Studies on restricting parameters of sexual reproduction in the moss *Racomitrium lanuginosum*

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Abstract

To clarify which reproductive processes limit sexual reproduction in populations of the bryophyte *Racomitrium lanuginosum* (Hedw.) Brid., the following three studies were conducted:

1. an analysis of the reproductive parameters of sexual reproduction of *R. lanuginosum* along an altitudinal gradient;
2. a clarification of the effects of environmental factors on phenological factors associated with the sexual reproduction of *R. lanuginosum*; and
3. a comparative study of morphological parameters of male and female *R. lanuginosum* individuals.

First, changes in reproductive parameters associated with the sexual reproduction of *R. lanuginosum* along an altitudinal gradient in the alpine zone on Mt. Fuji were investigated. Sporophyte production was occurred only below 3000 m alt. and aborted sporophytes at the EOI stage were found mainly at 3000 m alt. The number of antheridia per shoot and per inflorescence decreased with increasing altitude, while the number of

archegonia per shoot and per inflorescence were almost the same at all altitudes, and most of the gametangia of each sex matured. Possible reasons for the limited sporophyte production included the limitation of fertilization by the absence of sex expression, the lack of male gametangia, low temperatures, and/or the shortened growth period for sporophyte development. The conceivable causes for reduced antheridia production are: low temperature and shortened growth period.

Next, a comparative study was conducted to determine the effect of the snow cover as length of growth period on the reproduction process of *R. lanuginosum*. In this study, the phenology of gametangia and sporophytes was compared between two distinct habitats; a seasonally snow-covered site (*ca.* 2200 m alt. on Mt. Fuji, called Okuniwa) and a snow-free site (*ca.* 645 m alt. at Mt. Mihara). At both sites, antheridia took longer to mature than archegonia, and antheridia and sporophytes developed during winter. At Okuniwa, although the development of antheridia and sporophytes stopped under the snow cover for 4.5 months, maturation of male and female gametangia and spore dispersal occurred synchronously in June. These results showed that the responses of phenology to the growth environment differed between males and females. Some notable phenological patterns were the dispersal of sperm from antheridia occurred before archegonia matured, and the occurrence of spore dispersal during winter only at Mt. Mihara.

To explore the reasons for the different responses of males and females to the growth environment, the morphological parameters related to sexual reproduction of *R. lanuginosum* were investigated in detail. The branching pattern of *R. lanuginosum* was monopodial and its inflorescence formation was cladocarpy. Compared with females, males had significantly higher numbers of second and subsequent branches per shoot, and significantly more gametangia per shoot and per inflorescence. The different responses of some morphological parameters such as the number of archegonia per shoot, to sites between male and female were found.

Together, these results supported that the sexual reproduction of *R. lanuginosum* is limited along an altitudinal gradient on Mt. Fuji. The main cause of this limitation was suggested to be the decrease in frequency of male gametangia with increasing altitude, along with the low temperatures and shortened growth period. In the margin of the limits of sexual reproduction, males and females showed some critically different responses to the environment in several reproductive parameters, such as the number of gametangia and the timing of their maturation.

Chapter 1
Introduction

Reproduction is the biological process by which parents produce new individuals (offspring). It is the basis of the survival and sustenance of species and populations (Ramawat et al., 2014). There are two main types of reproductive systems; sexual and asexual reproduction. Each reproductive system has developed different reproductive strategies among species, according to their life histories and growth environment (Bengtsson and Ceplitis, 2000; Obeso, 2002).

Sexual reproduction involves genetic recombination and enables the species to adapt to changes in its environment (Ramawat et al., 2014). Sexual reproduction has several advantages; it allows organisms to adapt to changes in their environment, and advances evolution via DNA repair and the elimination of deleterious genes (Ohara, 2010). However, the cost of sexual reproduction is two-fold that of asexual reproduction because male and female individuals are required (e.g., Charlesworth, 1989; Iwasa, 1987, 1988).

Trade-offs between investment in sexual reproduction and vegetative growth imply that the cost of sexual reproduction is decreased growth, survival, and/or future reproduction (Ehrlén, 1997; Reznick et al., 2000; Roff, 1992; Rydgren et al., 2010; Syrjänen and Lehtilä, 1993; Williams, 1966).

All plant species have limits to their distribution, and their populations demarcate margins and demonstrate the end-point of adaptation to environmental changes (Crawford, 2008). The limits of plant distribution were related to a failure to grow, or an inability of reproduction. In many cases, a failure to reproduce may be a more common response to environmental limitations than a failure to grow, probably because reproductive success requires more than just the development of viable seeds (Crawford, 2008). Terrestrial plant populations located at the margins of species' distribution 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 *Betula glandulosa*, which can reproduce both sexually and asexually, less than 0.5% of the seeds are viable and populations are maintained by asexual reproduction (Weis and Hermanutz, 1993). One hypothesis to explain this phenomenon is that the decline in sexual reproduction at the margins of species' distribution is associated with environmental depression of the energetic costs to produce reproductive organs (Fisher, 2011). An inability of sexual reproduction may arise from numerous causes, including failure to accomplish flowering, fertilization, viable seed reproduction, or germination of individuals of both sexes.

Mosses (Bryophyta), liverworts (Marchantiophyta), and hornworts

(Anthocerotophyta), commonly known as bryophytes, are represented by approximately 18,700 species worldwide. Bryophytes are terrestrial atracheophyte plants that can reproduce both sexually and asexually. In addition to sexual reproduction by dispersal of spores, asexual reproduction generally occurs by means of gemmae (often lacking) and vegetative gametophyte fragments. Spores and asexual propagules play different roles in the ecology and life history of bryophytes (Kimmerer, 1991; Laaka-Lindberg et al., 2003; Longton, 1994; Newton and Mishler, 1994). Spores disperse further and germinate better on previously uncolonized substrates, making them more suitable for long-distance dispersal (Miles and Longton, 1987, 1990; Mishler and Newton, 1988; Stoneburner et al., 1992). Gemmae or vegetative gametophyte fragments can be produced under more stressful conditions, disperse more locally, germinate more rapidly than spores, and are more successful at becoming established in contact with other moss colonies (Mishler and Newton, 1988).

Reproductive failure, as a result of constraints to one or several reproductive events, is the driving force for the extinction of populations and species (Ramawat et al., 2014). Most flowering plants can reproduce only sexually, and the restricting factors of their sexual reproduction at the marginal areas in their distribution have been investigated (e.g. Eckert, 2002; Pigott, 1981; Weis and Hermanutz, 1993). On the other hand, almost all bryophytes can reproduce both sexually and asexually (e.g., Maciel-Silva and Pôrto, 2014). Therefore, they can distribute 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 (Fig. 1).

Several studies on the distribution of sexually reproducing bryophytes have reported a lower frequency of sporophytes towards the distributional limits (Longton, 1988; Longton and Schuster, 1983). Moreover, reproductive success in some species, e.g. *Polytrichum alpestre* Hoppe, is known to decline sharply towards the limits of its geographical range (Longton and Greene, 1967). However, there is only limited information on the reproductive parameters of sexual reproduction such as the frequency of gametangia, sporophytes, and the size of gametangia, around the limits of sexual reproduction and in the extended distribution area (e.g., Maciel-Silva et al., 2012; Longton, 1988).

The aim of this study was to clarify which reproductive parameters restrict sexual reproduction around the limits of sexual reproduction in the bryophyte *Racomitrium lanuginosum* in great detail. To achieve this aim, three studies were conducted:

1. an analysis of the reproductive parameters of sexual reproduction of *R. lanuginosum* along an altitudinal gradient;
2. a clarification of the effects of environmental factors on phenological factors associated with the sexual reproduction of *R. lanuginosum*; and
3. a comparative study of morphological parameters of male and female *R. lanuginosum* individuals.

Chapter 2

Materials and Study sites

Section 1: Materials

1-1: Materials

Racomitrium lanuginosum (Hedw.) Brid. (Fig. 2) is a moss in the family Grimmiaceae. This species forms vast mats on sand or rock at open sites (Noguchi, 1988). The leaf consists of a long triangular green lamina and long hyaline hair-points.

R. lanuginosum is one of the most common and abundant mosses (Herzog, 1926; Noguchi, 1988) and is widely distributed in the marine Arctic (Steere, 1954) including Ellesmere Island (Brassard, 1971), Greenland (Holmen, 1960), Spitsbergen (e.g. Störmer, 1940), and the Siberian Arctic (Lazarenko, 1957). It is also widely distributed in temperate zones, particularly in regions with an oceanic climate and in mountainous areas world-wide (Herzog, 1926; Tallis, 1958). It is found in Hokkaido, Honshu, Shikoku, and Kyusyu in Japan

(Noguchi, 1988). Iwatsuki (2001) reported that this species distributed in sub-alpine zone and alpine zone in Japan.

R. lanuginosum is a dioecious moss (Noguchi, 1988). Its male shoot, female shoot, non-sex expressing (asexual) shoot, sporophytes, male inflorescences, antheridia, and archegonia are shown in Fig. 3. The term "inflorescence" is defined as a cluster of sex organs (gametangia) and the specialized leaves or bracts surrounding them (Bill and Malcolm, 2006). In this species, the bract is present only on the male plant. Here, the term "inflorescence" is also used for the cluster of gametangia and somewhat specialized leaves on female plants. In this species, sporophytes are very rare (e.g., Noguchi, 1988). The phenology of this species in England was investigated by Tallis (1959b), but only the timings of capsule development and spore dispersal were recorded.

The *R. lanuginosum* plant consists of an elongated main stem and many short lateral branches (Tallis 1959a); this unit is defined as the "shoot" (Fig. 4). Male and female inflorescences form at the apices of the branches. This type of inflorescence formation is known as cladocarpy (Farge-England, 1996).

1-2: Reproductive cycle of bryophytes

The lifecycle of bryophytes is an alternation of generations, with a green and perennial gametophyte and an ephemerous dependent sporophyte (Goffinet and Buck, 2013; Schofield, 1985; Vanderpoorten and Goffinet, 2009). The lifecycle of dioecious mosses is depicted in Fig. 5, following Vanderpoorten and Goffinet (2009). Bryophytes reproduce sexually to produce new individuals via spores. After the spores germinate, they produce a filamentous protonema. Leafy buds form on the protonema and give rise to many genetically identical leafy gametophytes (Nishida, 1978; Nehira, 1983; Duckett et al., 1998). Each gametophyte of both sexes produces inflorescences with gametangia, which consist of gametes enclosed by a wall composed of one or more sterile cell layers. Antheridia (male gametangia) have numerous antherozoids or sperm (motile male gametes), and archegonia (female gametangia) contain a single oosphere or egg (a non-motile female gamete). Fertilization can occur when an antherozoid reaches, mainly via water, an oosphere. After that, a zygote develops into an embryo inside the archegonium. The embryo gives rise to the sporophyte with a foot, seta or stalk (except in hornworts), and a capsule (sporangium). After meiosis inside the sporophyte, spores are released from the capsule and are dispersed usually by wind.

Section 2: Study sites

Mt. Fuji and Mt. Mihara were selected as the study sites.

2-1: Mt. Fuji

Mt. Fuji, a volcano located in central Japan (35°21′ N, 138°43′ E), is the highest mountain in Japan (peak, 3776 m alt.; Fig. 6 and 7). Its most recent eruption was in 1707 (Adachi et al., 1996; Tsuya, 1971). The ground between the summit and the hillside of this mountain is covered with a thick layer of basaltic scoria formed by past eruptions. In general, the timberline is located at around 2400 to 2500 m alt. (Masuzawa and Suzuki, 1991). The vegetation around the timberline is composed of *Alnus maximowiczii* Callier, *Betula ermanii* Cham., *Salix reinii* Franch. et Sav. ex Seemen, and *Larix kaempferi* (Lamb.) Carrière (Masuzawa, 1985; Sakio and Masuzawa, 1988). In the alpine zone (above the timberline), the slope is thinly 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 punctata* Lam. var. *hondoensis* (Kitam.) Ohwi, and *Arabis serrata* 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 and Suzuki, 1991).

Several studies have been conducted on the moss flora in the alpine zone on Mt. Fuji (e.g., Hayata, 1911; Masuzawa, 2002; Minami and Sugimura, 2003; Takaki, 1951, 1954, 1971; Takaki and Watanabe, 1987; Umemura, 1923). These studies reported *ca.* 40 bryophyte species in total. Populations of *R. lanuginosum* grow on bare ground, which is composed of erupted product, at 1000 to 3776 m alt. on Mt. Fuji (Takaki, 1971).

2-2: Mt. Mihara

Mt. Mihara (peak, 764 m alt.) is a caldera volcano located on Izu Oshima Island, which is located at about 110 km southwest of Tokyo in the Sagami Sea (34°43′ N; 139°23′ E; Fig. 6 and 8). The island consists mainly of the cone, known as the Oshima Volcano, with an outer caldera rim measuring about 3.5 to 4.5-km in diameter. Inside the outer rim there exists a cinder cone, Mt. Mihara, which rises to *ca.* 150 to 160 m above its base and has an inner rim with a summit crater (Isshiki, 1984). Its most recent eruption was in 1986 (Koyama and Hayakawa, 1996). The ground on the summit side of the mountain is covered with a thick basaltic rock and a scoria layer formed by past eruptions (e.g., Shimoda and Fujimoto, 2005). Izu Oshima Island has a rich flora with about 550 species of vascular plants (Ohba, 1990). The vegetation on Mt. Mihara mainly consists of *F. japonica* (Houtt.) Ronse Decr. var. *hachidyoensis* (Makino) Yonek. et H.Ohashi and *Carex okuboi* Franch. (Ohba, 1990). Furuki and Higuchi (2006) recognized 144 species of bryophytes on Izu Oshima Island. Populations of *R. lanuginosum* are distributed all around Mt. Mihara (Furuki and Higuchi, 2006).

2-3: Study sites

The three study sites were as follows:

Chapter 3: Alpine zone of Mt. Fuji (between 2400 m and 3700 m alt.; Fig. 9

and 10).

Chapter 4: Okuniwa (35°23′ N, 138°42′ E; *ca.* 2200 m alt.; Fig. 11 and 12) on Mt. Fuji and the summit area on Mt. Mihara (34°43′ N, 139°23′ E; *ca.* 645 m alt.; Fig. 13 and 14).

Chapter 5: All three sites described above.

Section 3: Environment

3-1: Weather conditions

The nearest official weather station to Mt. Fuji is at Lake Kawaguchiko (35°30′ N, 138°46′ E), *ca.* 17.5 km northeast, and the nearest to Mt. Mihara is at Motomachi on Izu Oshima Island (34°45′ N, 139°22′ E), *ca.* 4.3 km northwest. The climatic data were obtained from the website of the Japan Meteorological Agency (http://www.jma.go.jp/jma/index.html). The mean monthly air temperature and monthly total precipitation at Lake Kawaguchiko and Motomachi on Izu Oshima Island are shown in Tables 1 and 2, and Fig. 15 and 16.

3-2: Micro climates

3-2-1: Methods

The mean daily air temperature and relative humidity at ground level at the study sites were measured at 1-h intervals and the data was collected using data loggers (Tid bit and Hobo pro, Onset Computer Corp., Bourne, MA, USA) housed in a plastic box (Fig. 17).

3-2-2: Alpine zone on Mt Fuji

The mean daily air temperature at the study sites on the north route on Mt. Fuji (every *ca.* 200 m alt. between 2400 m and 3700 m alt.) were measured from 17 July 2014 to 26 August 2015. The mean daily relative humidity was measured at 2200 m, 3100 m, and 3700 m alt. (Fig. 18 and 19). The mean annual air temperature and number of days when the mean daily air temperature was below zero at each site are shown in Table 3. Table 4 shows the mean monthly air temperature.

3-2-3: Okuniwa on Mt. Fuji

The mean daily air temperature and relative humidity at ground level were measured at the study sites at Okuniwa on Mt. Fuji from 15 June 2014 to 13 July 2015 (Fig. 20). Table 5 shows the mean monthly air temperature.

3-2-4: Mt. Mihara

The mean daily air temperature and relative humidity at ground level were measured at the study sites on Mt. Mihara from 15 June 2014 to 13 July 2015 (Fig. 21). Table 6 shows the mean monthly air temperature.

Chapter 3

Reproductive parameters of sexual reproduction of *R. lanuginosum* along an altitudinal gradient

Section 1: Introduction

In all plant groups, there is a general decrease in species richness with altitude (e.g., Vetaas, 1997; von Haller, 1742). This decrease in diversity is a pattern conceptually and ecologically related to the often-cited biotic depauperation from the Equator towards the poles (Körner, 2000; Lomolino, 2001). Species richness approaches zero in the most extreme environments in alpine zones, as well as in the Arctic (Bruun et al., 2006). It has been reported that species at high altitudes invest more resources in growth than in reproduction (a conservative approach), while species at low altitude 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 more severe conditions in the high mountains, where low temperatures and a duration of snow cover lead to low productivity (Körner, 2007).

The strong influence of both temperature and snow cover on the distribution and abundance of plants has resulted in numerous studies on these relationships (see Arft et al., 1999). Snow cover directly controls soil and canopy temperatures during winter (Semenchuk et al., 2013). Snow cover influences the distribution and abundance of plants both directly and indirectly by affecting their establishment, growth, reproduction, and phenology (e.g., Arft et al., 1999; Körner, 1999). Snow distribution determines the length of the growth period (e.g., Billings and Bliss, 1959; Gjærevoll, 1956; Schaminée, 1992), protects against extreme cold, strong winds, and drought (Billings and Bliss, 1959; Körner, 1999), influences nitrogen availability (Bowman, 1992), induces respiration during winter (Benedict, 1990; Friend and Woodward, 1990), and influences soil development (Johnson and Billings, 1962).

Mountainous environments are unique, as the physical distances between high and low altitude sites are short, but environmental conditions and topography may differ greatly (Korpelainen et al., 2012). Altitudinal gradients combine decreasing temperature, length of the growth period, and substrate stability, and increasing radiation and wind (Körner, 2003).

As high altitudes represent marginal habitats of sexual reproduction of *R. lanuginosum*, I hypothesized that the value of reproductive parameters of sexual reproduction of this species change at high altitudes by environmental effects such as low temperature and snow cover. The aim of this study was to clarify the changing of reproductive parameters of sexual reproduction along an altitudinal gradient.

Section 2: Materials and methods

2-1: Study sites

I selected study sites on a north trail route (called as the Yoshida trail route) every 200 m alt. between 2400 m and 3700 m alt. on Mt. Fuji (see chapter 2, Fig. 6, 7, 9 and 10). Details of study site are described in chapter 2.

2-2: Sampling and methods

On 16 July 2014, 10 patches of *R. lanuginosum* were collected by hand or spatula from each study site and allowed to dry. The size of the patches was *ca.* 100 cm^2 (Fig. 22). After sampling, every shoot of patches were dissected. A total of 13115 shoots were found from each site. 1529 shoots at 2400 m alt., 2416 shoots at 2800 m alt., 1067 shoots at 3000 m alt., 4167 shoots at 3200 m alt., 2110 shoots at 3500 m alt., and 1826 shoots at 3700 m alt., were collected (Table 13). Each shoot were separated into sexual status as male (with antheridia), female (with archegonia or sporophytes) and non-sex expressing (asexual; without gametangia or/and sporophytes). Five reproductive parameters on sexual reproduction as size of shoots, branching of shoots, sex ratio, number and developmental stage of sporophytes and number and developmental stage and size of gametangia were investigated. These parameters were observed under the binocular microscope on each shoot. At first, the number and developmental stage of sporophytes (Table 7) were observed on the sporophytes above Late calyptra in perichaetium (LCP) stage on every shoot in each altitude. After observation of sporophytes, 100 shoots were randomly selected in each study site, and the sex ratio were recorded.

Finally, the size and branching of shoots and number, developmental stage and size of gametangia were recorded on randomly selected 5 shoots in each sex in each study site. All the observations were carried out under a binocular microscope (Olympus SZ61-ILST, Olympus, Tokyo, Japan). The developmental stages of the gametangia and sporophytes were identified and described according to Ayukawa et al. (2002; Table 7).

The statistical analyses of relationship between value of reproductive parameters and altitudes were carried out with R (v. 3. 0. 1, Foundation for Statistical Computing, Vienna, Austria). The *lm* function in the *lme4* package was used in the liner model.

Section 3: Environment

3-1: Precipitation

The amount of monthly air temperature and precipitation at Lake Kawaguchiko are shown in Table 1 and Fig. 15. The results show that the relatively high monthly air temperature were recorded in July and August and the relatively low monthly air temperature were recorded in January and February on each location. The highest monthly total precipitation was recorded in October, and relatively high monthly precipitation was recorded in June and July.

3-2: Air temperature

The mean daily air temperatures at ground level in each site are shown in Fig. 18. Also, the mean annual air temperature in each site are shown in Table 3 and 4. The data show that the mean annual air temperatures decrease along with increasing altitude and the mean daily air temperatures dropped to below zero in each site at maximum during October and May. The number of days of which mean daily air temperature is below zero in each site increase along with increasing altitude (Table 3).

3-3: Relative humidity

The relative humidity at the ground level at three study sites (2200 m, 3100 m and 3700 m alt.) are shown in Fig. 19. These were almost the same in each site.

Section 4: Results

4-1: Shoot size

Table 8 summarizes the size of male and female shoots. No male shoots were found at 3500 m alt., and no female shoots were found at 3200 m alt. The size of shoots of each sex remained almost the same along the altitudinal gradient (*lm*, $P < 0.5195$ for male shoots, $P < 0.1610$ for female shoots; Tables 9–11).

4-2: Branching of shoots

The branching patterns are summarized in Table 9–11. The number of first branches per female shoot increased with increasing altitude (*lm*, $P < 0.0061$; Table 11). The number of first branches (*lm*, $P < 0.7387$; Table 10) and second and subsequent branches per male shoot (*lm*, $P < 0.9880$; Table 10) and the number of the second and subsequent branches per female shoot (*lm*, $P < 0.2580$; Table 11) remained almost the same along the altitudinal gradient.

4-3: Sex ratio

The sex ratio at each site is shown in Table 12 and Fig. 23. The frequency of sex expression was lower than 50% at every site, but sex expression was observed at every site along the altitudinal gradient.

4-4: Number and developmental stage of sporophytes

The shoots contained sporophytes and gametangia of each sex from the present year and the previous year. The number and developmental stage of sporophytes along the altitudinal gradient are shown in Tables 13 and 14. Since no sporophytes were found above 3000 m alt., it is likely that spore dispersal occurred only below 3000 m alt. The highest frequency of sporophyte maturation was at 2400 m alt.

4-5: Number, size and maturation ratio of gametangia

The total number of male and female inflorescences, antheridia, and archegonia at each site are shown in Table 15. The numbers of male and female inflorescences and gametangia per shoot and per inflorescence at each site are shown in Fig. 24. The number of male inflorescences per shoot (*lm*, $P < 0.2330$; Table 10) was almost the same along the altitudinal gradient. The number of antheridia per shoot (*lm*, $P < 0.0351$; Table 10) and the number of antheridia per inflorescence (*lm*, $P < 0.0015$; Table 10) decreased with increasing altitude. The number of female inflorescences per shoot (*lm*, $P < 0.9668$; Table 11) and the numbers of archegonia per shoot (*lm*, $P < 0.9241$; Table 11) and per inflorescence (*lm*, $P < 0.3820$; Table 11) were almost the same at all sites.

Table 16 summarizes the size of antheridia and archegonia. The size of antheridia (*lm*, $P < 0.6990$; Table 10) and archegonia (*lm*, $P < 0.8750$; Table 11) were not affected by altitude. The developmental stages and maturation ratios of antheridia and archegonia are shown in Tables 17 and 18. Most of the male and/or female gametangia matured at every site.

Section 5: Discussion

5-1: Environment

The mean annual and monthly air temperatures decreased with increasing altitude (Tables 3 and 4). It is assumed that the air temperature also decreased with increasing altitude.

The length of the growth period is thought to be restricted by several environmental factors, such as temperature and water availability. The number of days which of mean daily air temperature is below zero increased with increasing altitude (Table 3). It is assumed that as the altitude increased, the length of the growth period shortened and the environment became less suitable for growth.

5-2: Reproductive parameters on sexual reproduction

The size of shoots of both sexes did not change with increasing altitude (Tables 8 and 10). However, the number of first branches per female shoot increased with increasing altitude (Table 9 and 11). Maciel-Silva et al. (2012) reported the opposite trend; that is, there were significant differences in the production of sexual branches between two sites, with more sexual branches per mass produced at sea level than at a mountain site.

Because sex determination is likely to be chromosomal, and sex chromosomes segregate at meiosis (Ramsay and Berrie, 1982), one would theoretically expect a 1:1 sex ratio among progeny. However, the lack of one sex, or different frequencies of males and females are often observed both at the population and the species levels (e.g., Longton and Schuster, 1983).

Populations of dioecious species sometimes show biased sex ratios, spatial separation of sexes, an absence of males, or failure of sex expression in males (e.g., Gemmell, 1950; Longton and Schuster, 1983; Bowker et al., 2000; Stark et al., 2010). In this study, no relationship between the sex ratio and altitude was detected (Table 12, Fig. 23).

Males and females were present at all study sites except at 3200 m and 3500 m alt. (Table 12, Fig. 23). Only male shoots were present at 3200 m alt., and only female shoots were present at 3500 m alt. This result indicated that the production of sporophytes was restricted by the lack of females at 3200 m and the lack of males at 3500 m alt.

Sporophytes were present only below 3000 m alt. Therefore, it is likely that spore dispersal occurred only below 3000 m alt. Sexual reproduction is known to decrease along environmental gradients in the margins of species' distribution (Fisher, 2011). Fisher (2011) investigated the reproductive situation along a geographical gradient. However, information on the changes in sexual reproduction parameters along an altitudinal gradient is very limited. Douglas (1981) reported that the number of successful inflorescences per shoot of *Mimulus primuloides* decreased with increasing altitude. The results of this study show that the success of sexual reproduction of *R. lanuginosum* decreased with increasing altitude.

The causes of limited sporophyte production are: limitation of fertilization, i.e., separated male and female shoots (Longton and Schuster, 1983); environmental constraints to the fertilization process, such as a lack of water (see Clark and Greene, 1970; Longton and MacIver, 1977; Schofield, 1972);

and environmental stress at the early stages of sporophyte development, i.e., abortion before detectable sporophyte appearance (Whitehouse, 1971). Kalliio and Heinonen (1973) reported that the optimum temperature for photosynthesis of *R. lanuginosum* between −10°C and 30°C was 5°C. In this study, air temperature decreased and the length of growth period shortened with increasing altitude (Tables 3 and 4, Fig. 18). This result suggested that sporophyte development was restricted due to low productivity by the low air temperature, and the shortened growth period at higher altitudes.

Most of the gametangia reached maturity at every site (Table 17 and 18), suggesting that environmental factors did not restrict the developmental processes required for gametangia to reach maturity. The number of male gametangia per shoot and per inflorescence decreased with increasing altitude (Table 10, Fig. 24). The number of gametangia was proposed to be an important factor to guarantee sufficient sperm for fertilization (Glime, 2007). The decrease in the number of male gametangia at higher altitudes is likely to be the reason for the limited sporophyte reproduction at high altitudes. The number of female gametangia did not decrease with increasing altitude (Table 11, Fig. 24). This difference in abundance between male and female gametangia may have been due to the different costs of the production and development of male and female gametangia. The production of male gametangia, compared with female ones, is more energetically expensive for bryophytes (Stark et al., 2000, 2005). The results suggest that the occurrence and development of male gametangia were restricted due to low productivity by low air temperature and the shortened growth period as a result of snow cover.

Consequently, the possible reasons for the limited sporophyte reproduction at the study sites are: limited fertilization because of the absence of sex expression and the lack of male gametangia; and the inhibitory effects of low temperature and the shortened growth period on sporophyte development. The possible causes for reduced male gametangia reproduction at higher altitudes are low temperatures and the shortened growth period.

At the sites studied here, the air temperature decreased and the length of the growth period shortened with increasing altitude. The decreased production of sporophytes and male gametangia at higher altitudes is likely caused by low temperature and the shortened growth period. In seasonal environments, appropriate timing of reproduction is critical for maximizing fitness (Reekie and Bazzaz, 1987; Kozlowski, 1992). Flowering phenology can strongly influence the number of seeds (Alatalo and Totland, 1997; Totland, 1999). In the next study, I focused if the effect of the length of the growth period, regulated by snow-cover, on the reproductive processes of *R. lanuginosum*.

Chapter 4

Effect of environmental factors on phenological parameters associated with sexual reproduction of *R. lanuginosum*

Section 1: Introduction

Phenology is the study of the seasonality of events related to reproduction and growth during the life cycle (e.g., Longton, 1979a, 1979b; Stark, 2002). Greene (1960) and Forman (1965) established methods to identify the stages in the moss reproductive cycle. Subsequently, many studies have focused on the reproductive phenology of bryophytes (e.g., Clarke and Greene, 1970, 1971; Laaka-Lindberg, 2005; Longton, 1979a, 1979b; Longton and Greene, 1969; Stark, 2002; Zehr, 1979). Bryophytes show a number of phenological patterns that reflect their adaptation to a wide variety of environments (e.g., Stark, 2002).

The length of the growth period is thought to be restricted by environmental factors such as temperature, water availability, and snow (Billings and Bliss, 1959; Gjærevoll, 1956; Schaminée, 1992). The timing of flowering and dormancy in alpine plants is strongly affected by the timing of snow appearance and/or disappearance (Holway and Word 1963, 1965; Bliss, 1971; Ram et al., 1988). Snow cover has been suggested as a principal factor controlling the length of the growth period (e.g., Wijk, 1986). Snow cover responds to modest fluctuations in climatic conditions such as temperature, precipitation, and wind (Johnson and Billings, 1962; Staanton et al., 1994; Walker et al., 1993). Snow protects against extreme cold, strong winds, and drought (Billings and Bliss, 1959; Körner, 1999), influences nitrogen availability (Bowman, 1992), induces respiration during winter (Benedict, 1990; Friend and Woodward, 1990), and affects soil development (Johnson and Billings, 1962). In this study, I focused on the effect of the length of the growth period regulated by snow cover.

Several previous studies have compared the reproductive responses of various moss species in distinct habitats (e.g., Clarke and Greene, 1971; Longton, 1979b). Clarke and Greene (1971) compared the reproductive responses of two species both within and between isolated populations in the Arctic, the north temperate region, and the sub-Antarctic. Longton (1979b) analyzed growth and reproduction in relation to the microclimates in boreal forest, sub-Arctic, and Antarctic populations of mosses. These studies reported that the development of antheridia and sporophytes was suspended under snow cover, and also that mosses developed faster in cold habitats than in warmer ones. Many reports have described the suspended development of antheridia and sporophytes under snow cover (Ayukawa et al., 2002; Laaka-Lindberg, 2005; Longton, 1966, 1972; Miles et al., 1989; Sagmo Solli et al., 1998). The phenological pattern of gametangia and sporophyte maturation in *R. lanuginosum* growing at Okuniwa has already been published (Maruo and Imura, 2016).

In this study, I compared the phenological patterns of gametangia and sporophyte maturation in *R. lanuginosum* between two sites: Okuniwa on Mt. Fuji (*ca.* 2200 m alt.) and Mt. Mihara (*ca.* 645 m alt.), to clarify the effect of snow cover.

Section 2: Materials and methods

2-1: Study sites

I selected study sites on a northwest-facing ridge called as Okuniwa (35°23′ N, 138°42′ E; Fig. 11 and 12) at *ca.* 2200 m alt. on Mt. Fuji which is seasonally snow-covered place, and on open site (34°43′ N; 139°23′ E; Fig. 13 and 14) at *ca.* 645 m alt. on Mt. Mihara which is a snow free place. Details of study site were described in chapter 2.

Overview of vegetation of study areas were described in chapter 2. The vegetation at Okuniwa was mainly consisted of *Larix kaempferi* (Lamb.) Carrière, *Abies veitchii* Lindl., *Tsuga diversifolia* (Maxim.) Mast., *Vaccinium vitis-idaea* L., *Rhododendron brachycarpum* D. Don ex G. Don. The forest floor was covered by a homogeneous population of *R. lanuginosum*. The vegetation on Mt. Mihara was consisted by *Miscanthus condensatus* Hack. and *Fallopia japonica* (Houtt.) Ronse Decr. var. *hachidyoensis* (Makino) Yonek. et H.Ohashi and homogeneous population of *R. lanuginosum*.

2-2: Sampling and methods

Samples of *R. lanuginosum* were collected every 2 weeks from 2 June to 15 November 2014 at Okuniwa on Mt. Fuji, and every 1 month from 28 March 2014 to 15 February 2015 at Mt. Mihara by hand or spatula, and allowed to dry. The size of sampling patches is *ca.* 100 cm^2.

After sampling, 10 male shoots and 10 female shoots were randomly selected in each sample. Each shoot were observed under a binocular microscope (Olympus SZ61-ILST; Olympus, Tokyo, Japan) and the following phenological parameters of sexual reproduction were recorded: number of inflorescences and number, size, and developmental stages of male and female gametangia and sporophytes. The measurement and description of the developmental stages for antheridia, archegonia and sporophytes (Table 7) were following Ayukawa et al. (2002). When determining the size of gametangia and sporophytes, only those in the active stage (un-dehisced) were measured by Microscale slide (Watson 177-401C, Watson, Tokyo, Japan).

Section 3: Growth environment

3-1: Okuniwa on Mt. Fuji

The mean daily air temperature and relative humidity at ground level at Okuniwa on Mt. Fuji are shown in Fig. 20. Both the mean daily air temperature and relative humidity were stable from mid-December, 2014, to the end of April, 2015. Consequently, I assumed that the site was covered by snow during this period. Table 1 and Fig. 15 show that the amount of monthly precipitation at Lake Kawaguchiko. The highest precipitation was recorded in October, and relatively high precipitation in June and July.

3-2: Mt. Mihara

The mean daily air temperature and relative humidity at ground level at Mt. Mihara are shown in Fig. 21. During winter, the stable period on daily air temperature and relative humidity were no observed. Consequently, it is assumed that the snow-covered period was not present through the whole year at Mt. Mihara. Table 2 and Fig. 16 show that the amount of monthly precipitation at Motomachi on Izu Oshima Island. The highest precipitation was recorded in October, and relatively high precipitation in June and July. It is assumed that the pattern of rain fall were almost the same on Mt. Fuji and Mt. Mihara.

Section 4: Results at Okuniwa

Development of antheridia, archegonia and sporophytes of *R. lanuginosum* at Okuniwa are shown in Table 19. Antheridia in the juvenile (J; abbreviations are shown in Table 7) stage were found at the beginning of August. Antheridia in the immature (I) stage were found in June and from August to November. Mature (M) stage antheridia appeared during June and July. Archegonia in the J stage were only found in June, and archegonia in the I and M stages were present at the same time. M stage archegonia were present from June to September. Swollen venter (SV) stage sporophytes appeared at the end of June. Early calyptra in perichaetium (ECP) stage sporophytes were found in August. Late calyptra in perichaetium (LCP) stage sporophytes were found at the beginning of September. Sporophytes at the early calyptra intact (ECI) stage were present from September to November and developed to the early operculum intact (EOI) stage by November. At the beginning of June, sporophytes in the EOI and the late operculum intact (LOI) stages were present. Sporophytes at the operculum fallen (OF) stage were present throughout the growth period.

Size of antheridia, archegonia and sporophytes are shown in Fig. 25. Antheridia were *ca.* 0.2 mm long in August and gradually developed to *ca.* 0.4 mm by November. After the duration of snow cover, the antheridia were *ca.* 0.5 mm long, and the mature antheridia were *ca.* 0.5 mm long. In June, the archegonia were *ca.* 0.4 mm long, and then decreased gradually to *ca.* 0.3 mm. The size of the sporophytes was *ca.* 1.0 mm in June, and they increased rapidly to *ca.* 6.0 mm long from mid-August to mid-September, and gradually elongated to *ca.* 8.0 mm long by mid-November. After the snow cover duration, the sporophytes were *ca.* 8.0 mm long, and mature sporophytes were *ca.* 8.0 mm long.

Section 5: Results at Mt. Mihara

Development of antheridia, archegonia and sporophytes of *R. lanuginosum* at Mt. Mihara are shown in Table 20. Antheridia in the J stage were found from September until December. The I stage antheridia were found from October to May. Antheridia in the M stage were present from at the end of January to June. I could not find J stage archegonia. Archegonia in the I stage present only in April. Archegonia in the M stage appeared during May and June. SV stage sporophytes appeared from May to September. Sporophytes in the ECP stage were present in September and developed to the late calyptra intact (LCI) stage by November. In December, sporophytes in the ECI, the LCI, the EOI, the LOI and the OF stages were present. Sporophytes in the OF stage were present during December and January, and from March to September.

Size of antheridia, archegonia and sporophytes are shown in Fig. 26. Antheridia were *ca.* 0.2-mm long in September and gradually increased to *ca.* 0.5-mm in mature stage. In April, the archegonia were *ca.* 0.2-mm long, and increased rapidly to *ca.* 0.6-mm by May. The Size of the sporophytes were *ca.* 1.0 mm in May, and gradually increased to *ca.* 2.0 mm by September. They increased rapidly to *ca.* 6.0 mm long from September to October, and gradually elongated to *ca.* 7.0 mm long by December, and the mature sporophytes were *ca.* 7.0 mm long.

Section 6: Discussion

The timing and stages of development of male and female gametangia at each site are depicted in Fig. 27 and 28, following Imura (1994).

The J-stage antheridia were present from August to November at Okuniwa (Table 19), and for a longer period (September to December) at Mt. Mihara (Table 20). It is assumed that antheridia were continuously produced during these periods at each site. Antheridia in the I stage were found in June and from August to November at Okuniwa. The antheridia were in the I stage when they were covered by snow, and resumed development in the early spring of the following year (Table 19). Antheridia in the M stage appeared at the end of January, and persisted until June at Mt. Mihara (Table 20). It is assumed that the sperm were dispersed continuously during this period. At Okuniwa, M-stage antheridia were present only in June and July (Table 19). The sperm dispersal period was longer at Mt. Mihara than at Okuniwa (Fig. 27 and 28). The antheridia took 4.5 (-10)-months to reach maturity at Mt. Mihara, compared with (7-) 10 months at Okuniwa (including the 4.5-months resting period under snow cover). Consequently, the duration of antheridia development was nearly the same at Okuniwa and Mt. Mihara.

The archegonia at Okuniwa took less than 1 month to mature (Table 19). Archegonia in the I stage appeared in April at Mt. Mihara (Table 20), and M-stage archegonia were present during May and June. Therefore, they took about 1.5-months to reach maturity. Consequently, the maturation period of archegonia was shorter at Okuniwa than at Mt. Mihara (Fig. 27 and 28). The tendency for a shorter archegonial maturation period at snow-covered sites than at snow-free sites has been reported in several other studies (Clarke and Greene, 1971; Longton, 1979a).

Sporophytes in the SV stage were present during May and September at Mt. Mihara (Table 20). The sporophytes gradually developed to the OF stage by December. The sporophytes at Mt. Mihara took 8.5-months to mature. The sporophytes at Okuniwa took 10 months to mature, including the 4.5-months resting period under snow cover (Table 19). Consequently, the sporophyte maturation period was shorter at Okuniwa than at Mt. Mihara (Fig. 27 and 28). This trend of a shorter sporophyte maturation period at snow-covered sites than at snow-free sites has been described in several studies (e.g., Clarke and Greene, 1971; Longton, 1979a).

At each site, antheridia took longer to mature than did archegonia (Fig. 27 and 28), a trend that has been described for several dioecious mosses (e.g., Ayukawa et al., 2002; Imura, 1994; Longton, 1972; Longton and Greene, 1967; Miles et al., 1989; Sagmo Solli et al., 1998). A possible explanation for this difference is that in dioecious species, male plants expend more resources on sex expression than do female plants (Stark, 2002).

Sporophytes in the SV stage appeared in May at Mt. Mihara (Table 20), and fertilization was presumed to occur during this period. At Okuniwa, SV-stage sporophytes appeared in late June (Table 19). The fertilization period at each site corresponded to the rainy season. This trend has been reported in numerous studies (e.g., Oliveira and Pôrto, 2001), and is related to the requirement of bryophyte sperm for water to reach the ovum (e.g., Wyatt, 1982).

The sizes of the antheridia and sporophytes were almost the same before and after the duration of snow cover at Okuniwa (Fig. 25). The sporophytes remained in the EOI stage during the duration of snow cover, and started to mature in the following spring. The duration of snow cover represented a resting period in the development of the antheridia and sporophytes. A resting period during sporophyte development has been reported for mosses in sub-Antarctic, sub-Arctic, and subalpine regions (Ayukawa et al., 2002; Longton, 1966, 1972, 1979b; Sagmo Solli et al., 1998), and for a few moss species growing in the lower mountain zones in Japan (Deguchi and Hidaka, 1987; Duguchi and Takeda, 1986). Therefore, it is assumed that snow cover is not a barrier to the reproduction of these species.

At Okuniwa, although the development of antheridia and sporophytes stopped under snow cover for 4.5 months, gametangia maturation and spore dispersal occurred in June, the usual season for fertilization and spore dispersal in bryophytes (Fig. 27).

An interesting phenological pattern observed at Mt. Mihara was that sperm were dispersed from antheridia before the archegonia matured (Fig. 28). This suggested that fertilization would be difficult because of the different maturation timing of male and female gametangia. Also, spore dispersal at Mt. Mihara mainly occurred during January and the following September, and especially in winter. Spore dispersal in winter has been reported previously (e.g., Nishimura, 1993), but is quite rare in bryophytes. These phenological patterns observed at Mt. Mihara indicated that this site was close to the limit of successful sexual reproduction, based on the discordance of phenological parameters between male and female shoots. In conclusion, this comparative study revealed notable phenological differences between males and females in two distinct habitats. Iwatsuki (2001) reported that *R. lanuginosum* distributed in subalpine and alpine zones in Japan. It is assumed that Mt. Mihara, a low mountain in temperate island, is unusual site of distribution for this species.

Chapter 5

Comparative study of morphological parameters between male and female individuals of *R. lanuginosum*

Section 1: Introduction

The number of male and female gametangia differed along the altitudinal gradient (Chapter 3), and the phenological patterns differed between male and female shoots (Chapter 4). Therefore, male and female shoots showed different sexual reproduction responses to the growth environment. Further analyses were conducted to identify the reasons for these differences.

Morphological characteristics provide basic information about organisms. A detailed study on morphology is required to determine the causes of the differences between males and females in terms of their sexual reproduction responses to the growth environment. Differences in morphology between the sexes can result in sex-specific responses to environmental factors (Gaber et al., 1999; Lande, 1980; Shine, 1989).

Several studies have reported differences in sex expression and sexual dimorphism in byrophytes. Rosengren and Cronberg (2014) reported on the frequency of dwarf males and fertilization, and Stark et al. (2001) investigated sex expression and sexual dimorphism by analyzing several parameters, such as biomass, branching, stem length, and number of inflorescences per shoot. Shaw and Gaughan (1993) reported sexual dimorphism based on differences in biomass, sex ratio, and bract length.

The aim of this study was to identify differences in morphological parameters between males and females of *R. lanuginosum*.

Section 2: Materials and methods

The study sites were described in Chapter 2, 3 and 4 (Fig. 7–14). In the alpine zone on Mt. Fuji, 25 male shoots (5 shoots at 2400m alt., 5 shoots at 2800 m alt, 5 shoots at 3000 m alt., 5 shoots at 3200 m alt., 5 shoots at 3700 m alt.) and 25 female shoots (5 shoots at 2400 m alt., 5 shoots at 2800 m alt., 5 shoots at 3000 m alt., 5 shoots at 3500 m alt., 5 shoots at 3700 m alt.) were collected and treated as one dataset. At Okuniwa, 10 shoots of each sex were collected on 18 July and 4 August in 2014. At Mt. Mihara, 10 shoots of each sex were collected on 26 July and 30 August in 2014.

Shoot size, branching pattern and number of branches, production of sex organs (number of inflorescences, number of gametangia per shoot and per inflorescence) and sex ratio were measured as the morphological parameters in this study. Shoots were observed under a binocular microscope (Olympus SZ61-ILST; Olympus, Tokyo, Japan). Shoot size was measured using a plastic ruler.

Statistical analyses were carried out with R (v. 3. 0. 1, Foundation for Statistical Computing, Vienna, Austria). The *Wilcox.exact* function in the *exactRankTests* package was used to conduct *t* tests. The *lm* function in the *lme4* package was used in the linear model.

Section 3: Results

3-1: Shoot morphology

The shoot morphology of *R. lanuginosum* is depicted in Fig. 29. The shoots consisted of a main stem and numerous short lateral branches (Fig. 29). The main stem elongated continuously. On all shoots, the first branches formed at almost the same height on the side of the main stem. Most of the first branches were short. When inflorescences formed at the apices of the first branches, the branches stopped developing and second branches formed beneath the inflorescences. The formation of inflorescences and subsequent branching occurred up to four times, leading to the formation of first, second, third and fourth branches.

Table 21 summarizes the shoot size, number of first, second and subsequent branches, inflorescences, and gametangia per shoot and number of gametangia per inflorescence at each site.

3-2: Sex ratio

The sex ratios at Okuniwa and Mt. Mihara are shown in Table 22 and Fig. 30. The ratio of male shoots:female shoots:shoots without sex expression was 38:29:33 at Okuniwa and 22:65:13 at Mt Mihara.

3-3: Differences on morphology of shoot between male and female

The branching pattern and formation of sexual organs on male and female shoots at each site are shown in Tables 23–28. In the alpine zone, there were no significant differences between males and females in terms of shoot size (*lm*, $P < 0.8810$; Table 26), number of first branches per shoot (*lm*, $P < 0.5850$; Table 26), and number of inflorescences per shoot (*lm*, $P < 0.0604$; Table 26). Compared with female shoots, male shoots had significantly higher numbers of second and subsequent branches per shoot (*lm*, $P < 0.0079$; Table 26), gametangia per shoot (*lm*, $P < 0.0059$; Table 26), and gametangia per inflorescence (*lm*, $P < 0.0004$; Table 26). Compared with small shoots, large shoots had significantly higher numbers of first branches per shoot (*lm*, $P < 0.2000\text{e-}09$; Table 26), inflorescences per shoot (*lm*, $P < 0.0122$; Table 26), and gametangia per shoot (*lm*, $P < 0.0406$; Table 26).

At Okuniwa on Mt. Fuji, there were no significant differences between males and females in terms of shoot size (*Wilcox exact*, $P < 0.1324$) and number of first branches per shoot (*lm*, $P < 0.3012$; Table 27). Compared with female shoots, male shoots formed significantly higher numbers of second and subsequent branches per shoot (*lm*, $P < 0.0160$; Table 27), inflorescences per shoot (*lm*, $P < 0.0332$; Table 27), gametangia per shoot (*lm*, $P < 0.0001$; Table 27), and gametangia per inflorescence (*lm*, $P < 0.2000\text{e-}15$; Table 27). Compared with smaller shoots, larger shoots formed higher numbers of first branches (*lm*, $P < 0.4870\text{e-}04$; Table 27), second and subsequent branches per shoot (*lm*, $P < 0.0270$; Table 27), inflorescences per shoot (*lm*, $P < 0.0277$; Table 27), and gametangia per shoot (*lm*, $P < 0.0361$; Table 27).

At Mt. Mihara, female shoots were significantly larger than male shoots (*Wilcox exact*, $P < 0.1500\text{e-}07$). Compared with female shoots, male shoots had higher numbers of first branches (*lm*, $P < 0.0004$; Table 28), second and subsequent branches (*lm*, $P < 0.0002$; Table 28), inflorescences (*lm*, $P <$

0.0002; Table 28), gametangia per shoot (*lm*, $P < 0.6930\text{e-}08$; Table 28), and gametangia per inflorescence (*lm*, $P < 0.2000\text{e-}15$; Table 28). Compared with smaller shoots, larger shoots had higher numbers of first branches per shoot (*lm*, $P < 0.5830\text{e-}08$; Table 28) and gametangia per inflorescence (*lm*, $P < 0.0277$; Table 28).

3-4: Morphological comparison among sites

The branching pattern and formation of sex organs at each site are summarized in Tables 29–33.

Comparing Okuniwa and the alpine zone on Mt. Fuji, there were no significant differences in the number of first branches per shoot (*lm*, $P < 0.2240$ for males; Table 30, $P < 0.1600$ for females; Table 33), number of second and subsequent branches per shoot (*lm*, $P < 0.3973$ for males; Table 30, $P < 0.9480$ for females; Table 31), and number of inflorescences per shoot (*lm*, $P < 0.8701$ for males; Table 30, $P < 0.6325$ for females; Table 31). There were no significant differences in the number of gametangia per male shoot (*lm*, $P < 0.7405$; Table 30) or in the number of gametangia per inflorescence (*lm*, $P < 0.1510$; Table 31) on female shoots. The shoot size (*Wilcox exact*, $P < 0.5960\text{e-}07$ for males, $P < 0.5960\text{e-}07$ for females) of both sexes was significantly larger at Okuniwa than in the alpine zone. The number of gametangia per inflorescence (*lm*, $P < 0.0014$; Table 30) on male shoots was higher at Okuniwa than in the alpine zone. The number of gametangia per female shoot (*lm*, $P < 0.0003$; Table 31) was higher in the alpine zone than at Okuniwa.

Comparing Okuniwa on Mt. Fuji and Mt. Mihara, there were no significant differences in the number of first branches per shoot (*lm*, $P <$ 0.0521 for males; Table 32, $P <$ 0.9037 for females; Table 33), number of second and subsequent branches per shoot (*lm*, $P <$ 0.0777 for males; Table 32, $P <$ 0.0507 for females; Table 33), number of inflorescences per shoot (*lm*, $P <$ 0.0693 for males; Table 32, $P <$ 0.8126 for females; Table 33), number of gametangia per inflorescence (*lm*, $P <$ 0.1080 for males; Table 32, $P <$ 0.3520 for females; Table 33), and number of gametangia per male shoot (*lm*, $P <$ 0.1524; Table 32). On the other hand, the shoot size (*Wilcox exact*, $P <$ 7.2540 for males, $P <$ 0.1386e-05 for females) of both sexes was larger at Okuniwa than at Mt. Mihara. The number of gametangia per female shoot (*lm*, $P <$ 0.0259; Table 33) was significantly higher at Mt. Mihara than at Okuniwa.

Effect of size of shoot presented on the number of first branches per shoot (*lm*, $P <$ 0.7250e-05 for males; Table 30, $P <$ 0.1250e-07 for females; Table 31), number of inflorescences per shoot (*lm*, $P <$ 0.0217 for male; Table 30, $P <$ 0.0032 for female; Table 31) and number of gametangia per shoot (*lm*, $P <$ 0.0142 for males; Table 30, $P <$ 0.0032 for females; Table 31) on both sexes, the number of second and subsequent branches per shoot (*lm*, $P <$ 0.0176; Table 30) on male in comparison between alpine zone and Okuniwa. Between Okuniwa and Mt. Mihara, the number of first branches per shoot (*lm*, $P <$ 0.1710e-04 for males; Table 32, $P <$ 0.1330e-05 for females; Table 33) on both sexes, the number of second and subsequent branches per shoot (*lm*, $P <$ 0.0038; Table 34) and number of inflorescences per shoot (*lm*, $P <$ 0.0100; Table 32) and number of gametangia per shoot (*lm*, $P <$ 0.0069; Table 31) on male were represented the effect of size of shoot.

Section 4: Discussion

The shoots of *R. lanuginosum* consist of a main stem and numerous short lateral branches (Table 21, Fig. 29), a structure consistent with that reported by Tallis (1959a). Farge-England (1996) recognized that *R. lanuginosum* has a monopodial branching pattern and cladocarpic inflorescence formation, consistent with the findings of this study.

The results of the sex ratio analysis showed that the sex expression rate (ratio of sexual shoots) was higher at Mt. Mihara than at Okuniwa on Mt. Fuji, and much lower in the alpine zone on Mt. Fuji (Table 22, Fig. 30). In addition, a female-biased sex ratio was found only at Mt. Mihara.

Analyses of the morphological parameters of this species showed that the number of second and subsequent branches per shoot, the number of gametangia per shoot, and the number of gametangia per inflorescence were significantly higher on male shoots than on female shoots (Table 21, 24–29). There were also more inflorescences per shoot on male plants than on female plants. That is, the male shoots produced more branches than did female shoots, and formed many inflorescences at the tips of branches, thereby producing more gametangia than did female shoots. This tendency for male shoots to produce more gametangia has been reported in several studies (e.g., Miles and Longton, 1989; Laaka-Lindberg, 2005; Stark et al., 2000). In dioecious mosses, it appears to be more energetically expensive to produce male gametangia than to produce female ones (Stark, 2002; Stark et al., 2000, 2005). The results of this study indicate that the male shoots allocated more resources than did female shoots for the production of numerous gametes.

Glime (2007) reported that numerous gametangia on male plants guarantee a higher fertilization rate because numerous sperm reach the archegonia. A possible explanation for the difference in the number of branches, inflorescences, and gametangia between male and female shoots of this species is that the male plants make a greater investment in sexual reproduction, and produce a large number of sperm to increase the chances of fertilization.

Morphological parameters such as shoot size and the number of gametangia per shoot and per inflorescence differed among sites (Tables 29–33). Also, some morphological parameters specific to male and female plants differed between sites. The number of female gametangia per shoot was significantly lower in Okuniwa than in the alpine zone and on Mt. Mihara (Tables 29, 31, and 33), and the number of male gametangia per inflorescence was significantly lower in the alpine zone than in Okuniwa (Tables 29 and 30). On the other hand, there were no major differences among sites in the number of male gametangia per shoot and the number of female gametangia per inflorescence.

In this study, detailed analyses revealed substantial differences in morphological parameters and the responses of reproductive organs to the growth environment between male and female plants. These differences are discussed in detail in Chapter 6.

Chapter 6
General discussion

The aim of this study was to clarify the restricting parameters for sexual reproduction around the limits of sexual reproduction of *R. lanuginosum*. Three studies were conducted to achieve this aim:

1. an analysis of the reproductive parameters of sexual reproduction of *R. lanuginosum* along an altitudinal gradient;
2. a clarification of the effects of environmental factors on phenological factors associated with the sexual reproduction of *R. lanuginosum*; and
3. a comparative study of morphological parameters of male and female *R. lanuginosum* individuals.

This chapter is a general discussion of the results of these three studies.

Chapter 3: Changes in sexual reproduction parameters along an altitudinal gradient

Production of sporophytes occurred only below 3000 m alt. in the alpine zone on Mt. Fuji (Tables 13 and 14). Aborted sporophytes at the EOI stage were found mainly at 3000 m alt. (Table 14). In males, the number of gametangia per shoot (lm, $P < 0.0351$; Table 10) and per inflorescence (lm, $P < 0.001472$; Table 10) decreased with increasing altitude (Fig. 24). However, in females, these parameters did not change significantly with increasing altitude (Table 11, Fig. 24). Most of the male and female gametangia reached maturity at each sites (Tables 17 and 18). Possible reasons for the limited sporophyte production at higher altitude on Mt. Fuji include the limitation of fertilization by the absence of sex expression, the lack of antheridia, and the abortion of sporophyte during the early developmental stage under the low temperature and shortened growth period at high altitudes.

Chapter 4: Effect of duration of snow cover on phenology

The effect of the growth environment, especially snow cover on the reproductive process of *R. lanuginosum* were studied in two distinct habitats; a seasonally snow-covered site (Okuniwa, located at *ca.* 2200 m alt. on Mt. Fuji) and a snow-free site (*ca.* 645 m alt. on Mt. Mihara). At both sites, antheridia took longer to mature than archegonia (Tables 19 and 20, Figs. 27 and 28), and antheridia and sporophytes developed through the winter. At Okuniwa, the development of antheridia and sporophytes was suspended under snow cover for 4.5 months, and maturation of both gametangia and spore dispersal occurred in the rainy season (Table 19, Fig. 27). Some notable phenological patterns included the dispersal of sperm from antheridia before archegonia matured, and spore dispersal during winter at Mt. Mihara (Table 20, Fig. 28). These results highlight the differences between males and females in sexual reproduction responses to the growth environment.

Chapter 5: Differences in morphological parameters between males and females

The shoots of *R. lanuginosum* exhibit the monopodial branching and cladocarpic inflorescence formation (Fig. 27). Analyses of parameters related to sexual reproduction showed that the number of second and subsequent branches per shoot, and the number of gametangia per shoot and per inflorescence were significantly higher in males than in females (Tables 21, 23–28). Additionally, the number of inflorescences per shoot was higher in males than in females.

Shoot size and the number of gametangia per shoot and per inflorescence (Tables 29–33) were found to differ between sites. In addition, some morphological parameters differed between males and females at different sites, such as the number of archegonia per shoot.

General discussion

As described in Chapter 3, sporophyte production occurred only below 3000 m alt. in the alpine zone on Mt. Fuji (Table 14). The number of antheridia per shoot (*lm*, $P < 0.0351$; Tables 9 and 10, Fig. 24) and per inflorescence (*lm*, $P < 0.0015$; Tables 9 and 10, Fig. 24) decreased with increasing altitude. According to the environmental data summarized in

Chapter 2, air temperature decreased with increasing altitude in the alpine zone on Mt. Fuji (Tables 3 and 4, Fig. 18). The low temperatures that occurred during the growth period are assumed to have restricted the production and development of sporophytes and male gametangia. However, the numbers of archegonia per shoot and per inflorescence were almost the same at each altitude, indicating that the archegonia growing in the alpine zone on Mt. Fuji were not affected by low temperatures (Tables 9 and 11, Fig. 24).

As shown in Chapter 4, temperature and relative humidity remained relatively stable during the winter at Okuniwa (Fig. 20), suggesting that the site was covered by snow throughout the winter. Several studies have reported that snow cover shortens the growth period but plays an important role in protecting the plant canopy from winter temperatures and desiccation (e.g., Billings and Bliss, 1959; Holway and Ward, 1963). Phenological observation of the population at Okuniwa (Table 19, Figs. 25 and 27) showed that the development of antheridia and sporophytes was suspended under snow cover. The high maturation rate of antheridia (Table 17) and the low number of aborted sporophytes (Table 14) suggested that these antheridia and sporophytes were covered by snow and protected from low temperatures during winter.

The temperatures was colder and both temperature and relative humidity were less stable in the alpine zone than Okuniwa on Mt. Fuji (Figs. 18 and 20). It is assumed that the snow cover in the alpine zone on Mt. Fuji was shallower because of strong winds. This shallower snow layer would not have protected the plants from low temperatures and desiccation in winter. Low temperatures during winter in the alpine zone on Mt. Fuji were likely responsible for the limited production of sporophytes, and lower numbers of antheridia per shoot and per inflorescence at that site. Furthermore, the sporophytes and antheridia in the alpine zone were exposed to blasts of cold air during development. These periods of low temperatures in winter may have damaged the developing sporophytes and gametangia.

The shorter growth period is another possible explanation for the limited production of sporophytes at high altitudes on Mt. Fuji. Phenological observation indicated that sporophytes needed 5.5–8.5-months and antheridia needed 4.5–10 months to reach maturity (Tables 19 and 20, Figs. 27 and 28). At high altitudes in the alpine zone on Mt. Fuji, the growth period was approximately 4 months, lasting from June to September (Tables 3 and 4). This short growth period in the alpine zone was likely insufficient for sporophytes and antheridia to reach maturity. Therefore, sporophytes and antheridia would need more than two growing seasons to reach maturity in the alpine zone.

The difference in the timing of maturation between male and female gametangia was another possible cause for the limited sporophyte production at high altitudes on Mt. Fuji. Phenological observation showed that the gametangia of both sexes remained at the mature stage for only 1–2 months (Tables 19 and 20, Figs. 27 and 28). Successful fertilization requires simultaneous maturation of male and female gametangia. The duration of the growth period decreased with increasing altitude, which affected the timing of male and female gametangia development. This effect, ultimately resulted in male and female gametangia reaching maturity at different times.

Although the development of sporophytes and antheridia was suspended under snow cover for 4.5-months at Okuniwa, male and female gametangia matured and spores were dispersed in June (Table 19, Fig. 27). This coincided with the rainy season, which is the main period of fertilization and spore dispersal for bryophytes (e.g., Stark, 2002). Notably, in the population on Mt. Mihara, sperm release began in winter before archegonia reached maturity (Table 20, Fig. 28); furthermore, spores were dispersed from winter to spring. These phenological features of the *R. lanuginosum* population on Mt. Mihara may have been induced by the continuous development of sporophyte and antheridia during the snow-free winter season. These results suggest that the phenological features of this species are well-fitted to the seasonally snow-covered site and that Mt. Mihara was close to the geographical limit of sexual reproduction for this species.

In the marginal area of sexual reproduction, sexual reproduction was restricted by several reproductive parameters, including fewer gametangia, altered timing of fertilization and abortion. Furthermore, male and female individuals showed different responses to environmental factors. The results of this study suggest that reduced number of antheridia played a particularly important role in restricting sporophyte production in the marginal area of sexual reproduction.

Future work

This study was carried out to determine the environmental limits on sexual reproduction in *R. lanuginosum*. Further investigations into the behaviors and environmental factors related to the limits on sexual reproduction and the subsequent limits of geographical distribution (Fig. 1) are required to clarify the process of restriction of sexual reproduction.

The current study focused on air temperature, growth period duration and relative humidity as environmental variables that changed along the altitudinal gradient. However, it has been reported that altitudinal gradients also affect substrate stability and increasing radiation and wind in addition to temperature and growth period (Körner, 2003). Thus, it will be necessary to also investigate the effects of other environment factors, such as radiation and wind, on reproductive parameters in future studies.

To gain a deeper understanding of the process by which sexual reproduction is restricted, physiological factors such as photosynthetic activity should also be investigated. In addition, the relationship between sexual reproduction-related behaviors and physiological factors must be better understood to clarify which reproductive parameters and environmental factors restrict sexual reproduction.

As described in Chapter 5, male shoots had a significantly higher number of second and subsequent branches per shoot and significantly more gametangia per shoot and per inflorescence than females. Thus, it appears that male shoots paid a greater cost than females to produce numerous gametes and increase the chance of fertilization. Chapter 3 describes the decreased production of antheridia along the altitudinal gradient. This decreased production suggests that male shoots shifted the relative allocation of resources between reproductive and vegetative growth according to their environment. However, the cost of producing gametangia for each sex was not measured in this study. In future studies, it will be necessary to determine the cost of producing sexual organs by both sexes to clarify their different strategies of resource allocation.

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Tables and Figures

Table 1. Mean monthly air temperature and total monthly precipitation at Lake Kawaguchiko, at the foot of Mt. Fuji, from July 2014 to June 2015.

	2014						2015						Total
	7	8	9	10	11	12	1	2	3	4	5	6	
Temp. (℃)	22.1	22.4	17.2	13.2	8.2	1.2	0.3	0.4	4.9	10.4	16.0	17.2	
Prec.	315.5	186.0	61.5	395.0	52.5	59.0	87.5	29.0	88.5	133.0	64.5	193.5	1665.5

Table 2. Mean monthly air temperature and total monthly precipitation at Motomachi on Izu Oshima Island from July 2014 to June 2015.

	2014						2015						Total
	7	8	9	10	11	12	1	2	3	4	5	6	
Temp. (℃)	24.8	25.6	22.4	18.8	15.1	9.0	7.5	7.8	11.0	14.3	19.2	20.6	
Prec.	362.0	114.0	83.5	454.5	128.0	192.5	165.0	163.0	245.0	315.1	101.0	312.0	2636.0

Table 3. Mean annual air temperature and number of days of which mean daily temperature is below zero at 2500 m, 2800 m, 3000 m, 3200 m, 3500 m and 3700 m alt. from July 2014 to June 2015.

Altitude (m)	Mean annual air temperature (℃)	Number of days of which mean daily air temperature is below zero (day)
3700	0.61	227
3500	0.83	219
3200	0.42	219
3000	3.01	218
2800	2.86	180
2500	5.88	158

Table 4. Mean monthly air temperature (℃) at 2500 m, 2800 m, 3000 m, 3200 m, 3500 m and 3700 m alt. from July 2014 to June 2015.

	2014						2015					
Altitude (m)	7	8	9	10	11	12	1	2	3	4	5	6
3700	8.3	7.4	6.1	0.5	-3.1	-5.2	-9.3	-10.8	-9.5	-0.8	0.2	6.4
3500	11.3	9.8	6.0	1.0	-4.2	-8.2	-10.7	-13.6	-10.4	-0.2	4.5	7.8
3200	11.9	10.7	6.9	1.4	-5.2	-13.0	-14.4	-14.8	-9.6	0.2	4.9	8.3
3000	14.9	13.3	9.5	3.6	-2.8	-9.0	-10.9	-13.0	-7.1	1.2	8.3	10.7
2800	15.5	14.2	9.4	4.3	-2.3	-11.6	-12.8	-13.1	-8.5	1.7	8.6	10.3
2500	16.6	16.0	11.0	6.5	0.5	-5.4	-7.9	-8.0	-3.4	4.0	11.5	12.2

Table 5. Mean monthly air temperature at Okuniwa on Mt. Fuji from July 2014 to June 2015.

	2014						2015					
	7	8	9	10	11	12	1	2	3	4	5	6
Temp. (℃)	15.3	15.6	10.4	6.9	1.1	-5.2	-3.3	-1.1	-0.3	2.7	9.1	10.5

Table 6. Mean monthly air temperature at Mt. Mihara from July 2014 to June 2015.

	2014						2015					
	7	8	9	10	11	12	1	2	3	4	5	6
Temp. (℃)	22.4	22.1	18.8	15.1	10.9	4.0	3.1	3.0	6.8	10.8	16.7	17.9

Table 7. Developmental stages of gametangia and sporophytes (following Ayukawa et al. 2002).

Stages	Explanations
Gametangia	
Juvenile (J)	Small gametangia up to half of full size
Immature (I)	Gametangia from half to full size, green
Mature (M)	Gametangia still green but with open apex. Spermatozoids are visible, in antheridia
Dehisced (D)	Gametangia brown. Antheridia empty and somewhat shrunken
Sporophytes	
Swollen venter (SV)	The lower part of the archegonium is swollen. This is the first visible sign that the archegonium is fertilized
Early calyptra in perichaetium (ECP)	Calyptra starts to emerge from the bracts
Late calyptra in perichaetium (LCP)	Calyptra from half to almost fully visible above the bracts. Seta no yet visible
Early calyptra intact (ECI)	Calyptra present. Seta ranging from just visible above the bracts to fully elongated. Capsule with the same diameter as the seta
Late calyptra intact (LCI)	Calyptra present. Capsule expanding or has attained full size
Early operculum intact (EOI)	Capsule changes color from green to brown but more than half the capsule is still green
Late operculum intact (LOI)	More than half the capsule is brown but operculum is intact
Operculum fallen (OF)	Operculum fallen and spores are dispersed
Empty and fresh (EF)	Capsules of current cycle with more than 75 % of the spores released

Table 8. Mean shoot size of male and female in each study site in alpine zone on Mt. Fuji (mean ± standard deviations (SD) (n=5).

Altitude (m)	Mean size ± SD (cm) (n=5)	
	Male	Female
3700	2.4 ± 1.1	2.4 ± 0.5
3500	-	3.2 ± 1.0
3200	2.3 ± 0.2	-
3000	1.4 ± 0.4	1.1 ± 0.8
2800	2.2 ± 0.8	2.6 ± 1.2
2400	2.8 ± 0.9	2.0 ± 0.4

Table 9. The effects of altitude on reproductive parameters of sexual reproduction. (+): increase along an altitudes, (-): decrease along an altitudes.

Reproductive parameters	Sexuality	Effect of altitude	Effect of size
Size of shoot	Male	No	-
	Female	No	-
Number of first branches / shoot	Male	No	Yes
	Female	Yes (+)	Yes
Number of second, and further branches / shoot	Male	No	No
	Female	No	No
Number of inflorescences / shoot	Male	No	No
	Female	No	Yes
Number of gametangia / shoot	Male	Yes (-)	No
	Female	No	No
Number of gametangia / inflorescence	Male	Yes (-)	No
	Female	No	No

Table 10. Parameter estimates for lm (with Gaussian distribution) fitted to the reproductive parameters on male towards altitude (as factor), size (as numeric) (n=25).

Reproductive parameter	Independent variable	Estimate	S.E.	T-value	P-value
Size of shoot	Altirude	-0.0003	0.0004	-0.6540	0.5195
Number of first branches / shoot	Altitude	-0.0007	0.0021	-0.3380	0.7387
	Size	3.9262	1.1256	3.4880	0.0021 **
Number of second, and further branches / shoot	Altitude	0.0001	0.0042	0.0150	0.9880
	Size	1.4310	2.2270	0.6420	0.5270
Number of inflorescences / shoot	Altitude	-0.0047	0.0039	-1.2260	0.2330
	Size	1.8415	2.0529	0.8970	0.3790
Number of gametangia / shoot	Altitude	-0.0574	0.0255	-2.2460	0.0351 *
	Size	14.1143	13.5967	1.0380	0.3105
Number of gametangia / inflorescence	Altitude	-0.0038	0.0011	-3.6320	0.0015 **
	Size	0.1133	0.5598	0.2020	0.8415
Size of gametangia	Altitude	-1487	4336	-0.4250	0.6990

Table 11. Parameter estimates for lm (with Gaussian distribution) fitted to the reproductive parameters on female towards altitude (as factor), size (as numeric) (n=25).

Reproductive parameter	Independent variable	Estimate	S.E.	T-value	P-value
Size of shoot	Altitude	0.0006	0.0004	1.4490	0.1610
Number of first branches / shoot	Altitude	0.0043	0.0014	3.0370	0.0061 **
	Size	5.2803	0.6559	8.0500	0.5320e-07 ***
Number of second, and further branches / shoot	Altitude	-0.0030	0.0026	-1.1620	0.2580
	Size	1.5459	1.1835	1.3060	0.2050
Number of inflorescences / shoot	Altitude	-0.0001	0.0024	-0.0420	0.9668
	Size	3.1440	1.0944	2.8730	0.0088 **
Number of gametangia / shoot	Altitude	-0.0008	0.0087	-0.0960	0.9241
	Size	11.5826	4.0057	2.8792	0.0085
Number of gametangia / inflorescence	Altitude	-0.0007	0.0008	-0.8910	0.3820
	Size	0.4317	0.3708	1.1640	0.2570
Size of gametangia	Altitude	1054	6147	0.1710	0.8750

Table 12. Number of male, female and non-sex expressing (asexual) shoots and sex ratio in each study site in alpine zone on Mt. Fuji.

Altitude (m)	Number of shoot (%)			
	Male	Female	Asexual	Total
3700	12 (12.0)	30 (30.0)	58 (58.0)	100 (100)
3500	0 (0.0)	44 (48.9)	46 (51.1)	90 (100)
3200	15 (50.0)	0 (0.0)	15 (50.0)	30 (100)
3000	13 (14.9)	26 (29.9)	48 (55.2)	87 (100)
2800	25 (25.0)	8 (8.0)	67 (67.0)	100 (100)
2400	20 (20.0)	14 (14.0)	66 (66.0)	100 (100)

Table 13. Frequency of sporophytes in each study site in alpine zone on Mt. Fuji.

Altitude (m)	Number of shoot	Number of shoot with sporophytes	Frequency of sporophytes (%)
3700	1826	0	0.00
3500	2110	0	0.00
3200	4167	0	0.00
3000	1067	27	2.53
2800	2416	5	0.21
2400	1529	21	1.37

Table 14. Developmental stages of sporophytes in each study site in alpine zone on Mt. Fuji. SV-EF: Developmental stages of sporophytes described in Table 7. Sporophyte in SV and ECP stages were not observed in this study.

Altitude (m)	SV	ECP	LCP	ECI	LCI	EOI	LOI	OF	EF
3700			-	-	-	-	-	-	-
3500			-	-	-	-	-	-	-
3200			-	-	-	-	-	-	-
3000			4	14	2	1	3	-	1
2800			-	1	-	1	-	-	1
2400			-	2	-	1	4	-	31

Table 15. Number of inflorescences and gametangia of both sexes in each study site in alpine zone on Mt. Fuji.

Altitude (m)	Total number of male inflorescences (n=5)	Total number of antheridia (n=5)	Total number of female inflorescences (n=5)	Total number of archegonia (n=5)
3700	26	103	10	30
3500	-	-	51	190
3200	35	120	-	-
3000	30	139	8	25
2800	58	458	35	81
2400	56	436	10	56

Table 16. Size of gametangia of both sexes in each study site in alpine zone on Mt. Fuji, (mean ± standard deviation (SD) (n=5).

Altitude (m)	Size of gametangia ± SD (mm) (n=5)	
	Male	Female
3700	0.41 ± 0.06	0.54 ± 0.05
3500	-	0.65 ± 0.09
3200	0.40 ± 0.08	-
3000	0.29 ± 0.08	0.53 ± 0.05
2800	0.43 ± 0.07	0.55 ± 0.08
2400	0.45 ± 0.07	0.59 ± 0.05

Table 17. Developmental stages and maturation ratio of antheridia in each study site in alpine zone on Mt. Fuji. (n=5 shoots). J-D: Developmental stages of gametangia described in Table 7.

Altitude (m)	Number of antheridia				Maturation ratio (%)
	J	I	M	D	
3700	0	0	2	22	100
3500	-	-	-	-	-
3200	0	0	0	25	100
3000	3	0	0	26	89.7
2800	1	3	3	51	93.1
2400	0	0	0	56	100

Table 18. Developmental stages and maturation ratio of archegonia in each study site in alpine zone on Mt. Fuji. (n=5 shoots). J-D: Developmental stages of gametangia described in Table 7.

Altitude (m)	Number of archegonia				Maturation ratio (%)
	J	I	M	D	
3700	0	0	5	5	100
3500	-	-	-	50	100
3200	-	-	-	-	-
3000	0	0	0	6	100
2800	0	1	5	20	96.2
2400	0	0	0	10	100

Table 19. Inflorescence number and development of antheridia, archegonia and sporophytes at Okuniwa on Mt. Fuji from 2 June 2014 to 15 October 2014. J-D, SV-EF: Developmental stages of gametangia and sporophytes described in Table 7.

Data		2014/6/2	6/14	6/29	7/18	8/4	8/24	9/6	9/18	10/4	10/16	11/1	11/15
No. of inflorescences		153	116	177	209	128	92	166	150	100	138	77	173
No. of	D	1243	802	1357	2299	1331	909	1515	1358	774	1054	366	1389
antheridia	M	119	287	415	9	-	-	-	-	-	13	-	-
	I	376	-	-	-	-	65	20	225	87	338	481	463
	J	-	-	-	-	4	56	72	41	31	73	4	19
No. of inflorescences		9	63	23	14	9	13	9	10	8	18	19	17
No. of	D	27	117	16	37	30	38	29	34	33	61	75	58
archegonia	M	-	73	8	5	-	6	2	5	-	-	-	-
	I	-	42	25	-	4	-	-	-	-	-	-	-
	J	-	7	-	-	-	-	-	-	-	-	-	-
No. of inflorescence		30	19	39	45	47	46	34	31	22	30	18	37
No. of	EF	9	13	22	26	26	33	12	11	12	2	3	13
sporophytes	OF	3	7	14	6	9	3	5	7	1	3	2	2
	LOI	16	-	-	-	-	-	1	-	-	-	-	-
	EOI	2	-	-	-	-	-	-	-	-	-	1	11
	LCI	-	-	-	-	-	-	-	-	2	6	11	12
	ECI	-	-	-	-	-	-	4	14	7	20	1	-
	LCP	-	-	-	-	-	-	8	-	1	-	-	-
	ECP	-	-	-	-	-	2	-	-	-	-	-	-
	SV	-	-	6	6	15	11	5	-	-	1	-	1

Table 20. Inflorescence number and development of antheridia, archegonia and sporophytes at Mt. Mihara from 28 March 2014 to 13 February 2015. J-D, SV-EF: Developmental stages of gametangia and sporophytes described in Table 7.

Data		2014/3/28	4/28	5/26	6/16	7/26	8/30	9/13	10/27	11/19	12/19	2015/1/29	2/13
No. of inflorescences		124	53	110	110	143	128	89	111	67	79	127	132
No. of antheridia	D	329	321	695	718	1001	1083	644	514	343	1054	670	573
	M	407	176	18	29	-	-	-	-	-	-	61	36
	I	172	4	41	-	-	-	-	90	217	276	400	526
	J	-	-	-	-	-	-	8	134	39	13	-	-
No. of inflorescences		4	6	25	31	13	19	12	10	18	54	30	100
No. of archegonia	D	20	3	66	131	40	71	48	32	69	234	132	510
	M	-	-	13	5	-	-	-	-	-	-	-	-
	I	-	12	-	-	-	-	-	-	-	-	-	-
	J	-	-	-	-	-	-	-	-	-	-	-	-
No. of inflorescence		21	13	30	23	39	30	26	25	23	27	21	19
No. of sporophytes	EF	5	9	14	9	15	15	12	12	-	1	6	2
	OF	2	2	1	4	3	6	7	-	-	1	2	-
	LOI	10	1	1	-	-	-	-	-	-	10	7	12
	EOI	2	-	-	-	-	-	-	-	-	11	2	2
	LCI	-	-	-	-	-	-	-	4	11	3	-	2
	ECI	-	-	-	-	-	-	-	6	8	2	1	-
	LCP	-	-	-	-	-	-	-	3	3	-	-	-
	ECP	-	-	-	-	-	-	2	-	-	-	-	-
	SV	-	-	17	9	22	11	6	-	2	-	-	-

Table 21. Morphological parameters on both sexes at alpine zone and Okuniwa on Mt. Fuji and Mt. Mihara, (mean ± standard deviations (SD) (n=5 shoots)

Morphological parameters	Sexually	Mean ± SD		
		Alpine zone	Okuniwa	Mt. Mihara
Size of shoot (cm)	Male	2.22 ± 0.83	4.90 ± 1.42	1.42 ± 0.38
	Female	2.26 ± 1.04	4.57 ± 1.00	2.82 ± 0.96
Number of first branches / shoot	Male	11.68 ± 5.44	17.90 ± 6.90	11.80 ± 3.38
	Female	11.24 ± 7.12	18.50 ± 4.10	13.35 ± 4.56
Number of second, and further branches / shoot	Male	10.60 ± 8.63	16.65 ± 16.84	10.35 ± 6.92
	Female	4.88 ± 5.83	6.25 ± 2.86	3.65 ± 2.35
Number of inflorescences / shoot	Male	8.20 ± 8.34	16.85 ± 14.62	13.70 ± 6.30
	Female	4.56 ± 6.08	8.65 ± 3.90	7.00 ± 2.96
Number of gametangia / shoot	Male	50.28 ± 59.80	183.00 ± 183.20	104.20 ± 51.22
	Female	15.28 ± 22.25	3.80 ± 3.62	5.85 ± 5.78
Number of gametangia / inflorescences	Male	5.66 ± 2.74	10.19 ± 1.64	7.55 ± 1.08
	Female	3.19 ± 1.80	2.33 ± 1.80	2.53 ± 1.93

Table 22. Number of male, female and non-sex expressing (asexual) shoots and sex ratio at Okuniwa on Mt. Fuji and Mt. Mihara.

Site	Number of shoot (%)			
	Male	Female	Asexual	Total
Okuniwa	38 (38)	29 (29)	33 (33)	100 (100)
Mt. Mihara	22 (22)	65 (65)	13 (13)	100 (100)

Table 23. Differences of morphological parameters between male and female and effect of size in alpine zone on Mt. Fuji.

Morphological parameters	Male ⇔ female	Effect of size
Size of shoot	=	-
Number of first branches / shoot	=	Yes
Number of second, and further branches / shoot	>	No
Number of inflorescences / shoot	=	Yes
Number of gametangia / shoot	>	Yes
Number of gametangia / inflorescence	>	No

Table 24. Differences of morphological parameters between male and female and effect of size at Okuniwa on Mt. Fuji.

Morphological parameters	Male ⇔ female	Effect of size
Size of shoot	=	-
Number of first branches / shoot	=	Yes
Number of second, and further branches / shoot	>	No
Number of inflorescences / shoot	>	Yes
Number of gametangia / shoot	>	Yes
Number of gametangia / inflorescence	>	No

Table 25. Differences of morphological parameters between male and female and effect of size at Mt. Mihara.

Morphological parameters	Male ⇔ female	Effect of size
Size of shoot	<	-
Number of first branches / shoot	>	Yes
Number of second, and further branches / shoot	>	No
Number of inflorescences / shoot	>	No
Number of gametangia / shoot	>	No
Number of gametangia / inflorescence	>	Yes

Table 26. Parameter estimates for lm (with Gaussian distribution) fitted to the morphological parameters on the differences between male and female in the alpine zone on Mt. Fuji towards sex type (as factor), size (as numeric) (n=50).

Morphological parameter	Independent variable	Estimate	S.E.	T-value	P-value
Number of first branches / shoot	Sex type	0.6453	1.1724	0.5500	0.5850
	Size	5.1322	0.6359	8.0710	0.2000e-09 ***
Number of second, and further branches / shoot	Sex type	5.7700	2.0790	2.7760	0.0079 **
	Size	1.2560	1.1270	1.1140	0.2711
Number of inflorescences / shoot	Sex type	3.7510	1.9490	1.9240	0.0604
	Size	2.7650	1.0570	2.6150	0.0120 *
Number of gametangia / shoot	Sex type	35.5630	12.3300	2.8840	0.0059 **
	Size	14.0810	6.6870	2.1060	0.0406 *
Number of gametangia / inflorescence	Sex type	2.4873	0.6554	3.7950	0.0004 ***
	Size	0.3561	0.3555	1.0020	0.3216

Table 27. Parameter estimates for lm (with Gaussian distribution) fitted to the morphological parameters on the differences between male and female at Okuniwa on Mt. Fuji towards sex type (as factor), size (as numeric) (n=50).

Morphological parameter	Independent variable	Estimate	S.E.	T-value	P-value
Number of first branches / shoot	Sex type	-1.5356	1.4646	-1.0480	0.3012
	Size	2.7928	0.6076	4.5970	0.4870e-04 ***
Number of second, and further branches / shoot	Sex type	9.2300	3.6560	2.5240	0.0160 *
	Size	3.4930	1.5170	2.3030	0.0270 *
Number of inflorescences / shoot	Sex type	7.1680	3.2400	2.2130	0.0332 *
	Size	3.0810	1.3440	2.2920	0.0277 *
Number of gametangia / shoot	Sex type	167.2700	39.4800	4.2360	0.0001 ***
	Size	35.6200	16.3800	2.1740	0.0361 *
Number of gametangia / inflorescence	Sex type	9.6263	0.3880	24.8120	<0.2000e-15 ***
	Size	0.1965	0.1609	1.2210	0.2300

Table 28. Parameter estimates for lm (with Gaussian distribution) fitted to the morphological parameters on the differences between male and female at Mt. Mihara towards sex type (as factor), size (as numeric) (n=50).

Morphological parameter	Independent variable	Estimate	S.E.	T-value	P-value
Number of first branches / shoot	Sex type	4.4620	1.1370	3.9230	0.0004 ***
	Size	4.2790	0.5690	7.5190	0.5830e-08 ***
Number of second, and further branches / shoot	Sex type	9.2830	2.2490	4.1270	0.0002 ***
	Size	1.8390	1.1260	1.6330	0.1109
Number of inflorescences / shoot	Sex type	8.9530	2.1540	4.1560	0.0002 ***
	Size	1.6040	1.0780	1.4880	0.1453
Number of gametangia / shoot	Sex type	117.7860	15.7850	7.4620	0.6930e-08 ***
	Size	13.8330	7.8990	1.7510	0.0882
Number of gametangia / inflorescence	Sex type	7.4027	0.3908	18.9440	<0.2000e-15 ***
	Size	0.4481	0.1955	2.2920	0.0277 *

Table 29. Comparison of morphological parameters between alpine zone on Mt. Fuji, Okuniwa on Mt. Fuji and Mt. Mihara, and effect of size on both sexes.

Morphological parameters	Sexuality	Effect of size	Alpine zone	⇔	Okuniwa	⇔	Mt. Mihara	Effect of size
Size of shoot	Male	-		<		>		-
	Female	-		<		>		-
Number of first branches / shoot	Male	Yes		=		=		Yes
	Female	Yes		=		=		Yes
Number of second, and further branches / shoot	Male	Yes		=		=		Yes
	Female	No		=		=		No
Number of inflorescences / shoot	Male	Yes		=		=		Yes
	Female	Yes		=		=		No
Number of gametangia / shoot	Male	Yes		=		=		Yes
	Female	Yes		>		<		No
Number of gametangia / inflorescences	Male	No		<		=		No
	Female	No		=		=		No

Table 30. Parameter estimates for lm (with Gaussian distribution) fitted to the morphological parameters on male on the comparison between alpine zone and Okuniwa on Mt. Fuji towards site (as factor), size (as numeric) (n=25 (alpine zone), 20 (Okuniwa).

Morphological parameter	Independent variable	Estimate	S.E.	T-value	P-value
Number of first branches / shoot	Site	2.8275	2.2922	1.2340	0.2240
	Size	3.3709	0.6586	5.1180	0.7250e-05 ***
Number of second, and further branches / shoot	Site	4.9240	5.7570	2.4720	0.0176 *
	Size	4.0880	1.6540	2.4720	0.0176 *
Number of inflorescences / shoot	Site	0.8499	5.1663	0.1650	0.8701
	Size	3.5395	1.4845	2.3840	0.0217 *
Number of gametangia / shoot	Site	-19.1800	57.5300	-0.3330	0.7405
	Size	42.3000	16.5300	2.5590	0.0142 *
Number of gametangia / inflorescence	Site	-3.7399	1.0941	-3.4180	0.0014 ***
	Size	0.2927	0.3144	0.9310	0.3571

Table 31. Parameter estimates for lm (with Gaussian distribution) fitted to the morphological parameters on female on the comparison between alpine zone and Okuniwa on Mt. Fuji towards site (as factor), size (as numeric) (n=25 (alpine zone), 20 (Okuniwa).

Morphological parameter	Independent variable	Estimate	S.E.	T-value	P-value
Number of first branches / shoot	Site	2.6753	1.8699	1.4310	0.1600
	Size	4.3028	0.6107	7.0460	0.1250e-07 ***
Number of second, and further branches / shoot	Site	0.1432	2.1761	0.0660	0.9480
	Size	0.6554	0.7107	0.9220	0.3620
Number of inflorescences / shoot	Site	1.0479	2.1751	0.4820	0.6325
	Size	2.2252	0.7104	3.1320	0.0032 **
Number of gametangia / shoot	Site	27.9100	6.9920	4.0010	0.0003 ***
	Size	7.1420	2.2830	3.1280	0.0032 **
Number of gametangia / inflorescence	Site	1.2139	0.8297	1.4630	0.1510
	Size	0.1544	0.2710	0.5700	0.5720

Table 32. Parameter estimates for lm (with Gaussian distribution) fitted to the morphological parameters on male on the comparison between Okuniwa on Mt. Fuji and Mt. Mihara towards site (as factor), size (as numeric) (n=20).

Morphological parameter	Independent variable	Estimate	S.E.	T-value	P-value
Number of first branches / shoot	Site	-5.4065	2.6938	-2.0070	0.0521
	Size	3.3017	0.6687	4.9380	0.1710e-04 ***
Number of second, and further branches / shoot	Site	-13.3060	7.3340	-1.8140	0.0777
	Size	5.6260	1.8200	3.0900	0.0038 **
Number of inflorescences / shoot	Site	-12.2840	6.5650	-1.8710	0.0693
	Size	4.4290	1.6300	2.7180	0.0100 **
Number of gametangia / shoot	Site	-113.6100	77.7600	-1.4610	0.1524
	Size	55.2100	19.3000	2.8600	0.0069 **
Number of gametangia / inflorescence	Site	1.4099	0.8570	1.6450	0.1080
	Size	0.3518	0.2127	1.6540	0.1070

Table 33. Parameter estimates for lm (with Gaussian distribution) fitted to the morphological parameters on female on the comparison between Okuniwa on Mt. Fuji and Mt. Mihara towards site (as factor), size (as numeric) (n=20).

Morphological parameter	Independent variable	Estimate	S.E.	T-value	P-value
Number of first branches / shoot	Site	-0.1665	1.3671	-0.1220	0.9037
	Size	3.0467	0.5290	5.7590	0.1330e-05 ***
Number of second, and further branches / shoot	Site	2.2919	1.1346	2.0200	0.0507
	Size	0.1765	0.4390	0.4020	0.6899
Number of inflorescences / shoot	Site	0.3508	1.4690	0.2390	0.8126
	Size	0.7445	0.5684	1.3100	0.1983
Number of gametangia / shoot	Site	-4.6393	1.9981	-2.3220	0.0259 *
	Size	1.4839	0.7732	1.9190	0.0627
Number of gametangia / inflorescence	Site	-0.7531	0.7991	-0.9430	0.3520
	Size	0.3194	0.3092	1.0330	0.3084

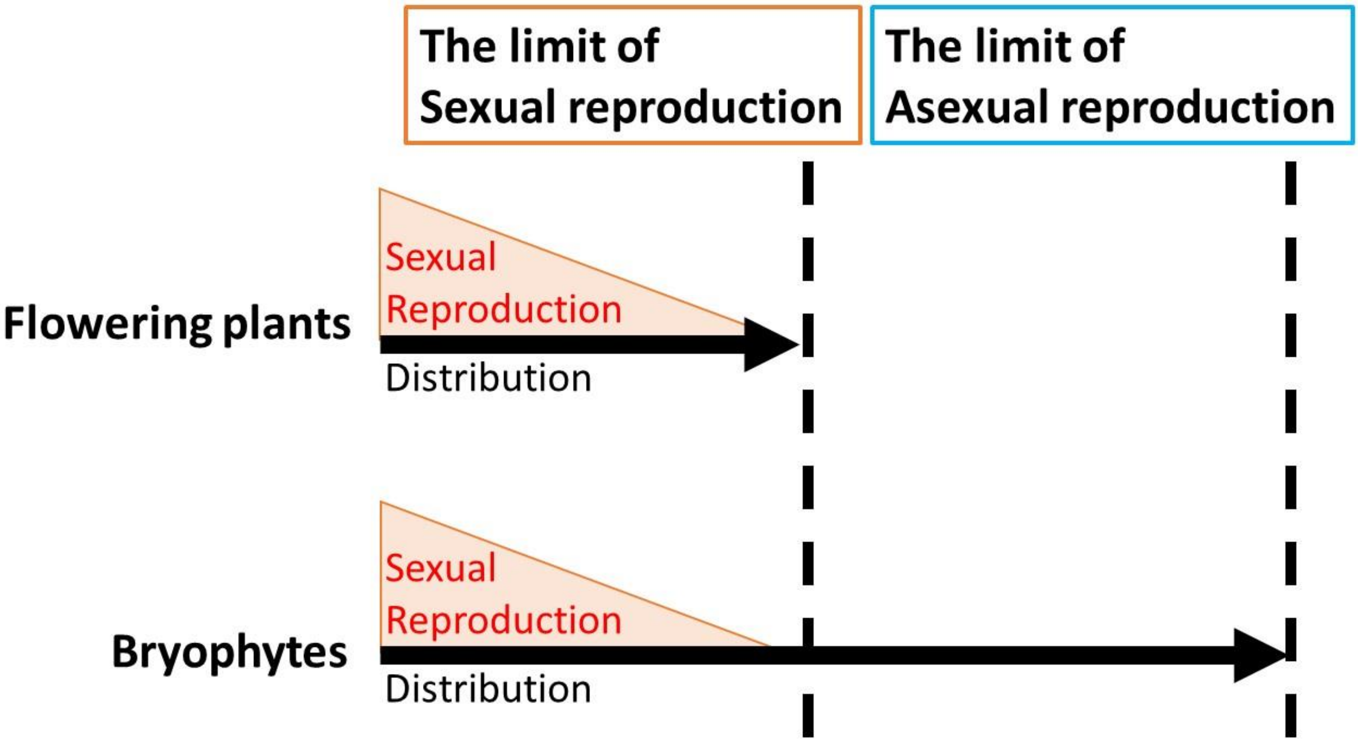

Fig. 1. Conceptual diagram of limits of sexual reproduction and asexual reproduction and distribution range of flowering plants and bryophytes.

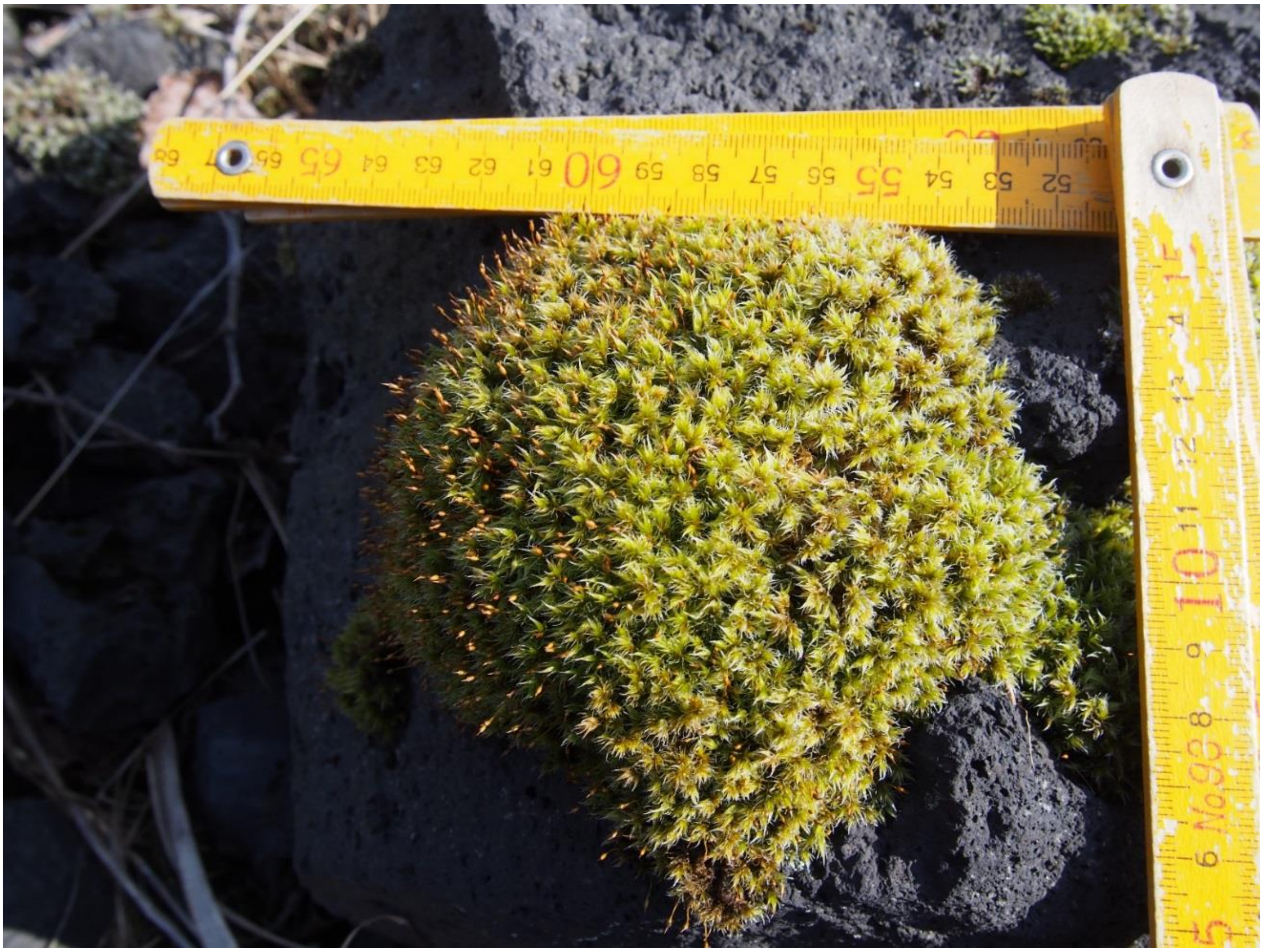

Fig. 2. Population of *Racomitrium lanuginosum* (Hedw.) Brid. at Mt. Mihara.

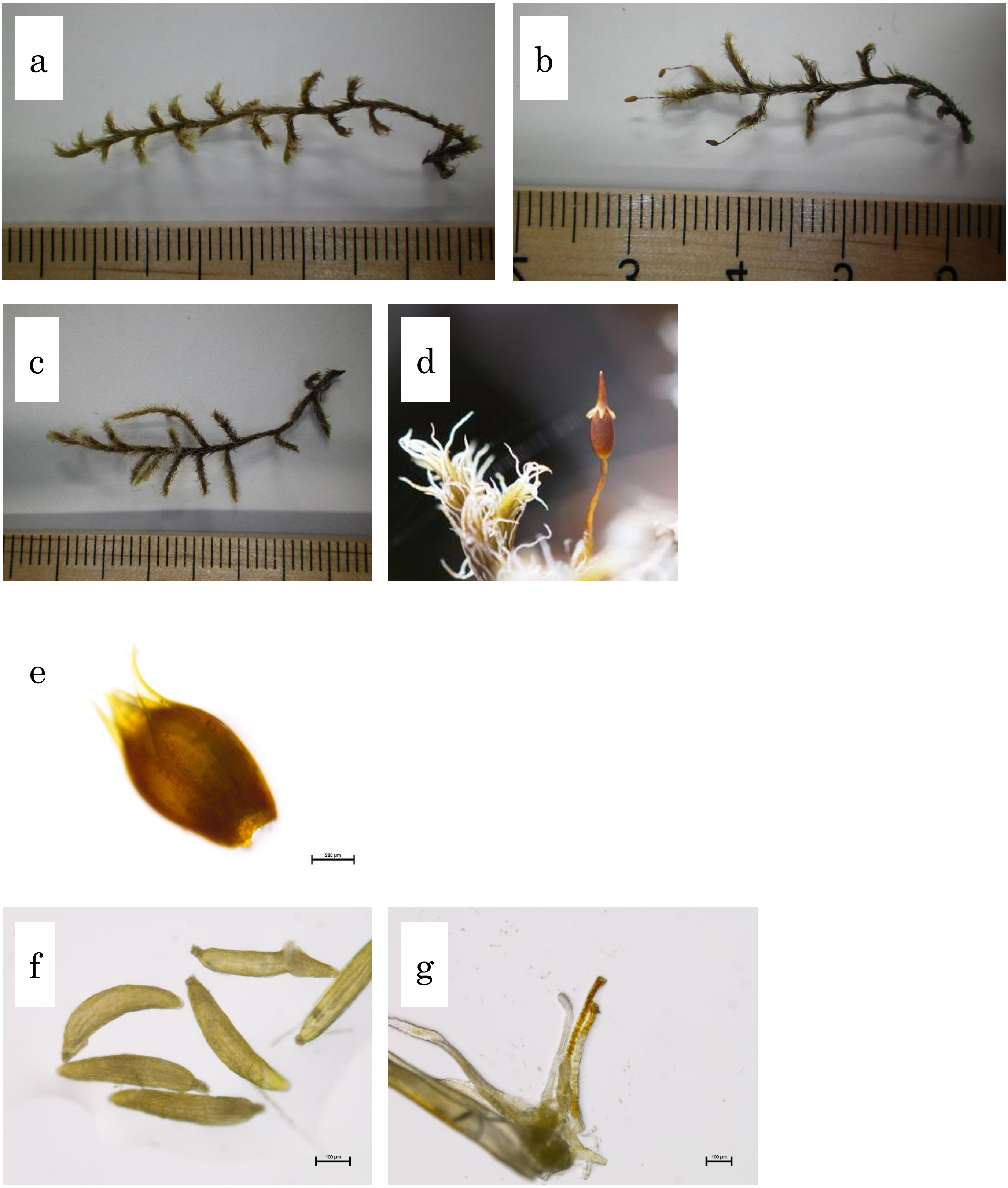

Fig. 3. Shoot and sexual organs of *R. lanuginosum*: a-male shoot, b-female shoot, c-non-sex expressing shoot (asexual), d-sporophyte, e-male inflorescence, f-antheridia (male gametangia), g-archegonia (female gametangia).

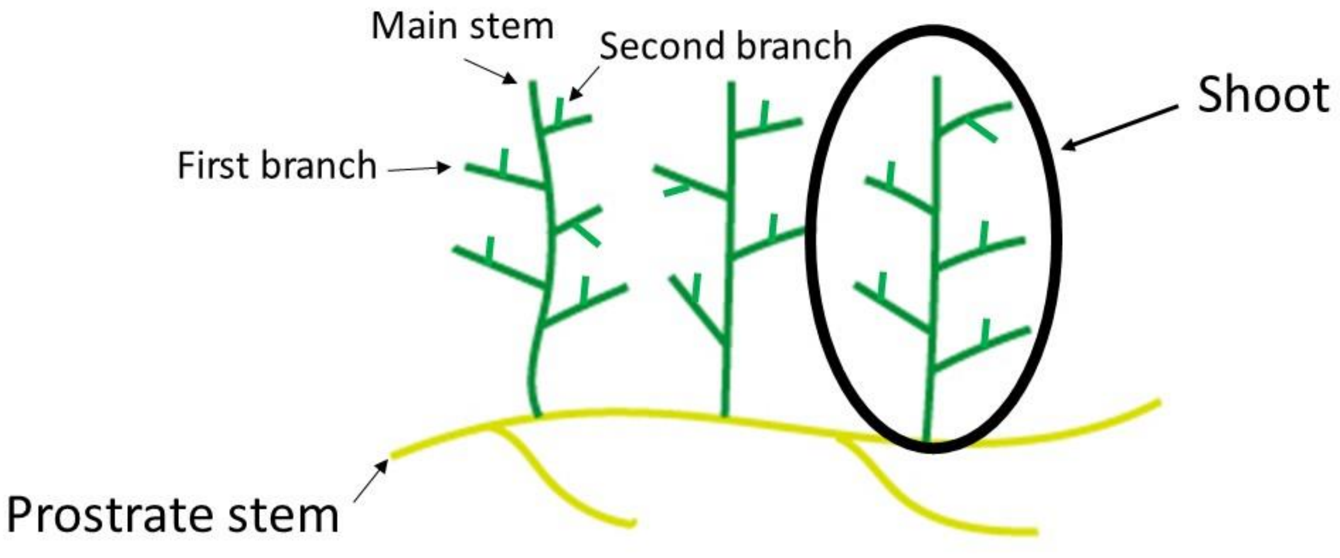

Fig. 4. Schematic diagram of structure of plant of *R. lanuginosum* and definition of the “shoot”.

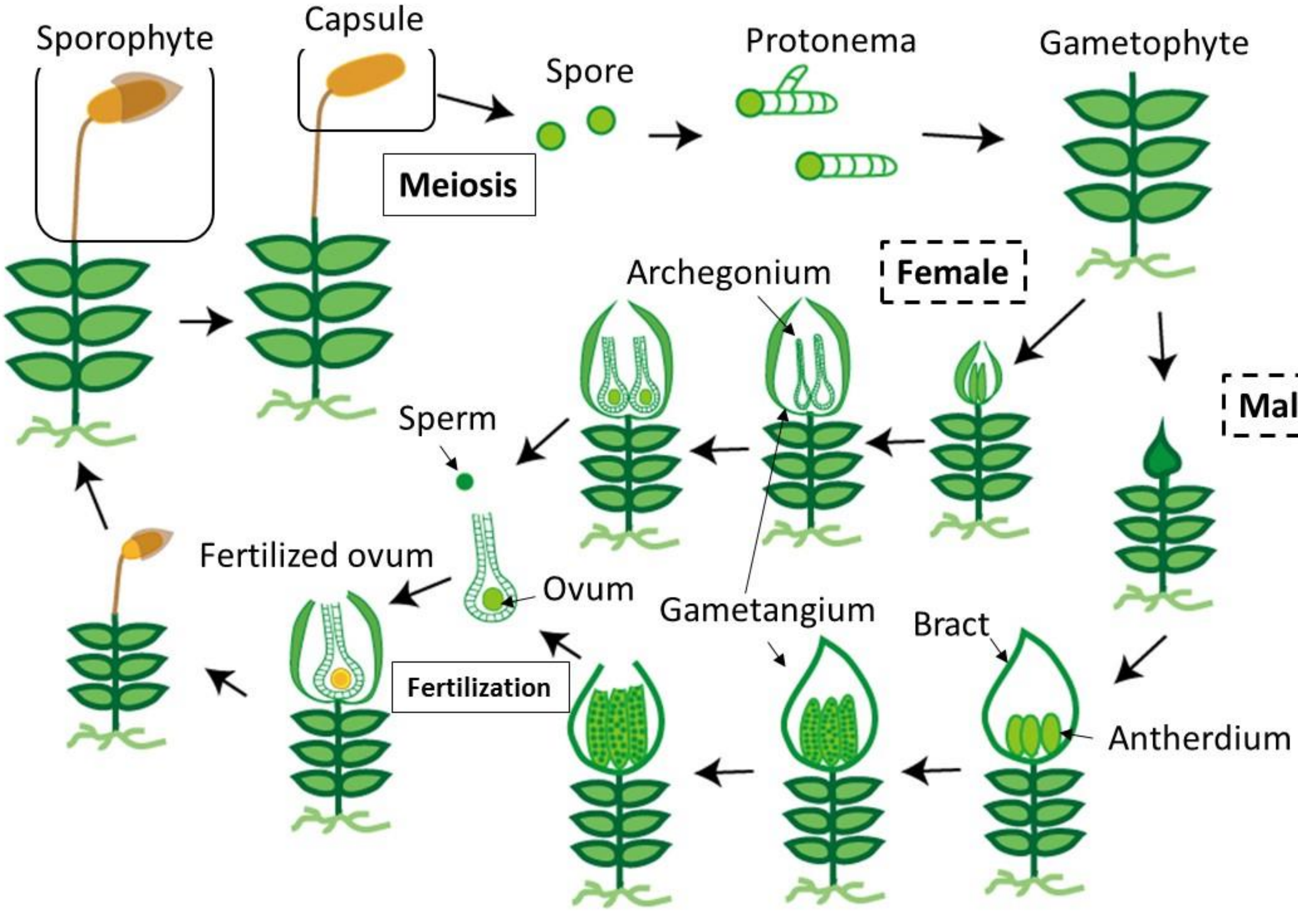

Fig. 5. Schematic diagram of sexual life cycle of *R. lanuginosum* (a dioecious mosses).

Fig. 6. Location of the study sites, Mt. Fuji (●) and Mt. Mihara (○), in Japan.

Fig. 7. Photograph of Mt. Fuji, 10 December 2011.

Fig. 8. Photograph of the summit area of Mt. Mihara, 16 June 2014.

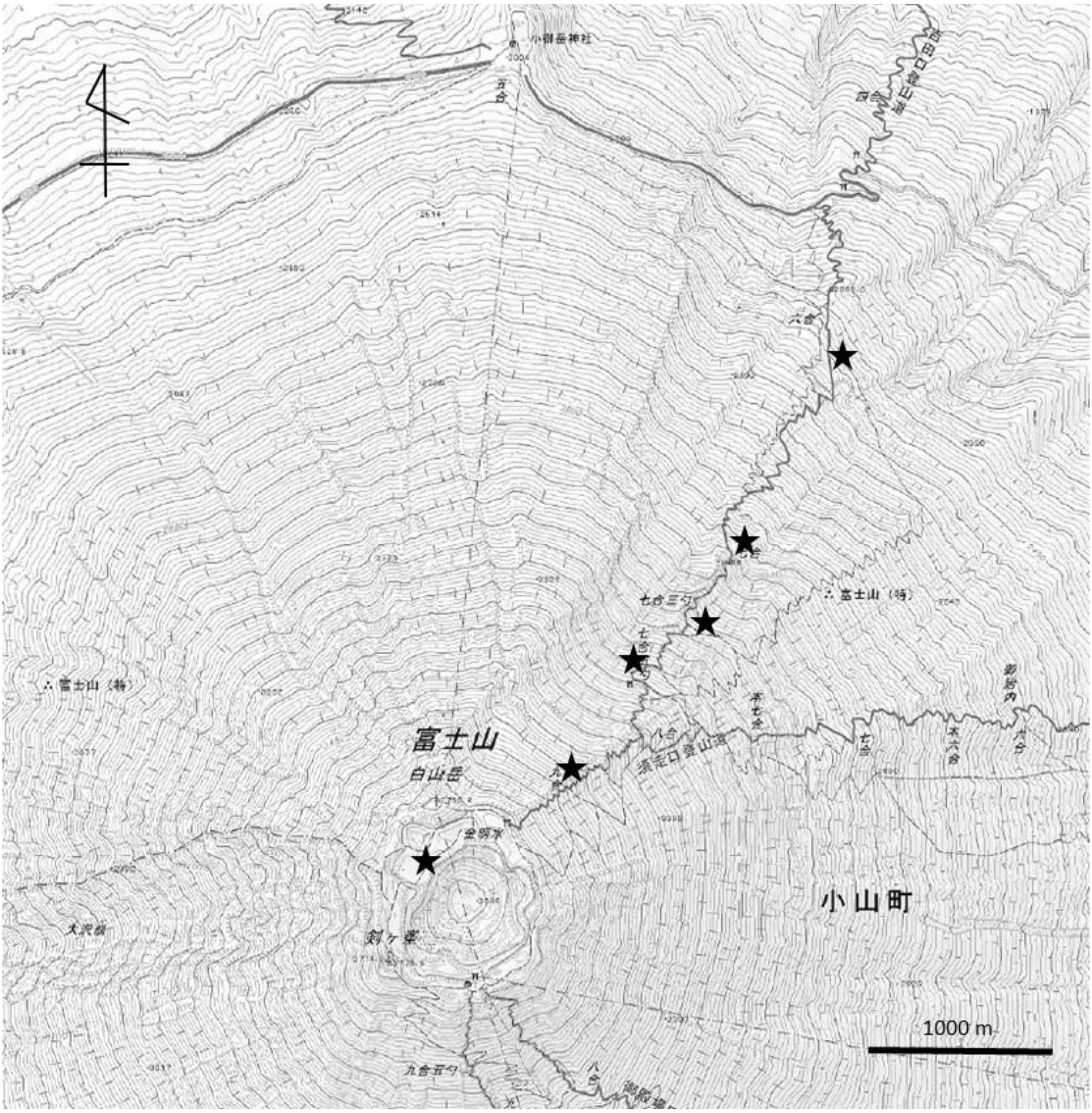

Fig. 9. Map showing alpine zone of Mt. Fuji with the location of each study site (★).

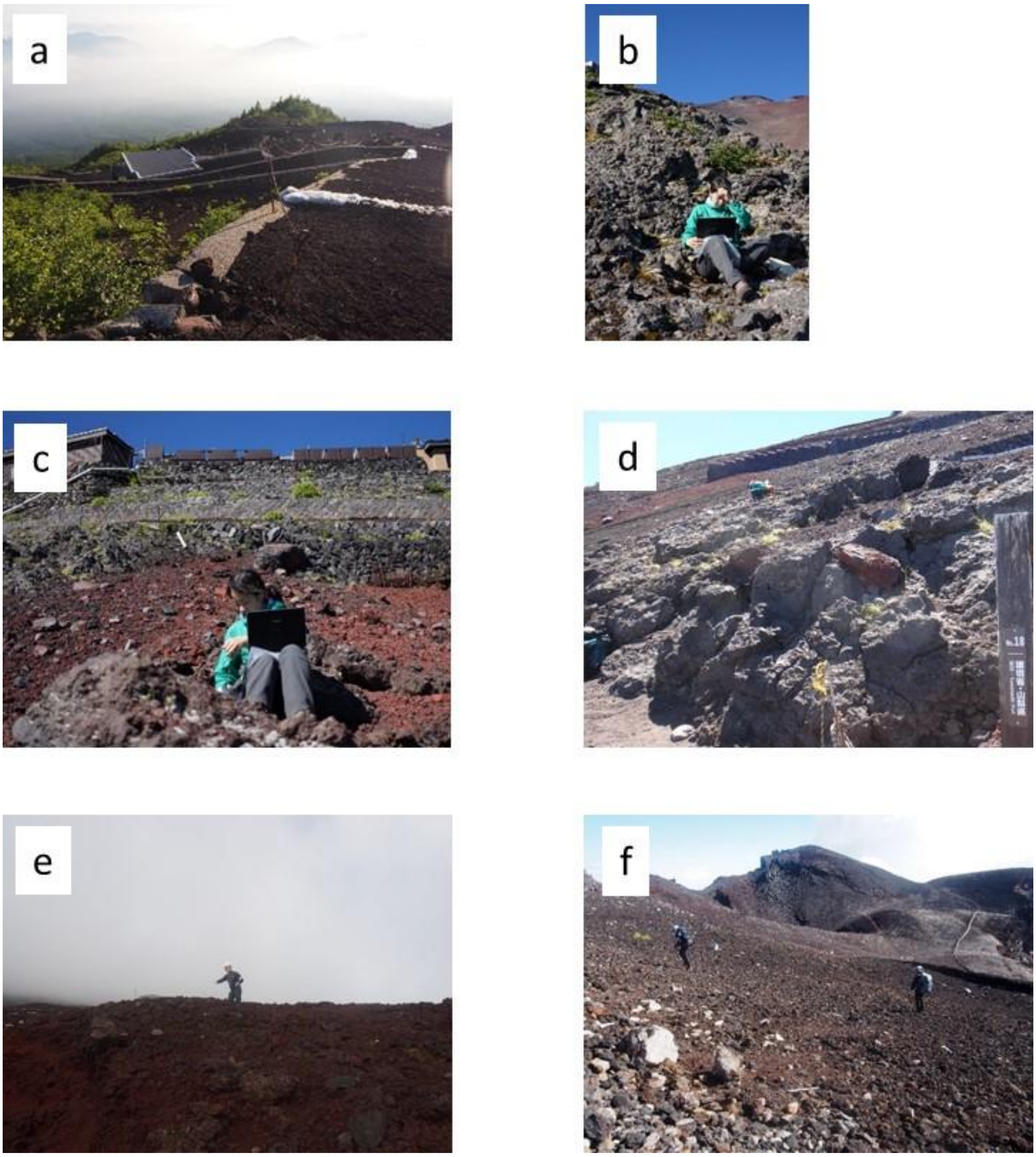

Fig. 10. Photographs of the six study sites representing an altitudinal gradient in alpine zone on Mt. Fuji: a-2400 m alt., b-2800 m alt., c-3000 m alt., d-3200 m alt., e-3500 m alt. and f-3700 m alt.

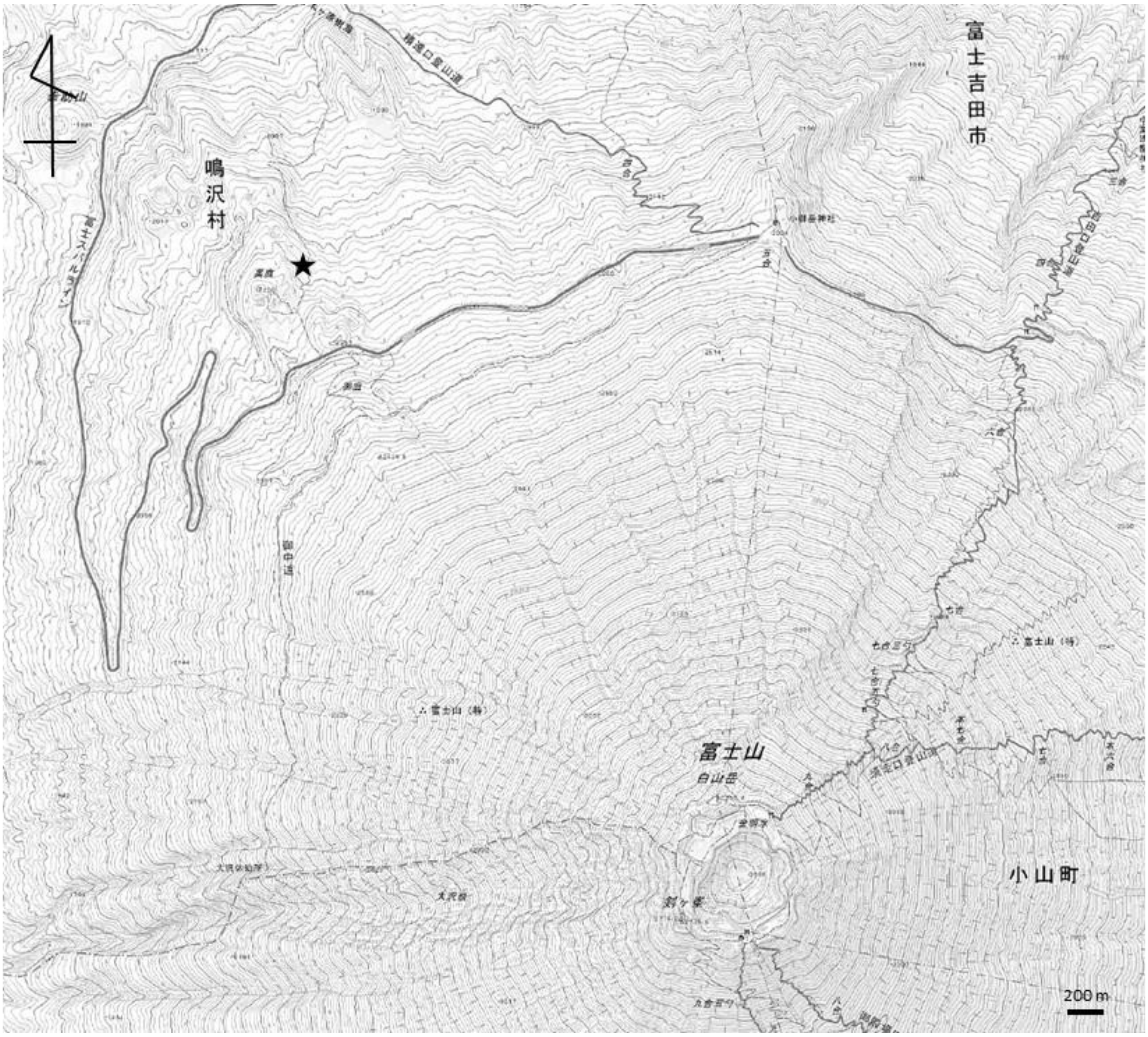

Fig. 11. Map showing Mt. Fuji with the location of study site at Okuniwa (★).

Fig. 12. Photograph of Okuniwa on Mt. Fuji, 4 October 2014.

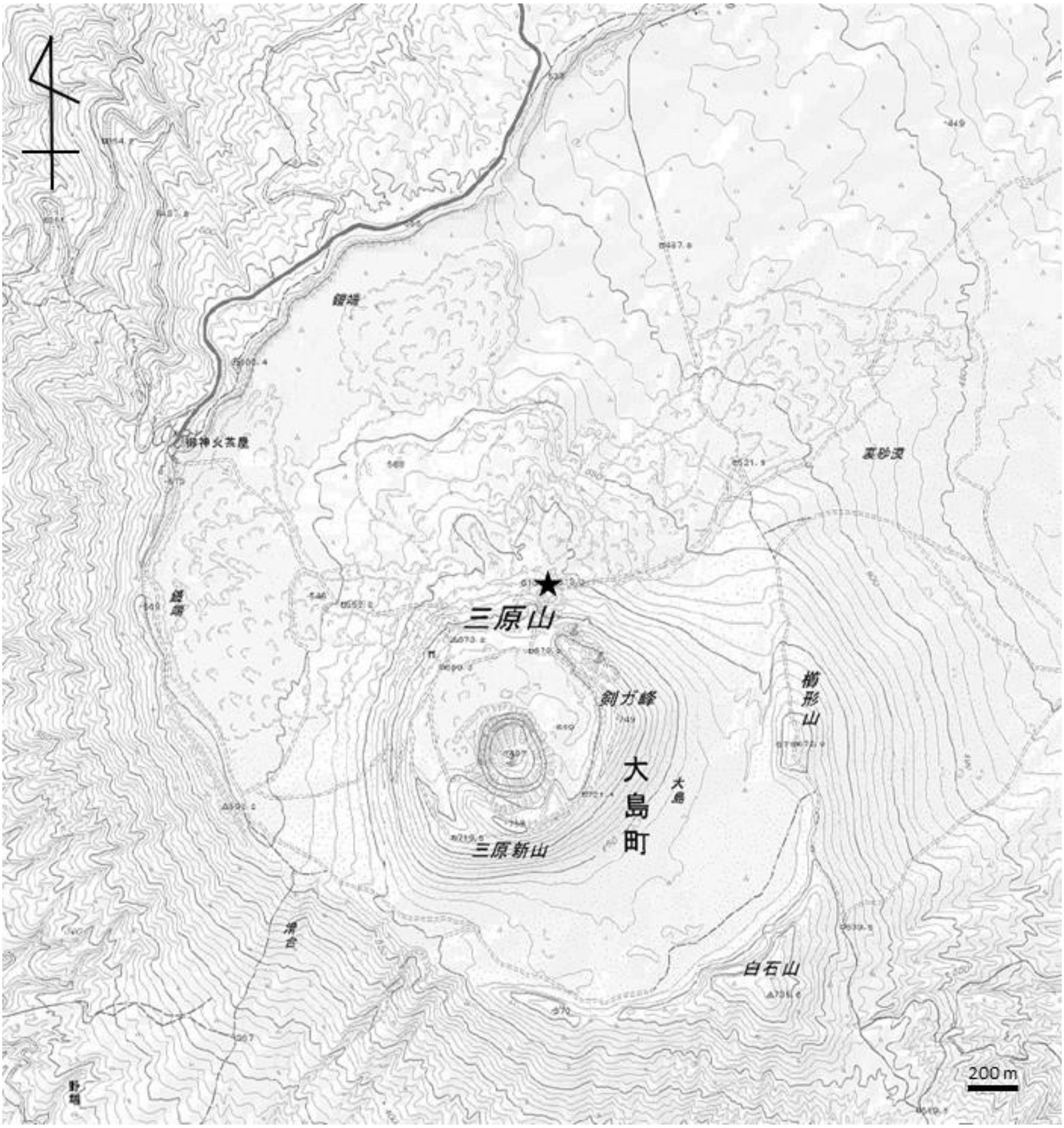

Fig. 13. Map showing Mt. Mihara with the location of study site (★).

Fig. 14. Photograph of Mt. Mihara, 17 May 2014.

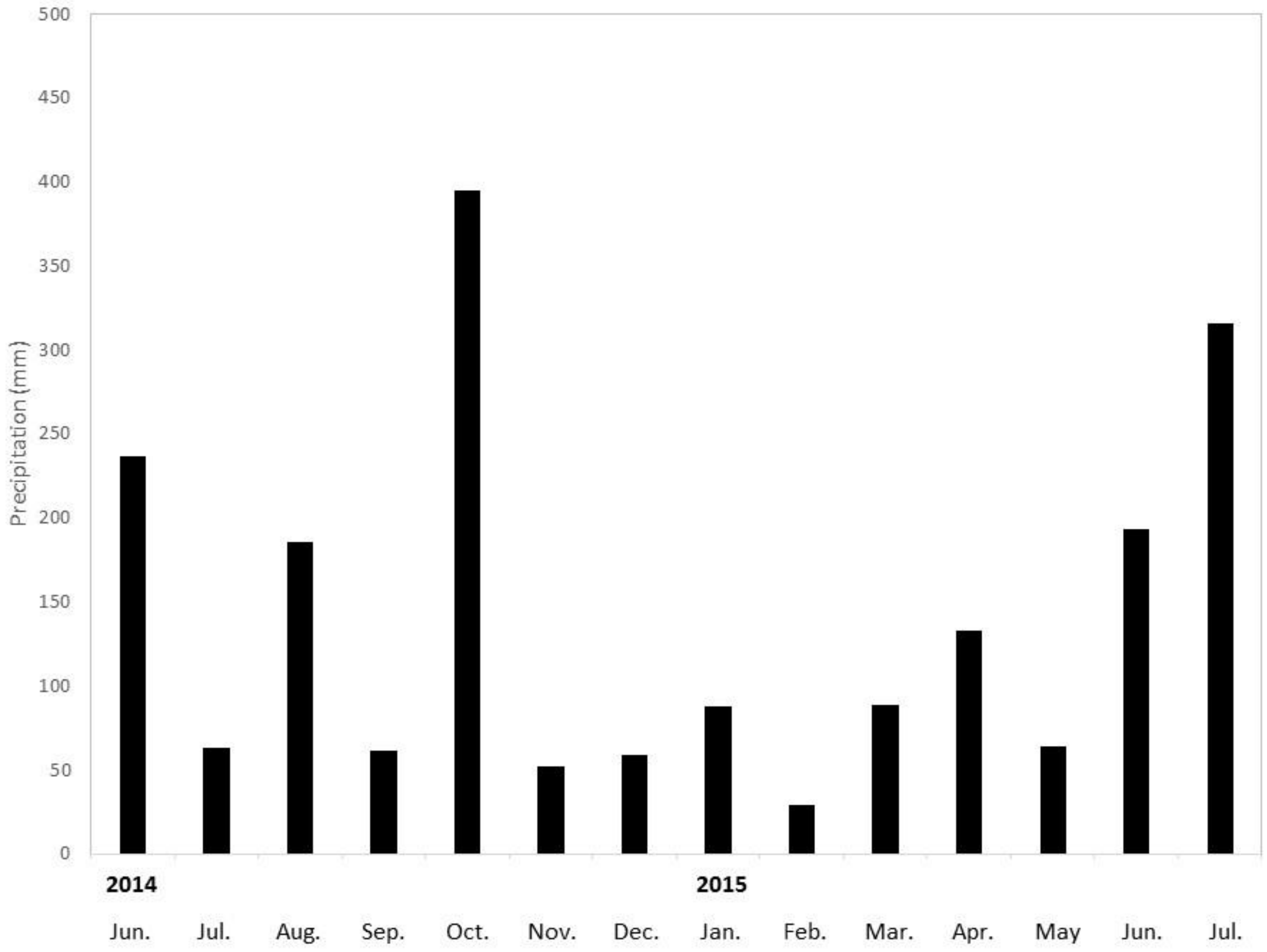

Fig. 15. Monthly precipitation (mm) at Lake Kawaguchiko from June 2014 to July 2015.

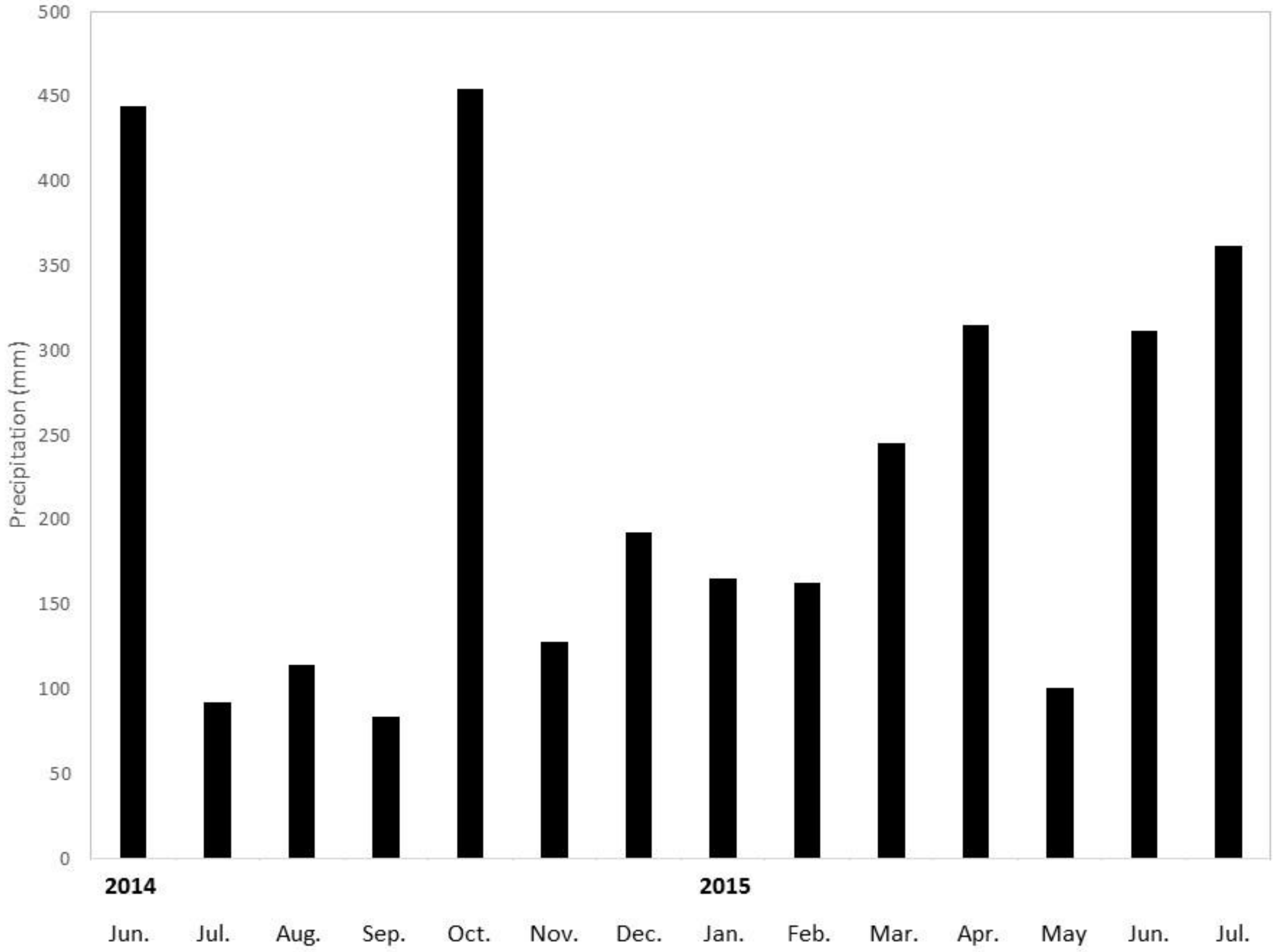

Fig. 16. Monthly precipitation (mm) at Motomachi on Izu Oshima Island from June 2014 to July 2015.

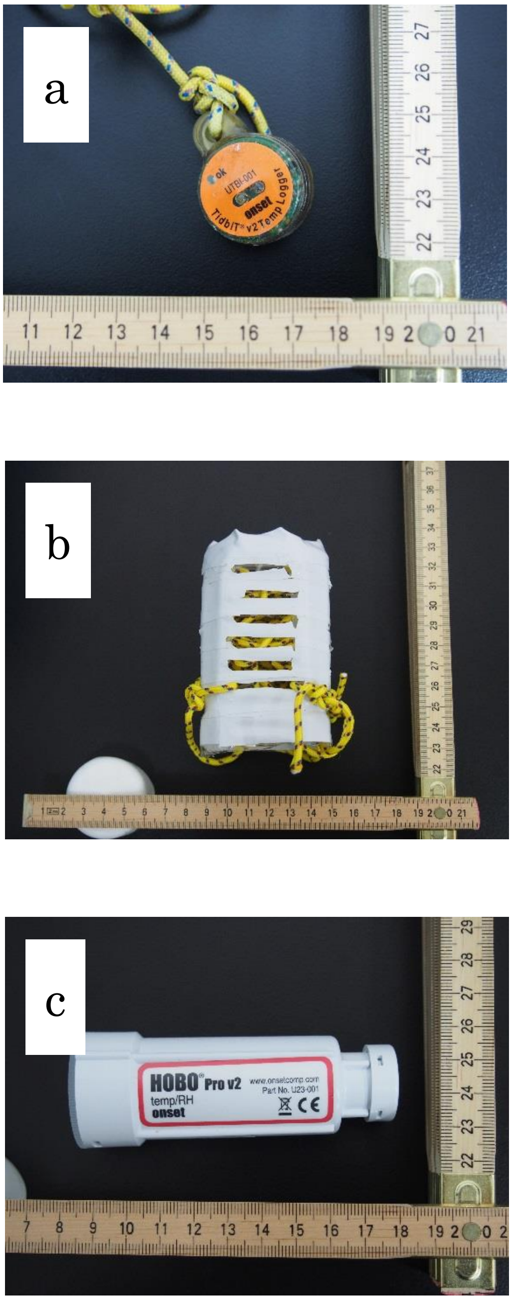

Fig. 17. Photograph of data loggers: a-Tid bit, b-Housing for Tid bit during summer, c-Hobo pro.

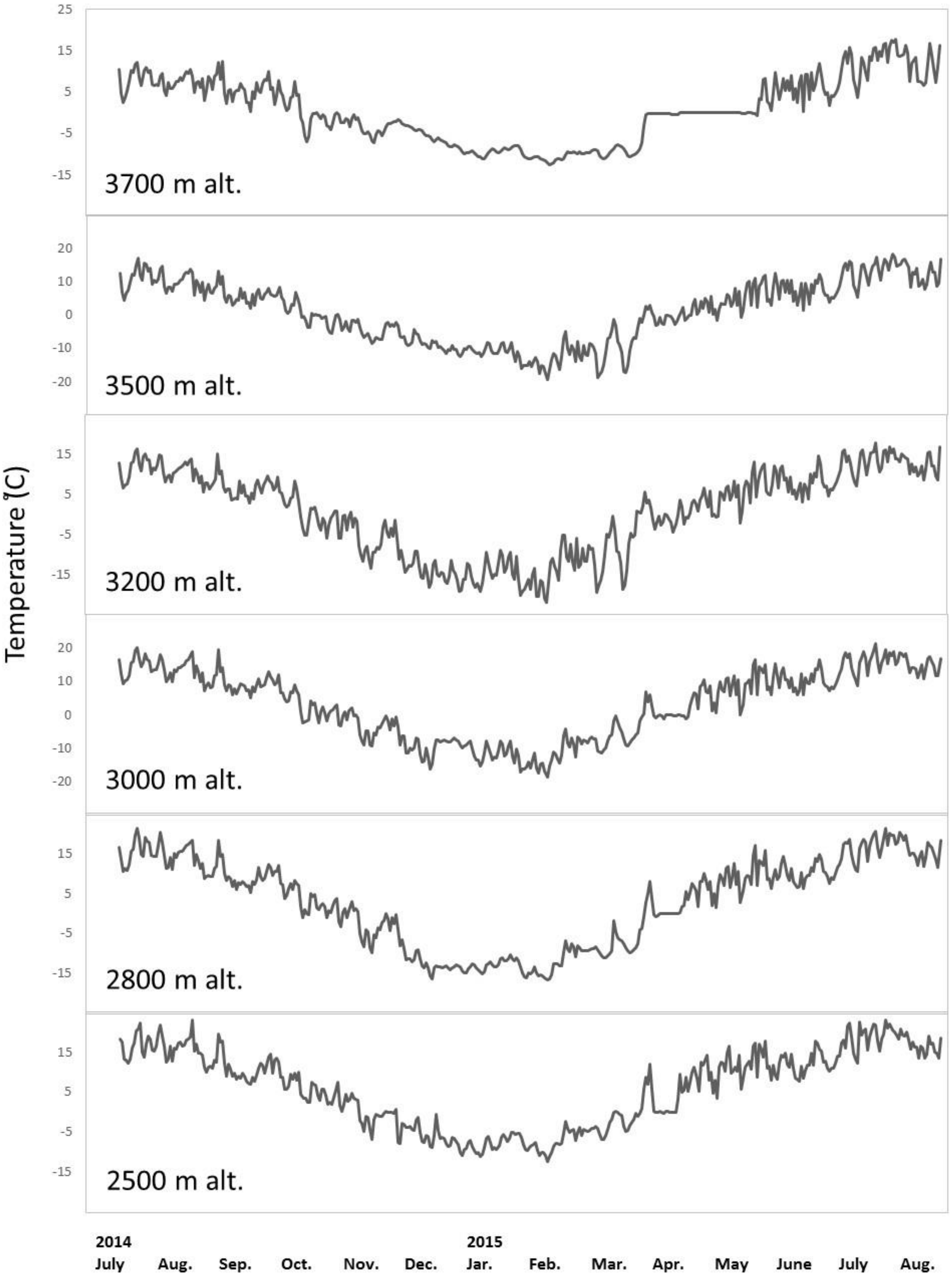

Fig. 18. Mean daily air temperature at ground level at 2500 m, 2800 m, 3000 m, 3200 m, 3500 m and 3700m alt. in alpine zone on Mt. Fuji, from 17 July 2014 to 26 August 2015.

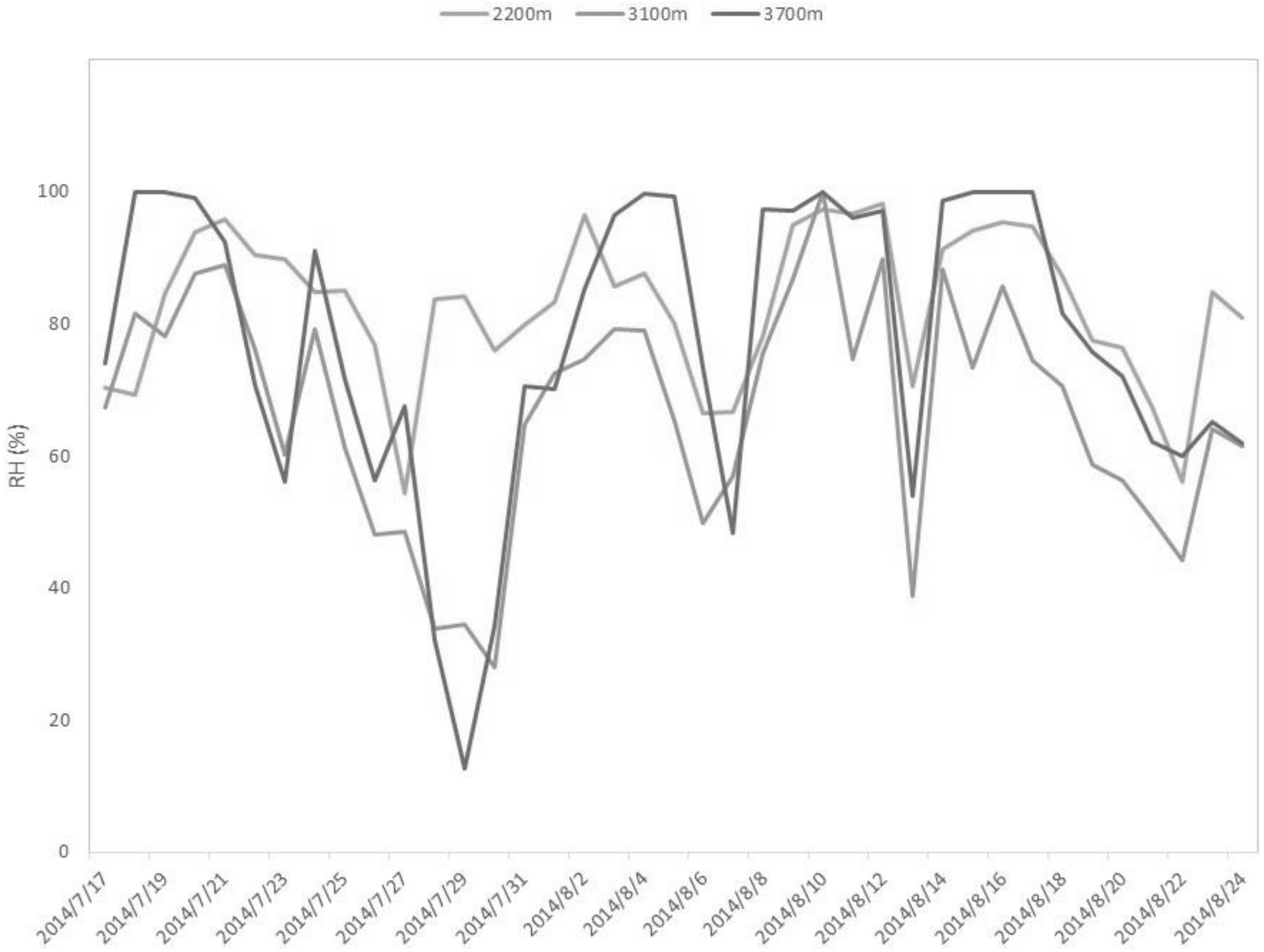

Fig. 19. Mean daily relative humidity at ground level of the three sites (2200 m, 3100 m and 3700 m alt.) in alpine zone on Mt. Fuji, from 17 July to 24 August 2014.

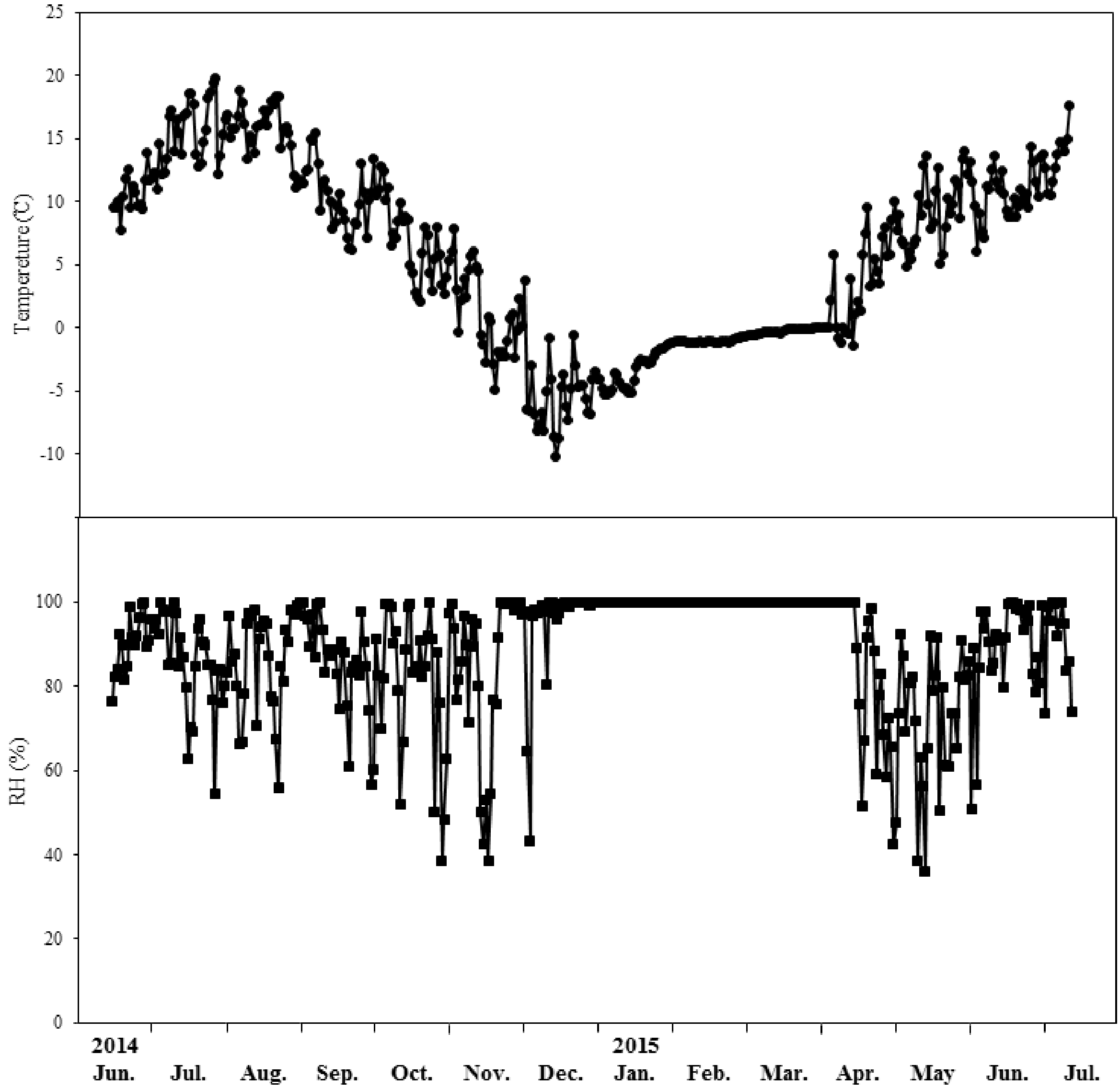

Fig. 20. Mean daily air temperature (●) and relative humidity (■) at ground level at Okuniwa on Mt. Fuji from 15 June 2014 to 13 July 2015.

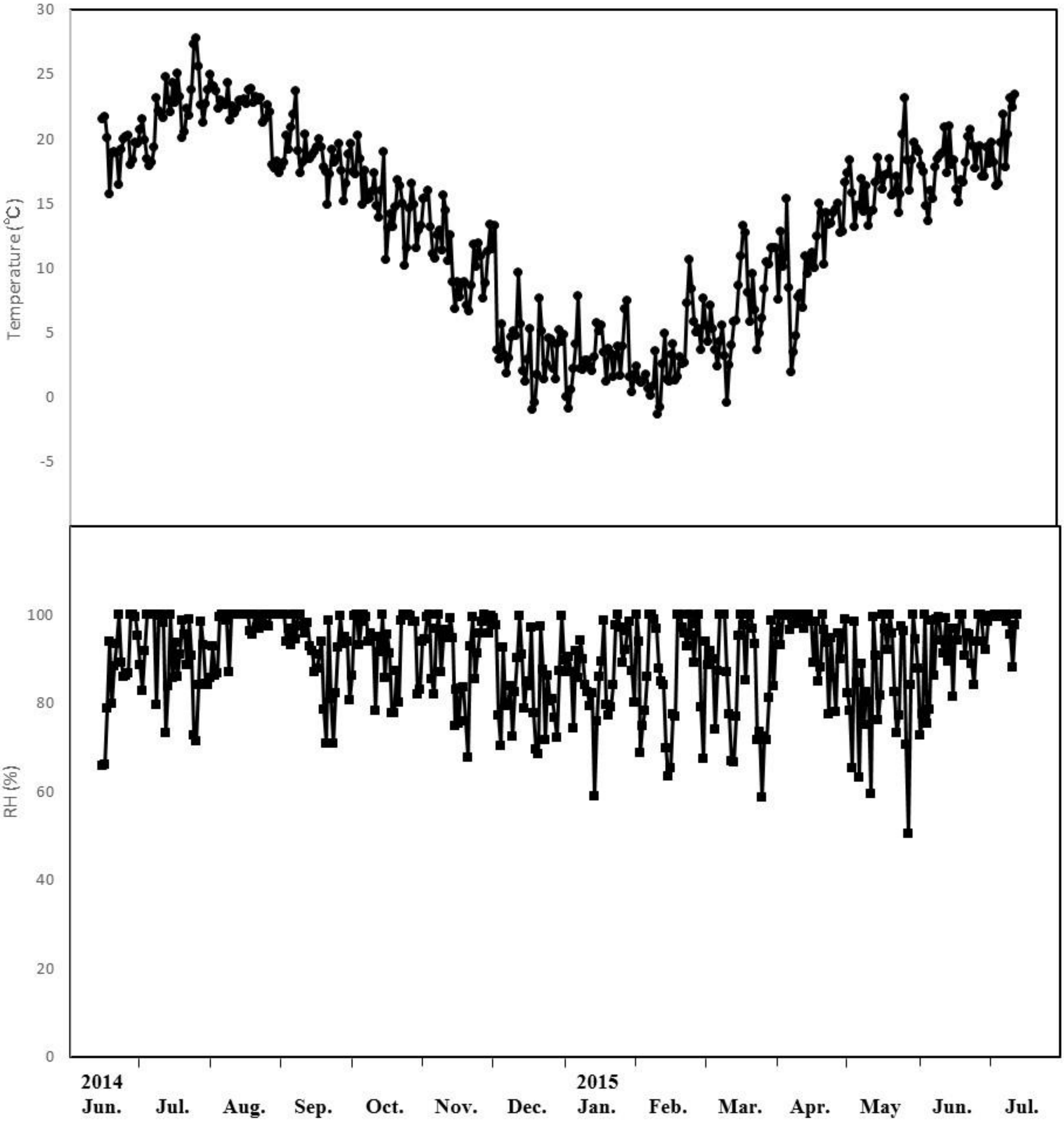

Fig. 21. Mean daily air temperature (●) and relative humidity (■) at ground level at Mt. Mihara from 15 June 2014 to 13 July 2015.

Fig. 22. Photograph of a patch of *R. lanuginosum*.

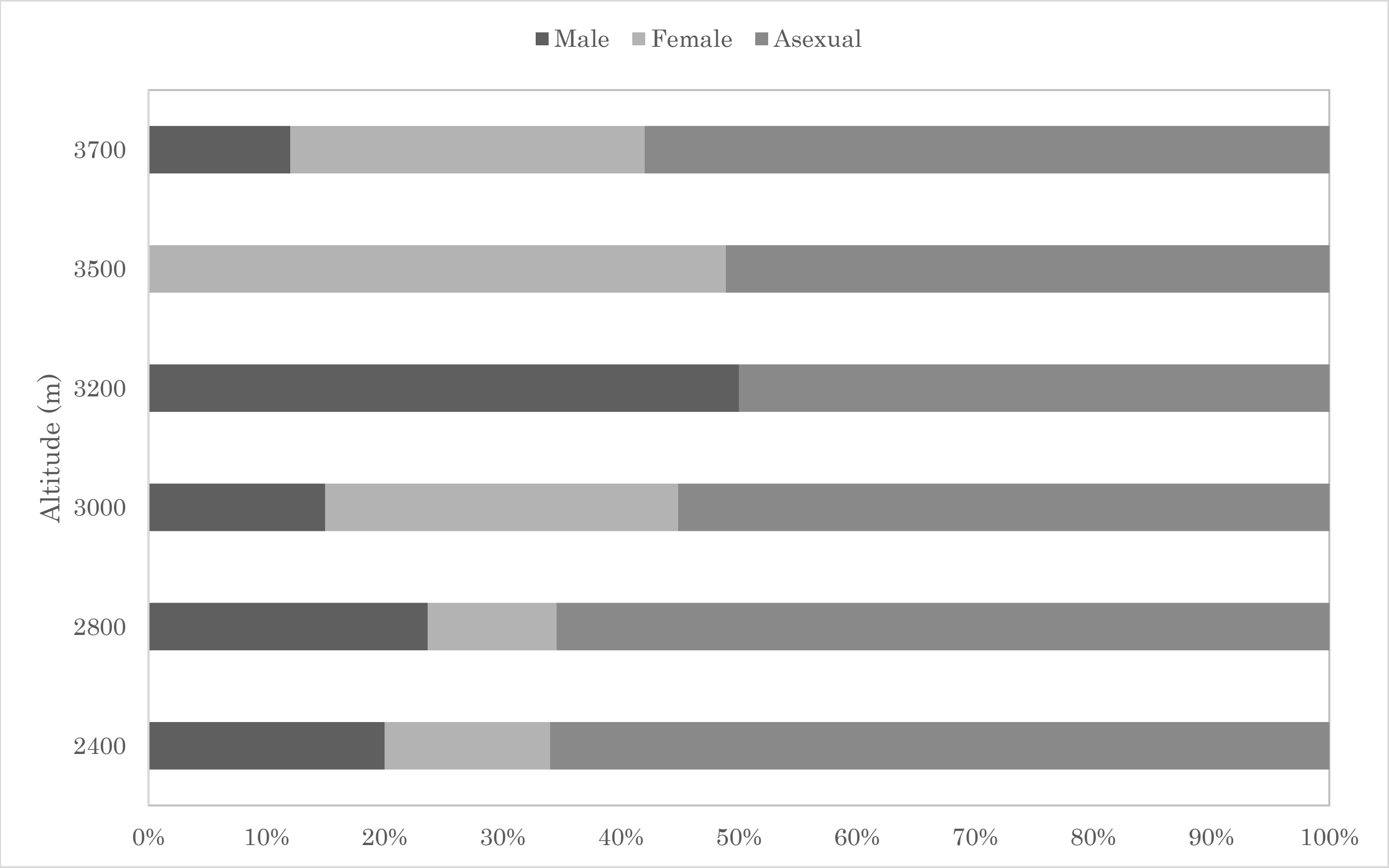

Fig. 23. Sex ratio at 2400 m, 2800 m, 3000 m, 3200 m, 3500 m and 3700 m alt. in alpine zone on Mt. Fuji: deep gray bar-male, pale gray bar-female, middle gray bar-non-sex expressing (asexual) shoot.

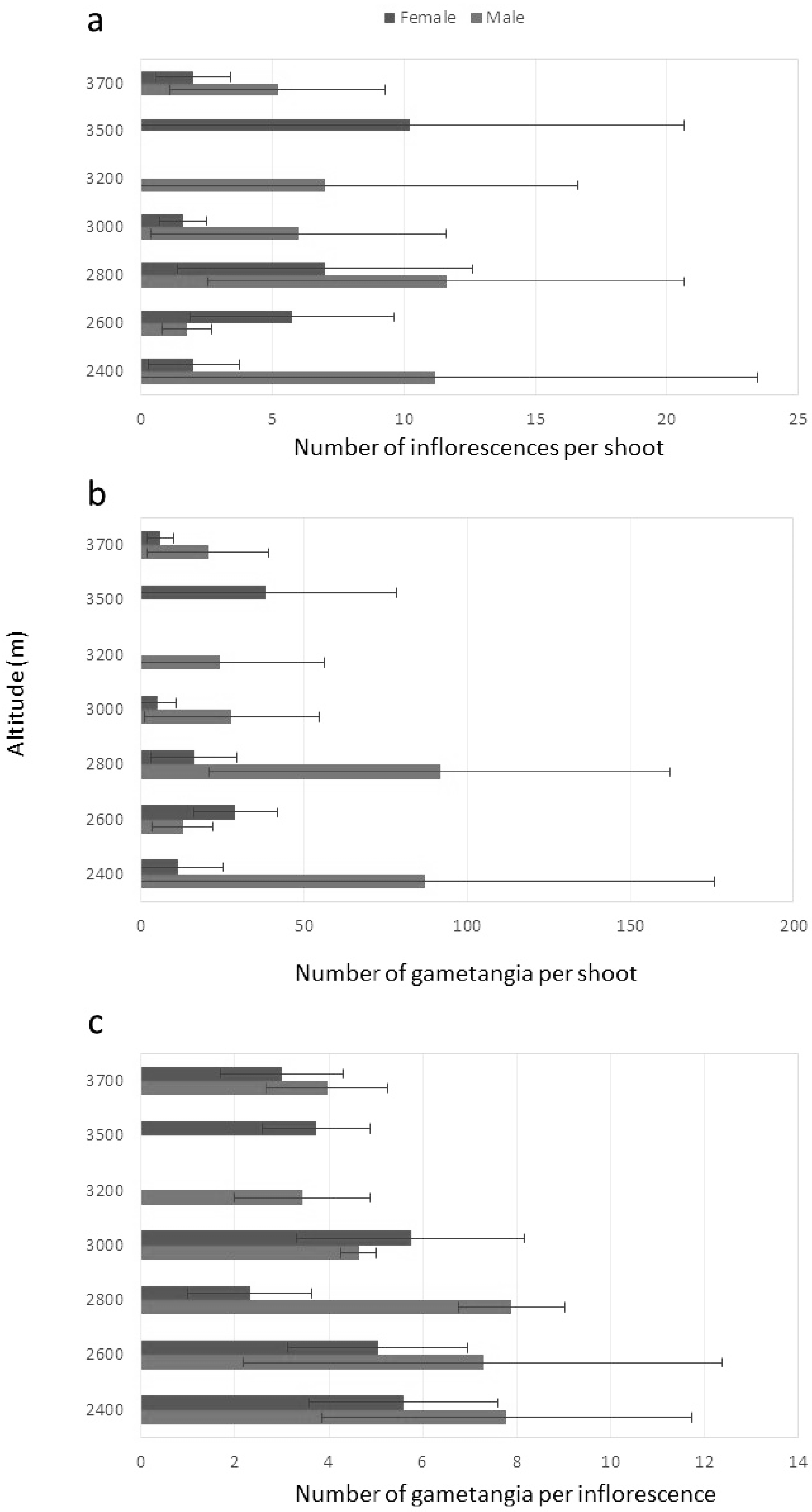

Fig. 24. Number of inflorescences and gametangia per shoot and per inflorescence in each sex in each study site in alpine zone on Mt. Fuji: a-number of inflorescences per shoot, b-number of gametangia per shoot, c-number of gametangia per inflorescence.

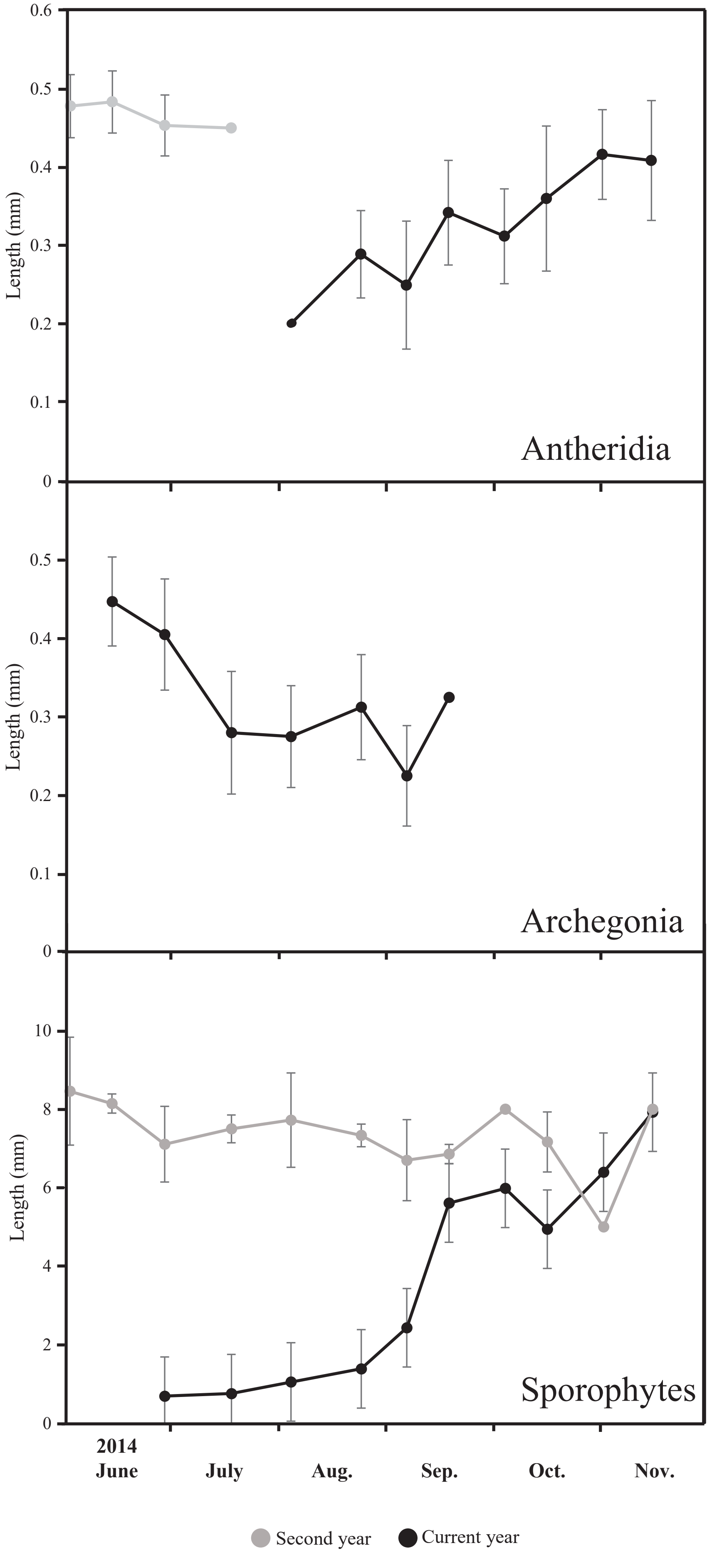

Fig. 25. Size of antheridia, archegonia and sporophytes at Okuniwa on Mt. Fuji. Values are the mean lengths of the antheridia, archegonia and sporophytes; bars indicate standard deviations (SD). The term “second year” refers to the antheridia and sporophytes initiated in the previous year, whereas the term “current year” refers to those initiated in the current year.

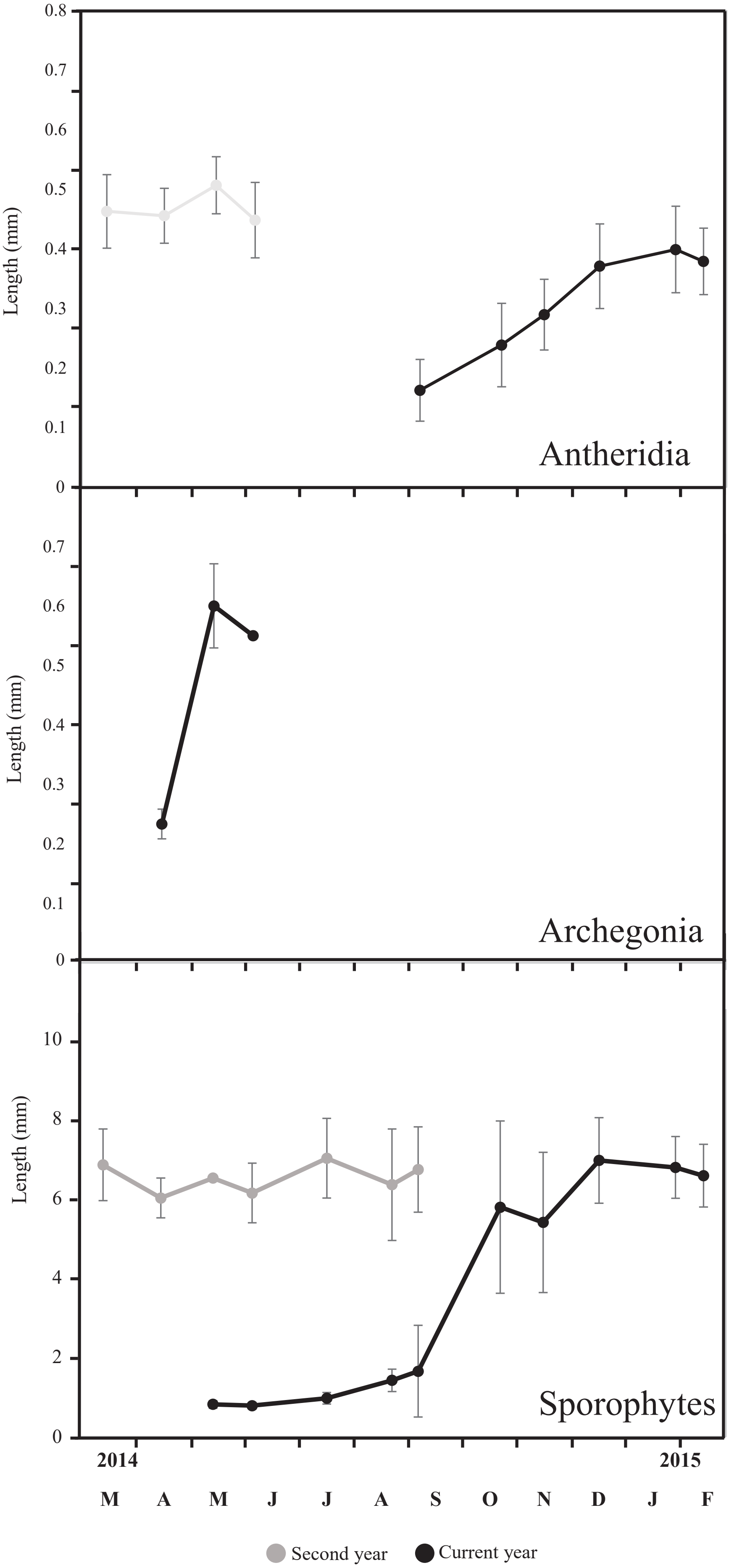

Fig. 26. Size of antheridia, archegonia and sporophytes at Mt. Mihara on Mt. Fuji. Values are the mean lengths of the antheridia, archegonia and sporophytes; bars indicate standard deviations (SD). The term "second year" refers to the antheridia and sporophytes initiated in the previous year, whereas the term "current year" refers to those initiated in the current year.

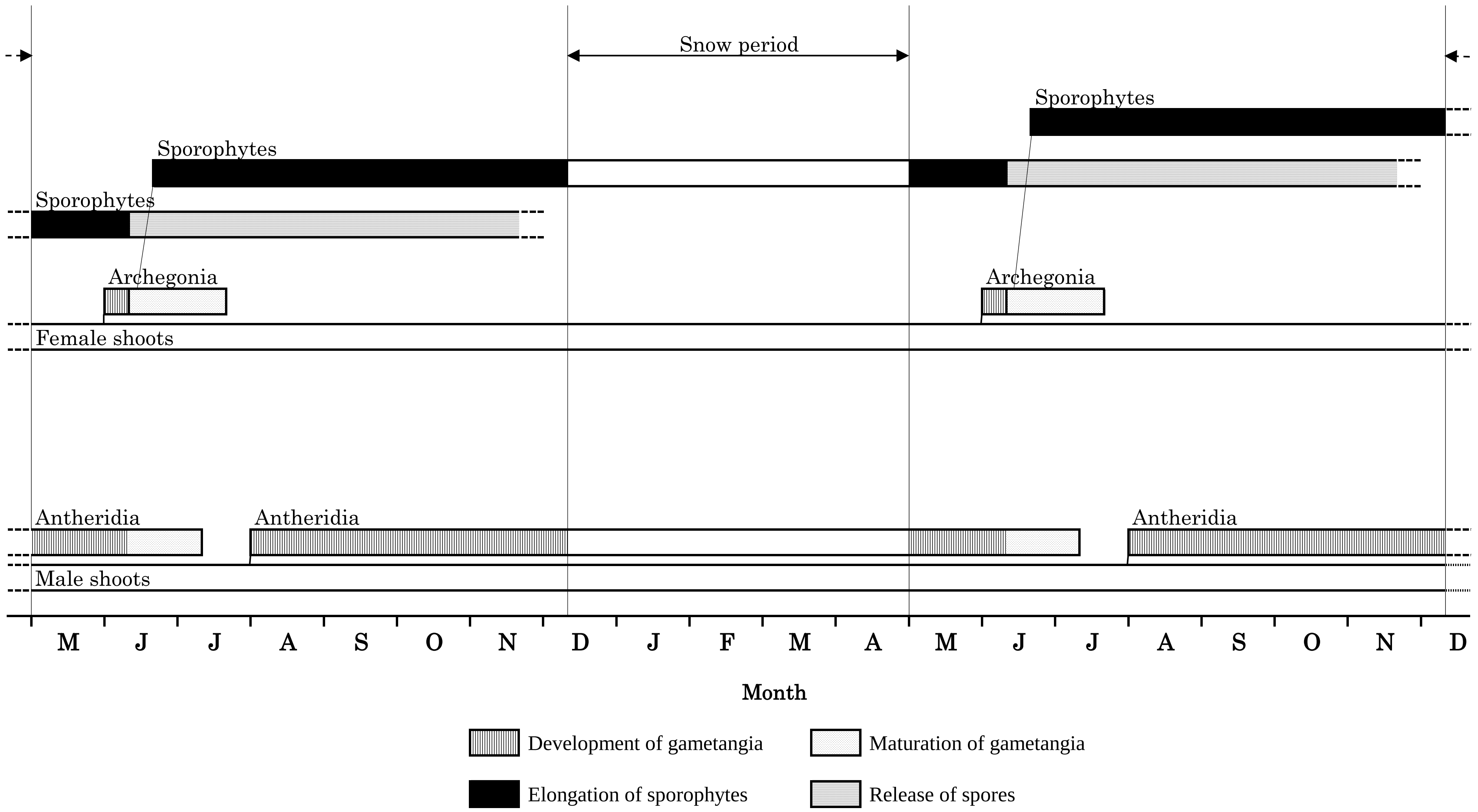

Fig. 27. Phenological spectra of *R. lanuginosum* at Okuniwa on Mt. Fuji.

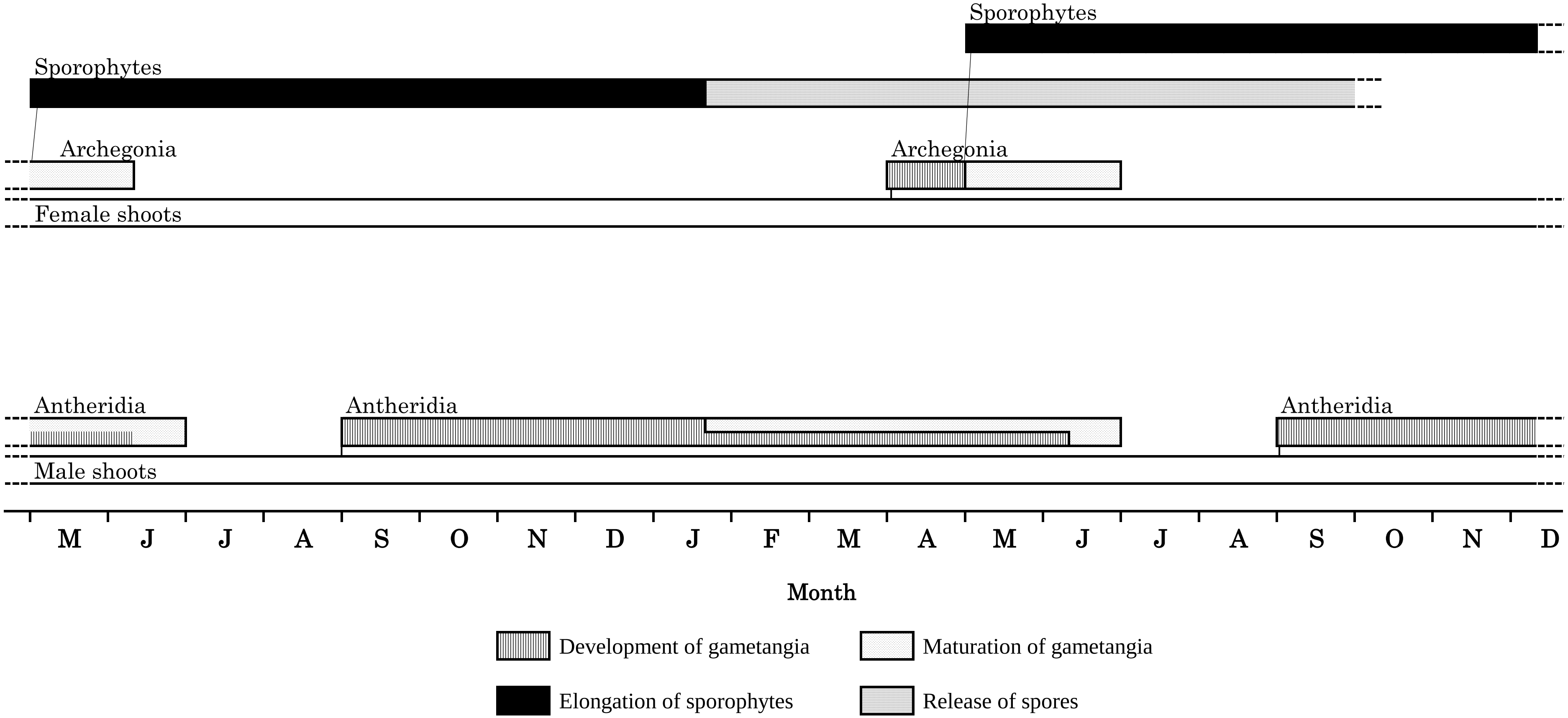

Fig. 28. Phenological spectra of *R. lanuginosum* at Mt. Mihara.

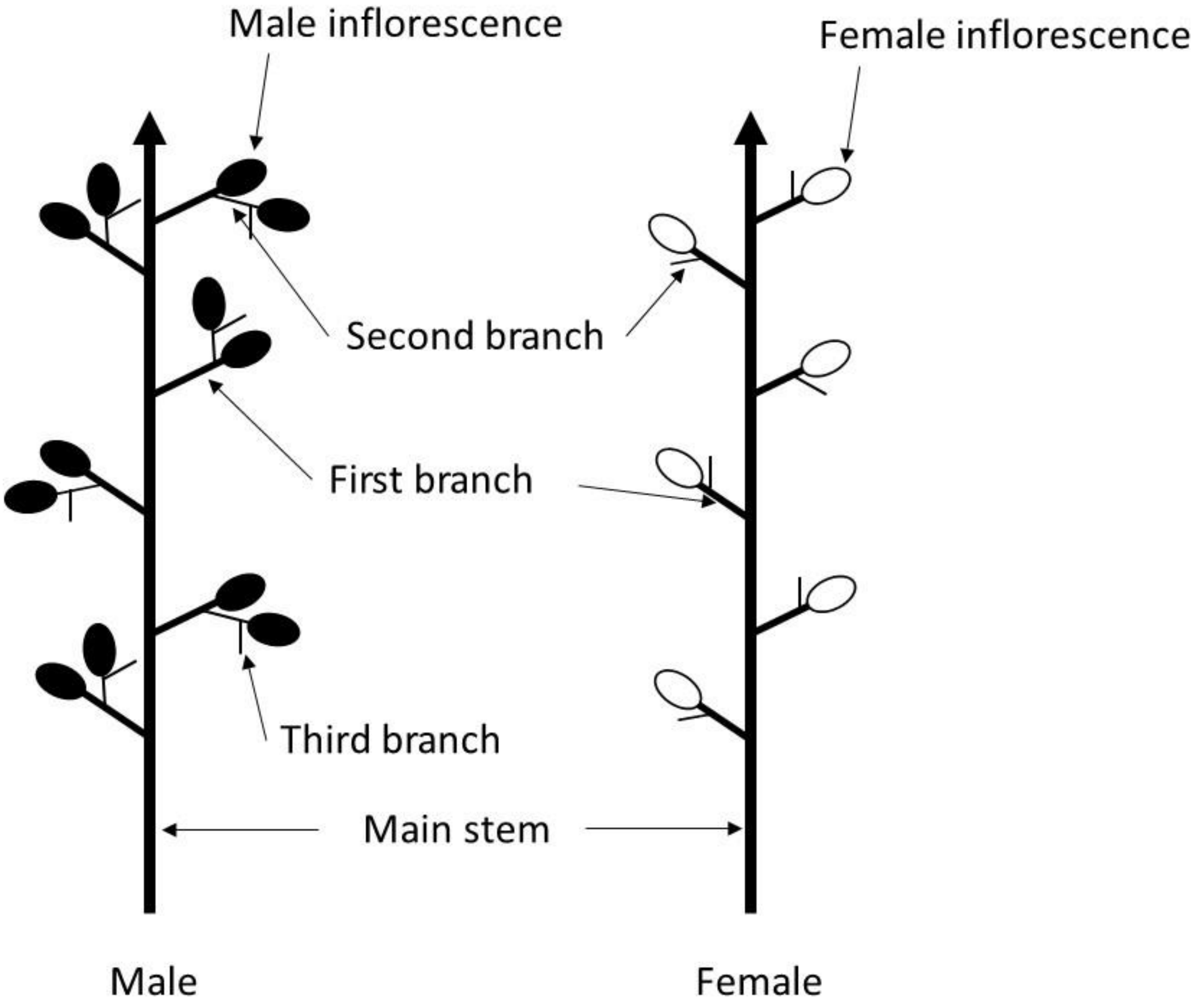

Fig. 29. Schematic diagram of branching and inflorescence formation on both sexes.

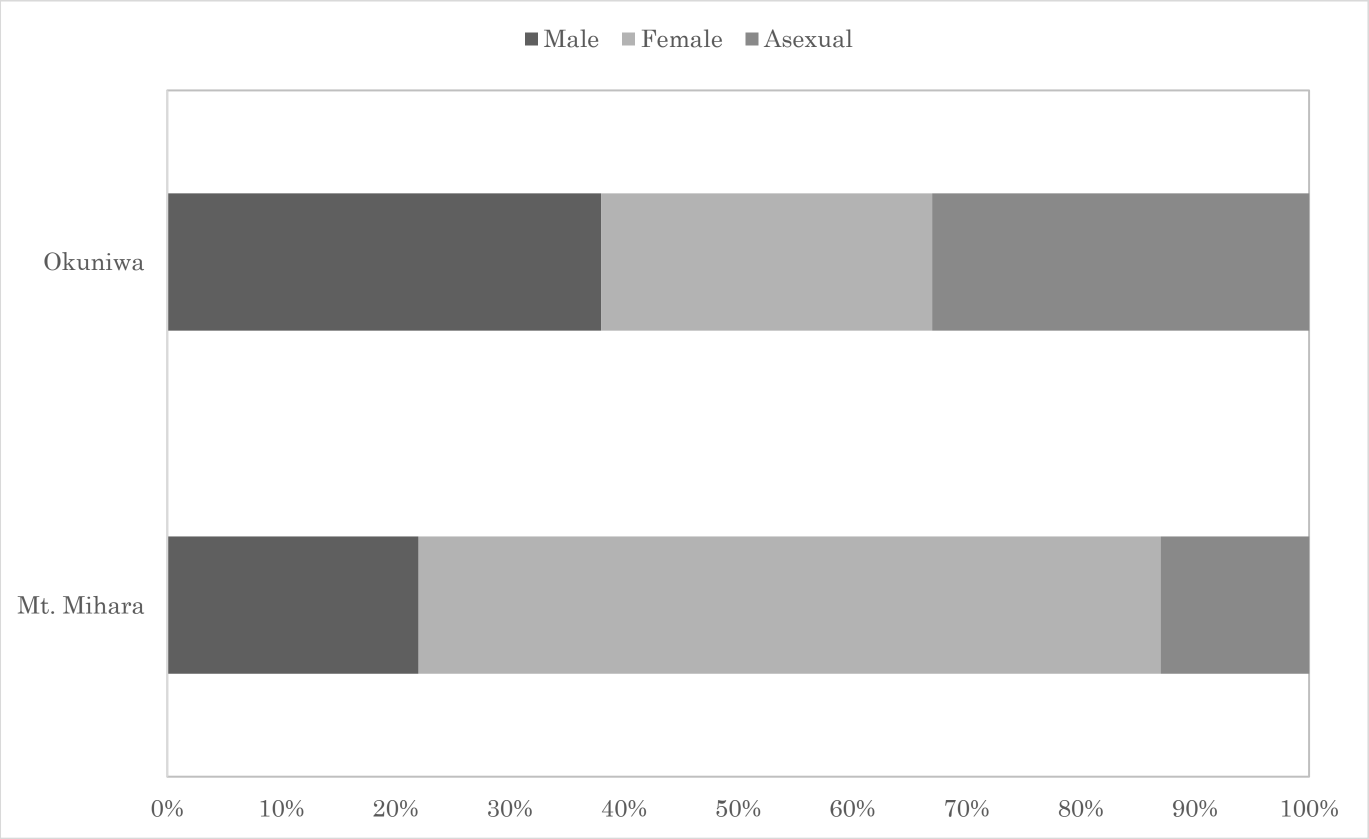

Fig. 30. Sex ratio at Okuniwa and Mt. Mihara: deep gray bar-male, pale gray bar-female, middle gray bar-non-sex expressing (asexual) shoot.