Restriction of sexual reproduction in the moss *Racomitrium lanuginosum* along an altitudinal gradient

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Abstract

1. Terrestrial plant populations located at the margins of species' distributions often display reduced sexual reproduction and an increased reliance on asexual reproduction. One hypothesis to explain this phenomenon is that the decline is associated with environmental effects on the energetic costs to produce reproductive organs. 2. In order to clarify the changing processes of sexual reproduction along an altitudinal gradient, we investigated the sexual reproductive parameters, such as the number of sporophytes and gametangia, in *Racomitrium lanuginosum*, a dioicous moss found on Mt. Fuji. Matured sporophytes were present only below 3000 m, and the number of sporophytes per shoot tended to be lower at higher altitudes. 3. The numbers of male inflorescences per shoot and antheridia per inflorescence and shoot significantly decreased with increasing altitude. In contrast, the numbers of female inflorescences per shoot and archegonia per inflorescence and shoot varied little across altitudes. 4. Synthesis. Our results suggest that the success of sexual reproduction in *R. lanuginosum* is restricted at higher altitudes on Mt. Fuji by decreases in male gametangia and the subsequent chance of fertilization. These differences between males and females may be caused by differences in the cost of production and development of gametangia, sensitivity to environmental stresses (low air temperature, shortened growth period, and environmental conditions in winter), and phenological patterns at higher altitudes.

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Key words

bryophytes, moss, sexual reproduction, gametangia, antheridia, archegonia, altitudinal gradient

1 Introduction

Reproduction is the basis of the survival and maintenance of species and populations (Ramawat et al. 2014). There are two main types of reproductive systems: sexual and asexual reproduction. Each reproductive system includes different productive strategies according to the life history and growth environment of each species (Bengtsson & Ceplitis 2000; Obeso 2002).

All plant species have limits to their distribution, and population margins demarcate the limits of adaptation to environmental changes. Terrestrial plant populations located at the margins of species' distributions often display reduced sexual reproduction and an increased reliance on asexual reproduction (e.g. Eckert 2002; Pigott 1981). For example, at the northern limits of the distribution of *Betula glandulosa* , which can reproduce both sexually and asexually, fewer than 0.5 % of seeds are viable, and populations are maintained by asexual reproduction (Weis & Hermanutz 1993). One hypothesis to explain this phenomenon is that the decline is associated with environmental depression on the energetic costs to produce reproductive organs (Fisher 2011).

Bryophytes are terrestrial atracheophytes that can reproduce both sexually and asexually (e.g. Maciel-Silva & Pôrto 2014). Therefore, they can disperse by asexual reproduction beyond the limits of sexual reproduction (Fisher 2011; Longton 1988). This extended distribution area maintained by asexual reproduction provides interesting opportunities to study the factors restricting sexual reproduction. Several studies on the distribution of sexually reproducing bryophytes have reported a lower frequency of sporophytes towards the distributional limits (Longton 1988; Longton & Schuster 1983). Moreover, reproductive success in some species, e.g. *Polytrichum alpestre* Hoppe, is known to decline sharply towards the distributional limits (Longton & Greene 1967). However, there is only limited information on the reproductive parameters of sexual reproduction, such as the frequency of gametangia and sporophytes, around the limits of sexual reproduction and in the extended distribution area.

Mountainous environments are unique, as the physical distances between high and low altitude sites are short, whereas differences in environmental conditions and topography are large (Korpelainen et al. 2012). High altitude environments are characterized by low temperature and long duration of snow cover (e.g. Ortiz et al. 2002), as well as increased radiation and wind speed (Körner 2007). It has been reported that species at high altitudes invest more resources in growth than in reproduction (a conservative approach), while species at low altitudes tend to invest more resources in reproduction than in growth (Hautier et al. 2009; von Arx et al. 2006). These findings are often related to the conditions at high altitudes in the mountains, where low temperature and a long duration of snow cover lead to low productivity (Körner 2007).

Racomitrium lanuginosum (Hedw.) Brid. is a dioicous moss in the family Grimmiaceae and is one of the most common and abundant mosses found in cold regions. It forms vast mats on sand or rock in open sites (e.g. Noguchi 1988). It is also widely distributed across temperature zones, particularly in regions with an oceanic climate and in mountainous areas (Herzog 1926; Tallis 1958). Iwatsuki (2001) reported that this species was distributed in the sub-alpine and alpine zones in Japan.

As high altitudes may represent marginal habitats of sexual reproduction for *R. lanuginosum* , we hypothesized that reproductive parameters of sexual reproduction in this species would change at high altitudes according to environmental stresses such as low temperature and snow cover. The aim of this study was to clarify the changing processes of reproductive parameters of sexual reproduction in *R. lanuginosum* along an altitudinal gradient.

2 Materials and methods

2.1 Study site

Mt. Fuji, the highest mountain in Japan (peak, 3776 m alt.), is a volcano located in central Honshu (35°21'N, 138°43'E). In general, the timberline is located at around 2400–2500 m alt. (Masuzawa & Suzuki 1991). The vegetation around the timberline is composed of *Alnus maximowiczii* Callier, *Betula ermanii* Cham., *Salix reinii*French. et Sav. ex Seemen and *Larix kaempferi* (Lamb.) Carrière (Masuzawa 1985; Sakio & Masuzawa 1988). The ground in the alpine zone (above the timberline) on this mountain is covered with a thick layer of basaltic scoria formed by past eruptions. In the alpine zone, the slope is patchy and covered with herbaceous perennials, such as*Aconogonon weyrichii* (F.Schmidt) H.Hara var. *alpinum*(Maxim.) H.Hara, *Artemisia pedunculosa* Miq., *Carex doenitzii* Boeck., *Fallopia japonica* (Houtt.) Ronse Decr. var.*japonica* , *Campanula punctate* Lam. var. *hondoensis*(Kitam.) Ohwi, and *Arabis serrta* Franch. et Sav. var.*serrata* . Patches of *A. weyrichii* and *F. japonica*are dominant near the timberline, and *A. serrata* grows at the highest altitude (Masuzawa & Suzuki 1991). Populations of *R. lanuginosum* are present on the bare ground at 1000–3776 m alt. (Takaki 1971). We selected study sites on Mt. Fuji on a northern trail route (the Yoshida trail route) every 200 m alt. between 2400 and 3700 m alt. (Fig. 1), except at 2600 m alt., at which we could not find an adequate population of *R. lanuginosum* .

2.2 Measurements

The mean daily air temperature at ground level at each site was derived from hourly measurements using data loggers (Hobo@Tidbit v2 Part No. UTBI-001, Onset Computer Corp., Bourne, MA, USA) housed in a plastic box at 2500, 2800, 3000, 3200, 3500, and 3700 m alt. from 17 July 2014 to 28 August 2015. The mean daily relative humidity at ground level was measured by data loggers (Hobo@ Pro v2 Part No. U23-001) at 2200, 3100, and 3700 m alt. during the summer of 2014.

On 16 July 2014, 10 patches (ca. 100 cm² each) of*R. lanuginosum* were collected by hand or spatula from each study site and allowed to dry. After sampling, every shoot in each patch was dissected. A total of 13,115 shoots were collected from across all sites: 1529 shoots at 2400 m, 2416 shoots at 2800 m, 1067 shoots at 3000 m, 4167 shoots at 3200 m, 2110 shoots at 3500 m, and 1826 shoots at 3700 m alt. Each shoot was classified by sexual status as male (with antheridia), female (with archegonia or sporophytes), or non-sex expressing (without gametangia and/or sporophytes) under a binocular microscope (Olympus SZ61-ILST; Olympus, Tokyo, Japan). First, the number and developmental stage of sporophytes were observed for all shoots at each study site. Then, 100 shoots were randomly selected from each study site, and the sex ratio of these shoots was recorded. Finally, the number, developmental stage, and size of gametangia were investigated for five randomly selected shoots from each sex from each study site. The developmental stages of the sporophytes and gametangia were identified and described according to Maruo and Imura (2016, 2018).

2.3 Statistical analysis

Statistical analyses of the relationships between reproductive parameters and altitude were carried out using R v.3.4.0. (R Core Team 2017). The *lme* and *glm* function in the *lme4* package were used to implement the linear mixed model and the generalized linear mixed model, respectively.

3 Results

3.1 Growth environment

The mean annual air temperature decreased with increasing altitude, and the number of days on which the mean daily air temperature was below 0 °C at each study site increased with increasing altitude (Table 1).

The relative humidity during the summer was similar across the three measured sites (Fig. 1).

3.2 Reproductive parameters

The percentage of shoots classified as male sex expressing was 20.0 % at 2400 m, 25.0 % at 2800 m, 14.9 % at 3000 m, 50.0 % at 3200 m, 0.00 % at 3500 m, and 12.0 % at 3700 m alt. The percentage classified as female sex expressing was 14.0 % at 2400 m, 8.0 % at 2800 m, 29.9 % at 3000 m, 0.00 % at 3200 m, 48.9 % at 3500 m, and 30.0 % at 3700 m alt. (Table 2). The total percentage of sex expressing shoots was 34.0 % at 2400 m, 33.0 % at 2800 m, 44.8 % at 3000 m, 50.0 % at 3200 m, 48.9 % at 3500 m, and 42.0 % at 3700 m alt. Thus, the total percentage of sex expressing shoots was lower than 50.0 % at each study site. There were no differences or trends in these percentages along the altitudinal gradient (Table 2).

Sporophytes were found at 2400 m, 2800 m, 3000 m, and 3700 m alt. (Table 3). At 3700 m alt., only the swollen venter (SV) and early calyptra in perichaetium (ECP) stages were present. Matured sporophytes [empty and fresh (EF) stages] were found below 3000 m alt., with most found at 2400 m alt. (Table 3). The formation ratio of sporophytes per shoot was 0.158 at 2400 m, 0.074 at 2800 m, 0.352 at 3000 m, 0.000 at both 3200 m and 3500 m, and 0.024 at 3700 m alt. (Table 4). As for the number of sporophytes per shoot, this tended to decrease with increasing altitude. At every site, the most abundant developmental stage was ECP (Table 3).

The numbers of male inflorescences per shoot (glm, $P = 0.018$), antheridia per shoot (glm, $P < 2 \times 10^{-16}$), and antheridia per inflorescence (glm, $P = 0.003$) significantly decreased with increasing altitude (Tables 4 and 5). In contrast, there were no significant differences in the numbers of female inflorescences per shoot (glm, $P = 0.573$), archegonia per shoot (glm, $P = 0.817$), or archegonia per inflorescence (glm, $P = 0.204$) according to altitude (Tables 4 and 5).

In terms of shoot size, no significant differences were observed between males and females (glm, $P = 0.922$) or along an altitudinal gradient (glm, $P = 0.449$). The sizes of antheridia (lme, $P = 0.347$) and archegonia (lme, $P = 0.724$) were almost identical at each study site, and there was no trend along the altitudinal gradient (Table 5). The maturation ratio of antheridia was 100.0 % at 2400 m, 3200 m, and 3700 m; 89.7 % at 3000 m; and 93.1 % at 2800 m alt. (Table 6). The maturation ratio of archegonia was 100.0 % at 2400 m, 3000 m, 3500 m, and 3700 m and 96.2 % at 2800 m alt. (Table 6).

4 Discussion

Our results present the success of sexual reproduction in *R. lanuginosum* is restricted at higher altitudes on Mt. Fuji by decreases in male gametangia and the subsequent chance of fertilization. Matured sporophytes were present only below 3000 m, and the number of sporophytes per shoot tended to be lower at higher altitudes. The numbers of male inflorescences per shoot and antheridia per inflorescence and shoot significantly decreased with increasing altitude. In contrast, the numbers of female inflorescences per shoot and archegonia per inflorescence and shoot varied little across altitudes.

Maruo & Imura (2016) reported a high frequency of matured sporophytes produced at 2200 m alt. on Mt. Fuji. In this study, the frequency of matured sporophytes was very low, and matured sporophytes (OF and EF stages) were present only below 3000 m and mainly found at 2400 m alt., which is the lowest altitude in the alpine zone. This suggests that the production of sporophytes and the dispersal of spores decreases with increasing altitude and that the environmental conditions in the alpine zone of Mt. Fuji are not suitable for the sexual reproduction of *R. lanuginosum* .

At the highest altitude (3700 m), only aborted sporophytes were found. While the number of sporophytes per shoot at higher altitudes tended to be lower than that at lower altitudes (Table 4), there were no significate differences along an altitudinal gradient. It is assumed that the success of sexual reproduction in terms of spore dispersal is restricted only at lower altitudes along an altitudinal gradient on Mt. Fuji. Ortiz (2002) reported that in *Juniperus communis* subsp. *alpina* (Suter) Ĉelak., cone reproduction and reproductive success decreased towards altitudinal distribution limits. Holm (1994) reported that the number of seeds per catkin decreased with increasing altitude in *Betula pendula* Roth and *B. pubescens* Ehrh. ssp. *pubescens* . Hegazy

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et al. (2008) found that the average number of seeds per individual in *Moringa peregrina* (Forssk.) Fiori significantly decreased along an altitudinal gradient. The causes of the decrease in sporophyte production are assumed to be: (1) limitation on sex expression; (2) limitation on the number of gametangia and underdevelopment of gametangia; (3) limitation on fertilization due to the distance between male and female shoots or the environmental constraints of the fertilization process, such as a lack of liquid water; and (4) limitation on sporophyte development due to environmental stress.

Sex expression is the key factor limiting sporophyte production, as the presence of both male and female individuals is essential for the success of fertilization. Populations of dioicous species sometimes show biased sex ratios, spatial separation of sexes, or the failure of sex expression in males (e.g. Bowker et al. 2000; Gemmell 1950). In extreme environments, greater reproductive effort can lead to a higher mortality rate in females and resulting male-biased sex ratios (e.g. Allen & Antos 1993). In bryophyte populations, sex ratios are predominately female-biased (Stark et al. 2010). In this study, the sex ratio at each altitude varied widely. However, a male-biased sex ratio was not observed, and there was no relationship between sex ratio and altitude detected. Notably, only male shoots were found at 3200 m and only female shoots were observed at 3500 m alt., and sporophytes were not produced at these altitudes. This suggests that the failure of sporophyte production is due to the sex expression of only a single sex at 3200 and 3500 m alt.

Most of the gametangia of this species reached maturity at every altitude, suggesting that environmental factors in the alpine zone did not restrict the development of gametangia. In contrast, we found interesting differences in the number of gametangia between males and females along an altitudinal gradient. The numbers of male inflorescences per shoot and antheridia per shoot and inflorescence significantly decreased with increasing altitude. In contrast, the numbers of female inflorescences per shoot and archegonia per shoot and inflorescence were virtually identical across the altitudinal gradient. This suggests that the production of gametangia results in different responses between males and females along an altitudinal gradient. This difference may be due to the different costs of the production and development of gametangia between males and females. Compared with female gametangia, the production of male gametangia is more energetically expensive for bryophytes (Stark et al. 2000, 2005). In addition, males are reported to be more sensitive to environmental stressors (Bisang & Hedenäs 2005; Cameron & Wyatt 1990; Longton 1985, 1988; Shaw et al. 1991). The results of this study are therefore consistent with these reports on bryophytes. It is assumed that the occurrence and development of male gametangia at higher altitudes are more strongly impacted than those of female gametangia by the low productivity caused by the low air temperature and limited water availability present at higher altitudes.

Maruo & Imura (2016) reported different phenological patterns in males and females, finding that the duration of male gametangia development was longer, continuing from summer to spring of the following year and including a resting period of development under the snow cover in winter. In contrast, female gametangia developed rapidly in spring and showed a shorter developmental duration than males. This suggests that the development of male gametangia may be more readily affected by low temperature and desiccation during the winter. The shortened growth period in the alpine zone is assumed to be another reason for the restriction of male gametangia development. The length of the growth period is thought to be restricted by several environmental factors, such as air temperature and water availability. In this study, the number of days on which the mean daily air temperature was below 0 °C increased along an altitudinal gradient. The length of the growth period, i.e. the number of days on which the mean daily air temperature was above 0 °C, was ca. 6.9 months at 2500 m and 4.6 months at 3700 m alt. Therefore, the growth period at 3700 m was ca. 1.3 months shorter than that at 2500 m alt. Thus, the length of the growth period was reduced and the environment became less suitable for plant growth at increasing altitudes. According to the phenological patterns of *R. lanuginosum* at 2200 m alt. (Maruo & Imura 2016, 2018), male gametangia require a longer developmental duration to reach maturity than female gametangia. This indicates that a shortened growth period may have a greater negative impact on male gametangia development than on female gametangia development. Consequently, we speculate that the occurrence and development of male gametangia at higher altitudes are restricted due to low air temperature, winter conditions, and a shortened growth period.

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Limitations on fertilization are thought to arise from the spatial segregation of male and female sex expression, since fertilization depends on sperm mobility (Van der Velde et al. 2001). Some previous studies have reported that the distance that sperm can disperse is on the order of centimeters to meters (Longton 1976; Van der Velde et al. 2001). In this study, spatial segregation of sex expression between males and females was observed at 3200 and 3500 m alt. This indicates that the males and females are separated by more than several meters at these sites. Consequently, it is thought that the spatial segregation of sex expression between males and females at these sites has a detrimental effect on fertilization.

The number of gametangia has also been proposed to be an important factor in guaranteeing sufficient sperm for fertilization (e.g. Glime 2007). It has been suggested that a decrease in the number of male gametangia at higher altitudes is likely to limit fertilization at higher altitudes. The phenological correspondence of maturation timing between male and female gametangia has also been considered a key factor in the success of fertilization (Maruo & Imura 2018). According to the number of days on which the mean daily air temperature is below 0 °C, it is assumed that there is a shorter growth period at higher altitudes. Thus, in this study, the negative impact of the shortened growth period on the phenology of male gametangia has already considered. In addition, a shift in the maturation timing of male gametangia induced by a phenological change due to the shortened growth period may occur. This change in the maturation timing of male gametangia is assumed to cause a discordance in the maturation timing between males and females, resulting in a low frequency of fertilization.

At every altitude in the alpine zone, the frequency of aborted sporophytes (younger than the OF stage) was very high. Most aborted sporophytes were found to be in the ECP stage at every altitude. This suggests that the ECP stage is an obstacle to maturity. Kallio and Heinonen (1973) reported that the optimum temperature for photosynthesis in *R. lanuginosum* is 5 , within the limits of –10 to 30 . In this study, air temperature decreased and the length of the growth period was reduced with increasing altitude. Mean annual air temperatures were below 5 at altitudes above 2800 m alt. These results suggest that sporophyte development was restricted due to the low productivity caused by the reductions in air temperature and the growth period. The phenological patterns of *R. lanuginosum* sporophytes may be another reason for limitations on sporophytes along the altitudinal gradient. At 2200 m alt., sporophytes emerged in the rainy season and developed from summer to spring of the following year, including a resting period of development under the snow cover in winter, with maturation in the spring (Maruo & Imura 2016, 2018). Maruo & Imura (2016, 2018) have also reported that the air temperature under the snow cover is ca. 0 , and the relative humidity is ca. 100 %. This suggests that snow cover protects the population of *R. lanuginosum* from extreme winter temperatures and desiccation. Some studies have indicated that snow cover shortens the growth period but plays an important role in protecting the plant canopy from winter temperature and desiccation (e.g. Billings & Bliss 1959; Holway & Ward 1963). In this study, the winter air temperature at each altitude fluctuated from –15 to –5 (Fig. 2), and winter relative humidity was stable. This indicates that the snow cover at each altitude in the alpine zone was thinned by strong winds and the sloping ground and that populations of *R. lanuginosum* were not protected from harsh winter conditions. Therefore, the development of sporophytes may be affected by low air temperatures throughout the year and desiccation in winter. Based on the above results, environments at higher altitudes, which have low air temperatures and shortened growth periods, are suggested to have a detrimental effect on the production and development of sporophytes.

In conclusion, the success of sexual reproduction of *R. lanuginosum* , as measured by the production of matured sporophytes and dispersal of spores, was restricted at higher altitudes in the alpine zone on Mt. Fuji. Reasons for this limitation are assumed to be the spatial segregation in sex expression between males and females, differences in the abundance of gametangia between males and females such as reductions in the numbers of male inflorescences per shoot and antheridia per shoot and inflorescence at higher altitudes, and limitations in sporophyte development that result in abortion. In particular, we found an interesting cause for the limited sexual reproductive success of this species, namely differences in the abundances of inflorescences and gametangia between males and females. We speculate that these differences are caused by differences in the costs of production and development of gametangia, sensitivity to environmental stressors,

and phenological patterns. Possible reasons for the decrease in male gametangia and inflorescences and the abortion of sporophytes are the inhibitory effects of low air temperature, a shortened growth period, and winter environmental conditions at higher altitudes.

Authors' contributions

F. M. and I. S. conceived of this study and collected the data and wrote the manuscript. F. M. carried out the statistical analyses. All authors agreed on the final manuscript.

Data Accessibility

All data generated or analyzed during this study are included in this published article.

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Figure and Table captions

Figure 1. Mean daily relative humidity (%) at ground level at 2200 (), 3100 (*), and 3700 ($^{\circ}$) *m alt. from 17 July to 24 August* 2014.

Figure 2. Mean daily air temperature () at ground level at 2500, 3200, and 3700 m alt. from 17 July 2014 to 28 August 2015.

Table 1. The mean annual air temperature and number of days on which mean daily air temperature was below 0 degC at 2500, 2800, 3000, 3200, and 3700 m alt. from July 2014 to June 2015.

Table 2. Number of male, female, and non-sex expressing (asexual) shoots and sex ratio (%) at each study site on Mt. Fuji.

Table 3. Developmental stages of sporophytes at each study site on Mt. Fuji on 16 July 2014. SV–EF: Developmental stages of sporophytes described in Maruo and Imura (2016, 2018).

Table 4. Numbers of shoots, sporophytes, sporophytes per shoot, male inflorescences, antheridia, female inflorescences, and archegonia at each study site on Mt. Fuji (n = 5).

Table 5. Parameter estimates for lme and glm [with Poisson (p) and Gaussian (g) distributions] fitted to the parameters for males and females along an altitudinal gradient on Mt. Fuji.

Table 6. Development and maturation ratios of antheridia (An) and archegonia (Ar) at each study site on Mt. Fuji. J–D: Developmental stages of gametangia described in Maruo and Imura (2016, 2018) (n = 5).

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11

Temperature (°C)

2500

3200

12