the square shows males and the left part shows females. Lines on the map are contour lines.

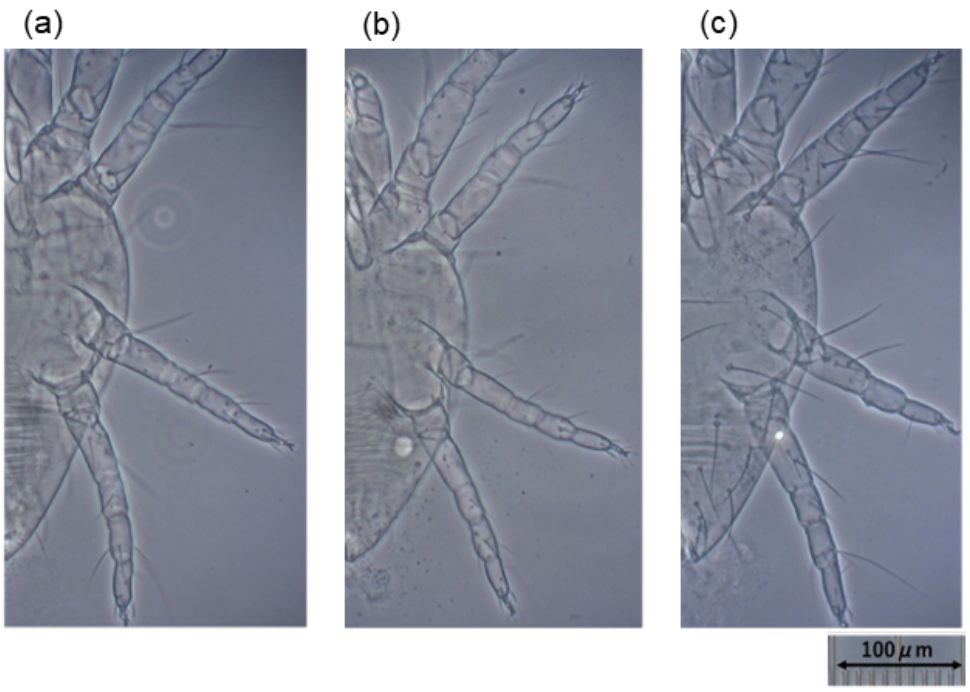

Figure 3

The third leg of a normal *S. sabelisi* male specimen (a), a normal *S. miscanthi* HG form male specimen (b), and an abnormal *S. miscanthi* HG form male specimen (c). The males of (a) and (b) were collected from 680 m (Amagi 20) and 20 m altitudes (Amagi 1), respectively, and the male of (c) was collected from 350 m altitude (Amagi 10) and identified as *S. miscanthi* HG form by measuring the other leg. Legs usually consist of four segments (tarsus, tibia, genu, and femur), as shown in (a) and (b); however, in (c), only three segments were present on one of the third legs.

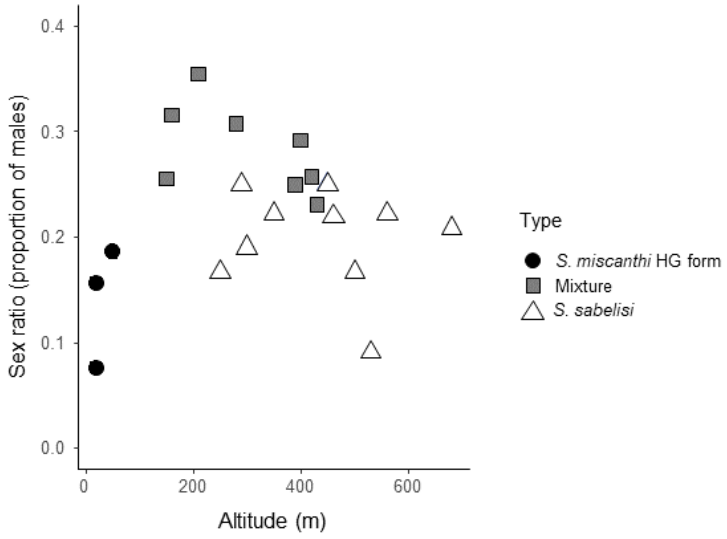

Figure 4

Sex ratio (proportion of males) in mite colonies along altitude. Blue squares show the mite colonies of pure *S. sabelisi*, red circles show the mite colonies of pure *S. miscanthi* HG form, and yellow squares show the mite colonies of a mixture of *S. sabelisi* and *S. miscanth*i HG form.

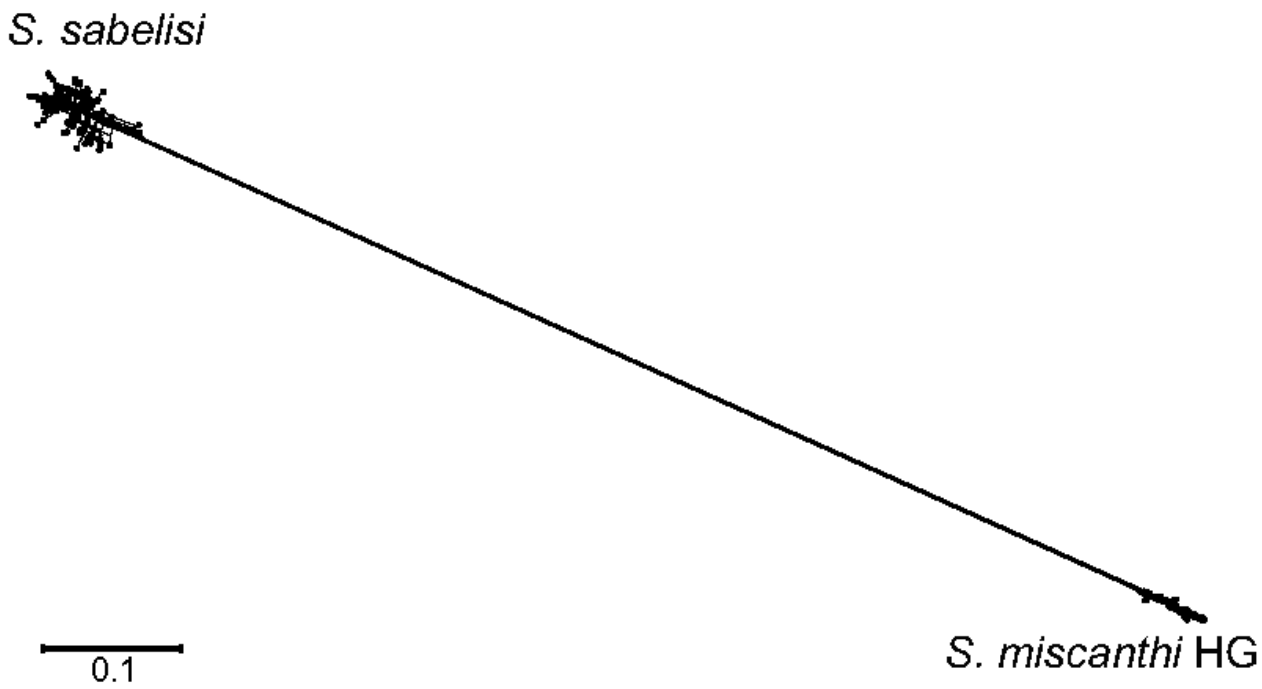

Figure 5

Neighbor-Net network of *Stigmaeopsis* reconstructed based on the uncorrected P distance. The filled cycle represents the samples.

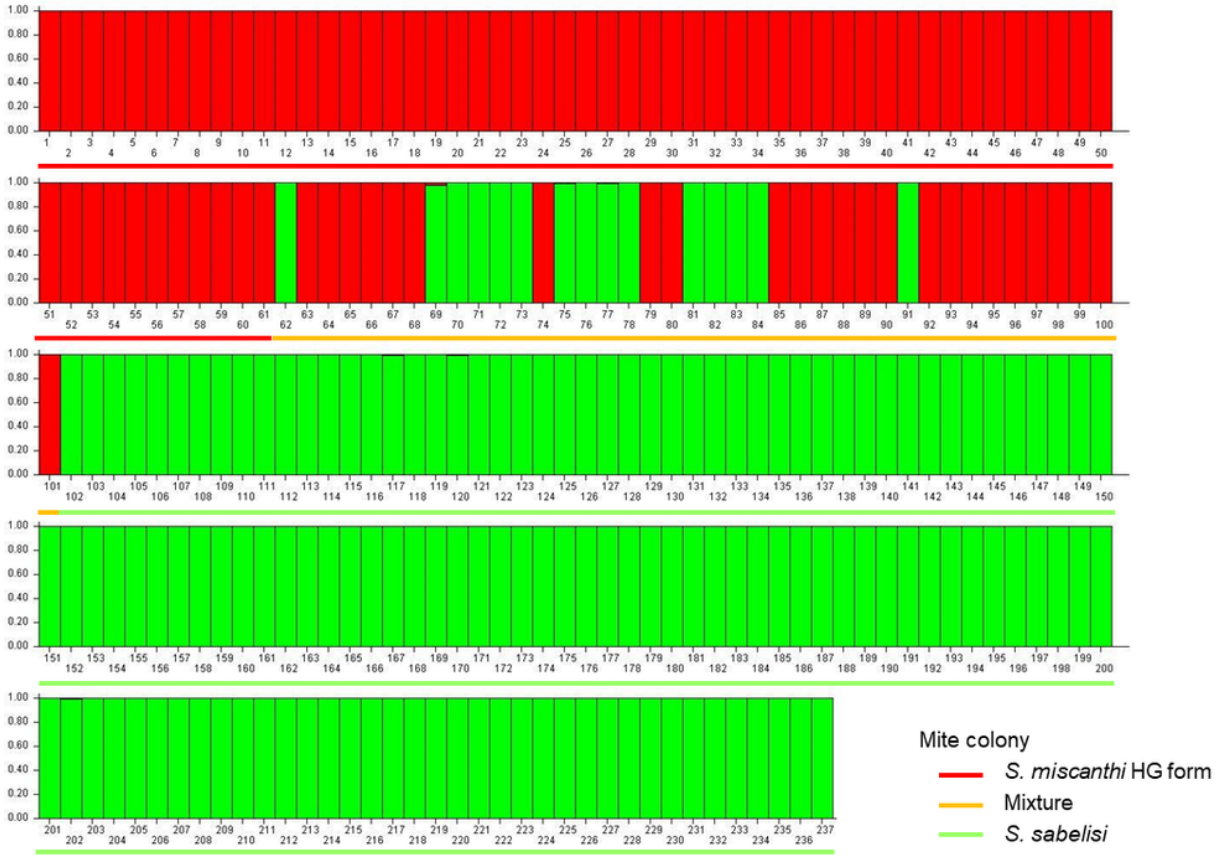

Figure 6

The proportion of the genome of every individual originating from each of the inferred clusters, K = 2: red is cluster 1 corresponding to *S. miscanthi* HG form, and green is cluster 2 corresponding to *S. sabelisi*. Individuals 1 to 61 were from the colonies where *S. miscanthi* HG form was found, individuals 62 to 101 were from the colonies where both *S. miscanthi* HG form and *S. sabelis*were found, and 102 to 237 were from the colonies where *S. sabelis* was found according to the results of identification by male morphology. Individuals 69, 75, 77, 117, and 120 have elements of *S. sabelisi*, as well as very few elements of *S. miscanthi* HG from.

Supplementary Files

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