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Research Article

Current and historical factors drive variation of reproductive traits in unisexual mosses in Europe: A case study

María Teresa Boquete[1*], Zulema Varela[2,3], José Angel Fernández[2], Juan Antonio Calleja[4,5], Cristina Branquinho[3], Antonina Chilà[2], Nils Cronberg[6], Ricardo Cruz de Carvalho[3], Cristiana Aleixo[3], Belén Estébanez-Pérez[4], Verónica Fernández-González[2], Andrés Baselga[7], Carola Gómez-Rodríguez[2], Juana María González-Mancebo[8], Sebastien Leblond[9], Javier Martínez-Abaigar[10], Nagore G. Medina[4,5], Encarnación Núñez-Olivera[11], Jairo Patiño[8,12], Rubén Retuerto[2], Antón Vázquez-Arias[2], Alain Vanderpoorten[13], Harald G. Zechmeister[14], and Jesús Ramón Aboal[2]

[1]Department of Evolutionary Ecology, Estación Biológica de Doñana, Consejo Superior de Investigaciones Científicas (CSIC), Sevilla, Spain
[2]Department of Functional Biology, CRETUS, Universidade de Santiago de Compostela, Santiago, Spain
[3]Global Change and Sustainablility Institute, cE3c - Centre for Ecology, Evolution and Environmental Changes & CHANGE, Faculdade de Ciências, Universidade de Lisboa, Lisboa 1749-016, Portugal
[4]Departamento de Biología (Botánica), Facultad de Ciencias, Universidad Autónoma de Madrid, Madrid, Spain
[5]Centro de Investigación en Biodiversidad y Cambio Global, Madrid, Spain
[6]Department of Biology, Lund University, Lund, Sweden
[7]Department of Zoology, Genetics and Physical Anthropology, CRETUS, Universidade de Santiago de Compostela, Santiago, Spain
[8]Department of Botany, Ecology and Plant Physiology, Unit of Botany, Universidad de la Laguna, San Cristóbal de La Laguna, Spain
[9]UMS Patrinat (OFB, MNHN, CNRS), Paris, France
[10]Department of Agriculture and Food, Unit of Botany, Universidad de la Rioja, La Rioja, Spain
[11]Department of Agriculture and Food, Unit of Plant Physiology, Universidad de la Rioja, La Rioja, Spain
[12]Island Ecology and Evolution Research Group, Instituto de Productos Naturales y Agrobiología (IPNA-CSIC), Tenerife, Spain
[13]Botany Institute, University of Liège, Liège, Belgium
[14]Department of Botany and Biodiversity Research, University of Vienna, Vienna, Austria
*Author for correspondence. E-mail: teresa.boquete@gmail.com; teresaboquete@ebd.csic.es
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Abstract Unisexual bryophytes provide excellent models to study the mechanisms that regulate the frequency of sexual versus asexual reproduction in plants, and their ecological and evolutionary implications. Here, we determined sex expression, phenotypic sex ratio, and individual shoot traits in 242 populations of the cosmopolitan moss *Pseudoscleropodium purum* spanning its whole distributional range. We tested whether niche differentiation, sex-specific differences in shoot size, and biogeographical history explained the spatial variation of reproductive traits. We observed high levels of sex expression and predominantly female-biased populations, although both traits showed high intraspecific variation among populations. Sex expression and sex ratio were partly explained by current macroscale environmental variation, with male shoots being less frequent at the higher end of the environmental gradients defined by the current distribution of the species. Female bias in population sex ratio was significantly lower in areas recolonized after the last glacial maximum (recent populations) than in glacial refugia (long-term persistent populations). We demonstrated that reproductive trait variation in perennial unisexual mosses is partially driven by macroscale and historical environmental variation. Based on our results, we hypothesize that sexual dimorphism in environmental tolerance and vegetative growth contribute to sex ratio bias over time, constraining the chances of sexual reproduction, especially in long-term persistent populations. Further studies combining genetic analyses and population monitoring should improve our understanding of the implications of the intraspecific variation in the frequency of sexual versus asexual reproduction in bryophyte population fitness and eco-evolutionary dynamics.

Key words: asexual reproduction, biogeographic distribution, phenotypic sex ratio, sex expression, sexual dimorphism, sexual reproduction

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1 Introduction

Reproduction mode (sexual vs. asexual) is a key life-history strategy with profound ecological and evolutionary implications for plants. From an ecological perspective, asexual reproduction promotes population expansion at relatively small spatial scales whereas sexual reproduction increases the potential for long-distance dispersal (Kimmerer, 1991; Rautiainen et al., 2004; but see Laenen et al., 2016). From an evolutionary perspective, asexual reproduction may help to maintain well-adapted phenotypes in environmentally stable habitats whereas genetic variation is recombined through sexual reproduction, which might enhance the chances of population survival during periods of environmental change (Niklas & Cobb, 2017). Many plant species can reproduce both sexually and asexually, and the relative importance of these two modes of reproduction frequently varies across populations within species (Eckert, 2002). This variability can be regulated by biotic and abiotic, genetic, and/or demographic factors (Ceplitis, 2001; Eckert, 2002; Tomita & Masuzawa, 2010; Chen et al., 2015; Yang & Kim, 2016), which may vary both temporally and spatially. Intraspecific variability in modes of reproduction has been mostly studied in vascular plants and/or at small spatial scales (but see Hedenäs & Bisang, 2019; Eckert et al., 1999). Nonetheless, assessing (i) how reproductive traits, such as sex expression and phenotypic sex ratio affect the relative frequency of sexual versus asexual reproduction, and (ii) how environmental and historical factors shape these key life-history traits across large distributional ranges, are crucial questions to better understand plant population dynamics.

Bryophytes, the second most diverse group of terrestrial plants, are especially interesting for this kind of study. First, many bryophyte species have broad geographical distributions spanning a wide range of environmental conditions (Vanderpoorten & Goffinet, 2009). Second, and unlike spermatophytes, about 60% of bryophyte species have separate sexes (Wyatt & Anderson, 1984; Villarreal & Renner, 2013; Laenen et al., 2016). Many unisexual bryophytes exhibit both sexual and asexual (either through specialized propagules, like gemmae, or vegetative propagation, i.e., fragmentation and/or clonal growth) reproduction. However, sexual reproduction is considered infrequent in many perennial species (Longton & Schuster, 1983; Frey & Kürschner, 2011; Glime & Bisang, 2017). Fertilization in bryophytes involves sperm cells swimming to the archegonia to fertilize the egg cell through a continuous film of water (but see Cronberg et al., 2006; Shortlidge et al., 2021). As sperm dispersal range has been proposed to average from a few centimeters to a few meters (Van Der Velde et al., 2001; Cronberg, 2002; Bisang et al., 2004); but see Pressel & Duckett (2019) (for fertilization distances up to 19 m in the liverwort *Marchantia polymorpha*), the chances of sexual reproduction in unisexual bryophytes are largely conditioned by the spatial arrangement of (non)expressing shoots within the population (Cronberg et al., 2006). Interestingly, unisexual bryophyte populations studied so far have been characterized by either low or highly variable sex expression levels (e.g., Bowker et al., 2000; Bisang & Hedenäs, 2005; Stark et al., 2005a) and strongly biased phenotypic sex ratios, usually toward females (Stark, 2002; Bisang & Hedenäs, 2005; Stark et al., 2005a, 2010). Consequently, these populations typically rely on asexual reproduction (During, 1979; Newton & Mishler, 1994; Frahm, 2007, but see Crawford et al., 2009); when this occurs through clonal propagation and/or fragmentation, it could increase the spatial segregation of sexes, and hamper the chances of sexual reproduction (Alonso-García et al., 2020). Nonetheless, clones can also become more strongly intermixed over time, increasing the chance for sex expressing male and female clones to grow within fertilization distance (Cronberg, 2002; Hedenäs et al., 2021).

Two hypotheses have been proposed to explain female dominance in unisexual bryophyte populations. The first one is based on the findings of field studies showing a consistent pattern of expressed male rarity, often linked to more exposed habitats with lower water availability and/or higher light exposure (Bowker et al., 2000; Stark et al., 2005a, 2010; Blackstock, 2015; Castetter et al., 2019; but see Fuselier & McLetchie, 2004; Groen et al., 2010). In light of this, authors proposed that higher female environmental tolerance leading to higher female spore germination rates, lower mortality rates, and/or higher success of establishment following dispersal events, could favor predominantly female-biased populations. A major limitation of these studies is that they are based on observed phenotypic sex ratios in populations with rather low sex expression levels making it impossible to decipher whether expressed male rarity was due to greater female environmental tolerance, or to sex-specific differences in the conditions required to express sex. Studies in which field plants were grown until sex expression in the laboratory, however, seem to support the first assumption (Castetter et al., 2019), and so do laboratory assays showing greater, though sometimes habitat-dependent, female stress tolerance (e.g., Marks et al., 2016, 2019; Silva-e-Costa et al., 2022; but see Stark et al., 2005b). It has also been recently demonstrated that higher female survival and/or clonal growth rates at the juvenile stage, that is, before sexual maturity, can also lead to shifts from male- to female-biased population sex ratios regardless of the environment (Eppley et al., 2018).

The second hypothesis states that higher pre-zygotic reproductive effort experienced by male plants could compromise their survival and growth rates, further contributing to the female bias. The development of reproductive structures is expensive in terms of nutrient resources (Chopra & Bhatla, 1983), and some studies have shown that males produce comparatively higher numbers of sexual organs or allocate more biomass to sexual organs than females (e.g., Longton & Greene, 1969; Stark et al., 2000; Horsley et al., 2011; Castetter et al., 2019), although this pattern might not be universal (Bisang et al., 2006). A greater male sexual reproductive investment is expected to contribute to the female-biased sex ratios through comparatively higher vegetative growth rates in expressed females before fertilization (reproductive costs in fertilized females might be greater as shown by Rydgren & Økland, 2002, 2003; Rydgren et al., 2010). This effect would be more pronounced in long-term persistent populations, where the effects of differences in vegetative growth rates might have been accumulating for a longer time. Hence, the climatic fluctuations of the Quaternary and the concomitant

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consequences on plant distributional ranges (Hewitt, 2000; Svenning & Skov, 2007; Giesecke et al., 2017) might also shed light on the current spatial variation in the reproductive traits of unisexual bryophytes (Cronberg et al., 2006; Kirchheimer et al., 2018; Blackstock, 2020; Bisang et al., 2020).

Here we implemented a macroecological approach to unravel the main drivers of sex expression and phenotypic sex ratio variation in bryophytes and their consequences for effective sexual reproduction. We sampled a unisexual pleurocarpous moss, *Pseudoscleropodium purum*, across its full native range in Europe to test the following predictions:

- Based on earlier studies on unisexual long-lived bryophytes (e.g., Bowker et al., 2000; Stark, 2002; Bisang & Hedenäs, 2005; Stark et al., 2005a, 2005b, 2010), we anticipated consistently low levels of sex expression (*H*1A) and strongly female-biased phenotypic sex ratios across its entire native range (*H*1B).

- Given that sex ratio and sex expression levels can differ across environments and species (Bisang et al., 2020), we hypothesized a significant association of sex expression and population sex ratio with environmental factors, due to niche differentiation among nonexpressed and expressed male and female plants of *P. purum* (*H*2).

- Since sex expression in individual shoots is resource-dependent (Chopra & Bhatla, 1983), we predicted that sex-expressed plants should be consistently larger than nonexpressed plants across populations (*H*3). In turn, we predicted that expressed females should be consistently larger than expressed males due to higher pre-zygotic resource allocation into sexual reproduction for males, and, as a result, relatively higher female investment into vegetative growth (*H*4).

- Sex-specific differences in reproductive investment, leading to higher pre-zygotic female vegetative growth rates, may also contribute to phenotypic sex ratio variation. If H4 held true, we expected to find a historical signal in the broad-scale geographic pattern of phenotypic sex ratio variation (Alonso-García et al., 2020). Accordingly, recently founded populations (i.e., after post-glacial recolonization) were expected to show more variable sex ratios than long-term persistent populations (i.e., glacial refugia), wherein we expected higher female biases due to the longer drift in sex ratio via asexual reproduction (*H*5).

2 Material and Methods

2.1 Study species

The moss *Pseudoscleropodium purum* (Hedw.) M. Fleisch. is a pleurocarpous unisexual robust moss that forms dense carpets of prostrate, sometimes ascending stems. It occurs in a wide range of habitats, from shaded environments, such as understory, forest clearings, and slopes to humid, open meadows, often in habitats characterized by some level of anthropic disturbances (Guerra, 2018). It is widely distributed across temperate regions in Europe and the Mediterranean, extending to Iceland in the North and the Canary Islands in the South (Fig. 1A). The species has been introduced in America, Oceania, and Africa (Fritz, 2010). *P. purum* exhibits mostly asexual reproduction by means of shoot fragmentation, through the decay of older shoots, resulting in ramet formation (e.g., Longton & Schuster, 1983; King, 2003), although it can also reproduce sexually (Fritz, 2010).

2.2 Sample collection

Specimens were collected from 242 populations in ten European countries spanning its distributional and latitudinal range from the Canary Islands to Scotland (Fig. 1), between April 2014 and January 2019 (Table S1). Along the Atlantic coast and Bay of Biscay, within the Iberian Peninsula, the sampling was carried out following a 30×30 km grid. We increased the sampling resolution to a 15×15 km grid in areas where we observed shifts from phenotypic female- to male-biased ratios. At each site, we collected approximately 30 subsamples (consisting of a handful of entire moss shoots of *P. purum*) of similar weight and uniformly distributed over an area of approximately 50×50 m (sampling method validated as explained in SM1). When the species was scarce, only ca. 100 moss shoots were collected. In the laboratory, intact shoots were separated from foreign material, air-dried for 3–5 days, and kept in plastic trays until processing.

2.3 Sex determination and plant morphological measurements

The proportions of expressed male, female, and nonexpressed shoots were estimated by sampling 100 moss shoots from each of the 242 populations. Entire shoots, including living and potentially dead tissues, were examined under a stereomicroscope (Olympus SZX7) and classified as sex-expressed males or females when bearing perigonia or perichaetia respectively, and as nonexpressed otherwise. Details about the location and the estimated permanence of gametangia on the moss shoots are shown in SM2. The sex expression level was calculated as the observed proportion of expressed shoots (no expressed shoots/no total shoots), and the phenotypic male/female expressed sex ratio as the proportion of expressed shoots of either sex (no females or males/[no females + no males]). Shoot weight of all sex-determined shoots from 237 populations (samples from five populations were lost), was determined using a Mettler Toledo NewClassic ML balance (1 mg precision), after drying the shoots to constant weight in an oven at 45°C. The length of the main stem (shoot length) was measured with a ruler (1 mm precision). We also estimated the ratio between the weight and the length of the main stem for each moss shoot ($mg \cdot cm^{-1}$), hereafter referred to as specific mass length.

2.4 Data analyses

2.4.1 Large scale patterns of sex expression and phenotypic sex ratio in *P. purum*

We used a chi-square test to assess whether the observed distributions of sex expression and female sex ratio ($n = 242$ data points for each variable) differed significantly from the uniform distribution (i.e., all levels of sex expression between 0 and 1 are equally likely to occur), and the binomial distribution (i.e., the sex ratio is not biased toward any sex)

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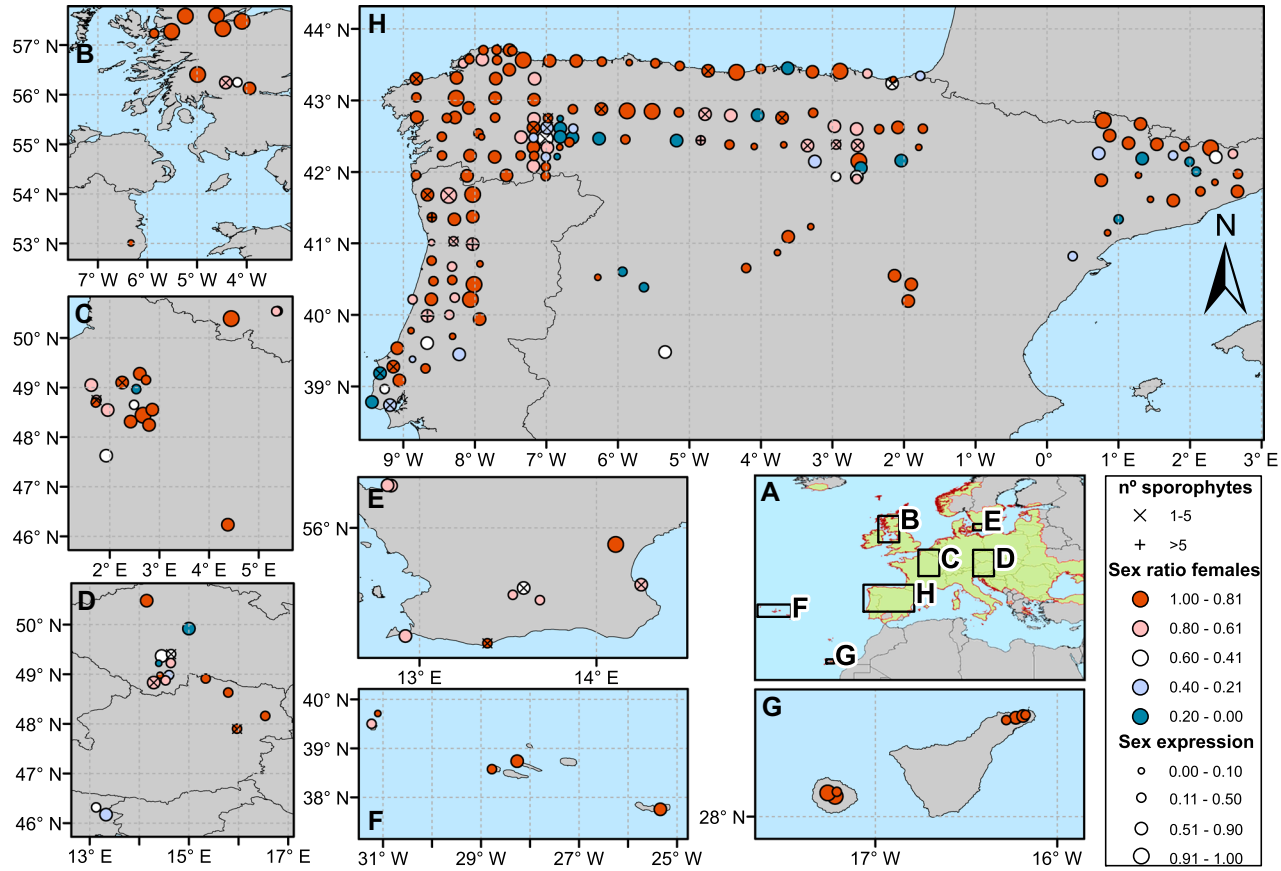

Fig. 1. Maps showing the location (WGS 84) of the samples of *Pseudoscleropodium purum* collected in this study. **A**, Overall view of the sampling area, including the geographic distribution of *P. purum* according to Fritz (2010), and modified in the Iberian Peninsula after Guerra (2018). **B**, Scotland and Ireland. **C**, France and Belgium. **D**, Czech Republic, Austria, and Italy. **E**, Scania—Sweden. **F**, Azores—Portugal. **G**, Tenerife and La Gomera—Canary Islands, Spain. **H**, Iberian Peninsula. Each dot contains information about the female phenotypic sex ratio (dot color), the proportion of sex-expressed shoots (dot size), and the number of sporophytes (additional + or × symbols) for each site.

(H1), respectively. We also looked for a significant association between sex expression and the female sex ratio using the Spearman rank correlation coefficient. Raw data for each population can be found in Table S1.

Additionally, we assessed whether sex expression and phenotypic sex ratio were spatially structured within the study area using semivariograms (SM3).

Finally, as we observed a considerable number of sporophytic females (i.e., shoots bearing the outcome of sexual reproduction), we performed a chi-square test to look for a significant association between the female sex ratio and the presence of sporophytes. We assigned our populations to four different categories (1 to 4) with sex ratios between 0–0.04, 0.05–0.50, 0.51–0.95, and 0.96–1 respectively. The number of females bearing sporophytes within each category was recorded.

2.4.2 Relationship between sex expression and phenotypic sex ratio variation and the environment

To test whether sex-specific differences in environmental tolerance were driving sex expression and phenotypic sex ratio variation in *P. purum* (*H2*), we studied the relationship between these two traits and environmental variation across sites. We used ArcGis 10.5.1® (Esri, 2011) to extract 106 environmental variables from digital maps, including climatic and bioclimatic data spanning 30 years between 1970 and 2000 (Fick & Hijmans, 2017) (full descriptions in Table S2). Elevation was extracted from a Digital Elevation Model (European Environment Agency, 2019). First, we used principal component analysis (PCA) to reduce the dimensionality of our environmental dataset (function *principal* from the R package psych with "varimax" rotation). Then, we performed two generalized linear models (GLMs) with "sex expression" and "female sex ratio" as response variables, and the first four principal components (PCs) of the PCA as predictors (function *glm* with the binomial distribution and the logit link function). Sex expression and female sex ratio were entered in the models as two-vector response variables (i.e., number of expressed and nonexpressed plants, and number of female and male plants, respectively) (Crawley, 2015). We subjected the full models to the *stepAIC* function (MASS package; Venables & Ripley, 2002) to perform a backward stepwise model selection (argument direction = "both"). For the phenotypic sex ratio, we performed this analysis on the complete dataset ($n = 235$

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as seven populations had no expressed plants), on a subset of the 67 populations with sex expression levels >75%, and on a reduced dataset including populations with female sex ratio >0.05 and <0.95 ($n = 119$) to exclude populations where environmentally driven shifts in sex ratio could not be assessed due to the nearly complete absence of one sex. Residuals were graphically inspected for normality and homoscedasticity. The statistical significance of the predictors was inspected using the *anova* function with likelihood ratio tests between the final model and the null model that only included the intercept.

In addition, we looked for evidence of niche differentiation between male, female, and nonexpressed plants as detailed in SM4. Briefly, we calculated a weighted probability density function on the frequency of presence for the three plant sex morphs in each of the first four PCs of the PCA and estimated the overlap between the pairwise probability density functions as the intersection between the areas under the curves.

2.4.3 Relationship between sex expression status and shoot morphological traits

To test whether sex expression occurred only after shoots reached a minimum size and whether sexes differed in their investment in the expression of sex (*H*3 and *H*4), we looked for significant differences in shoot length, weight, and specific mass length between expressed ($n = 11\,967$) and nonexpressed ($n = 10\,821$) shoots, and between female ($n = 9308$) and male ($n = 2659$) shoots, respectively. For this, we used two separate linear mixed models (*lmer* function in lme4 package; Bates et al., 2015) with log2-transformed-specific mass length, and log10-transformed shoot length and shoot weight as response variables, and sex expression (2 levels: expressed and nonexpressed) and sex (2 levels: female and male) as a predictor in the first and the second model, respectively. We included the population (237 levels) as a random effect to account for the potential within-population variation. Residuals were graphically inspected for normality and homoscedasticity. Statistical significance of the predictors was inspected with likelihood ratio tests using the *anova* function by comparing our models with null models that only included the intercept. Finally, we used the package *emmeans* (Lenth, 2020) to estimate the marginal means and the 95% upper and lower confident limits for each trait in each plant morph.

Additionally, we used linear models (*lm* function) to test whether the magnitude of the morphological differences between females and males depended on the female sex ratio and the environmental conditions (summarized as PCs1 to 4). We only included populations with at least five female and five male shoots ($n = 82$ populations; females: $n = 2957$; males: $n = 1741$). We ran three separate linear models with log10-transformed specific mass length, shoot weight, and shoot length as dependent variables and sex, PC1 to PC4, the female sex ratio, and the interaction between sex and all other variables as predictors. We graphically inspected the residuals for normality and homoscedasticity and tested the significance of the models with likelihood ratio tests using the *anova* function by comparing our models with null models that only included the intercept.

2.4.4 Relationship between phenotypic sex ratio variation and historical population persistence

To test whether sex-specific differences in long-term vegetative growth led to phenotypic sex ratio variation across sites (*H*5), we assessed the historical imprint of postglacial recolonization in the geographic pattern of populations' sex ratio in *P. purum*. We searched for differences in phenotypic sex ratio between glacial refugia and localities colonized after the deglaciation using a Wilcoxon test. The rationale behind this approach is that populations resulting from postglacial recolonization were expected to be younger than populations in glacial refugia. Thus, we expected a highly skewed distribution of phenotypic sex ratio (i.e., a larger proportion of females) in glacial refugia due to the isolation and the concomitant longer period to accumulate the effects of any sex-specific competitive advantage (i.e., sex-specific differences in vegetative growth), than in areas that had been recolonized during the current interglacial period (Alonso-García et al., 2020). Glacial refugia were estimated assuming that the current northern limit of the species distribution reflects its thermal limit. We downloaded all European presence records ($n = 111\,476$) from GBIF.org (24 March 2021, GBIF Occurrence Download https://doi.org/10.15468/dl.2m4zak), and extracted the mean annual temperature for all these localities from CHELSA climatic model (Karger et al., 2017). To avoid extreme values derived from geolocation errors or climatic model uncertainty, we computed the 0.005 quantile of the distribution of mean annual temperature in all the species presences, which yielded a threshold of 5.4°C. This threshold accurately captured the current northern limit in the species distribution (Fig. S5). We assumed that areas presenting mean annual temperatures below this threshold were unsuitable for the species in the last glacial maximum (LGM, ca. 21 000 years ago), and current presences in these areas can be considered as the result of a postglacial expansion. To classify our sampling localities as glacial refugia or recolonized areas since the LGM is based on the thermal threshold of 5.4°C (mean annual temperature), we considered four different models of paleoclimate: NCAR-CCSM4, MIROCESM, MPI-ESM-P and IPSL-CM5A-LR (Fig. S6). For each paleoclimatic scenario, we used a Wilcoxon test to assess whether the phenotypic sex ratio differed between glacial refugia and postglacial recolonized areas.

3 Results

3.1 Overall patterns of variation of sex expression and phenotypic sex ratio in *Pseudoscleropodium purum*

We found a total of 10821 nonexpressed and 11967 sex-expressed shoots. From the latter, we found 9308 females and 2659 males. Overall sex expression levels were high, with 52% of expressed shoots. At the population level, more than half of the populations ($n = 132$) had ≥ 50% of sex expression and more than a quarter of the populations ($n = 67$) had ≥75% of sex expression. The proportion of sex expressed shoots per population across the study area was uniformly distributed between 0 and 1, that is, did not differ significantly from the discrete uniform distribution ($\chi^2 = 5.3$, $p = 0.812$, Fig. 2A). Thus, all discrete sex expression classes

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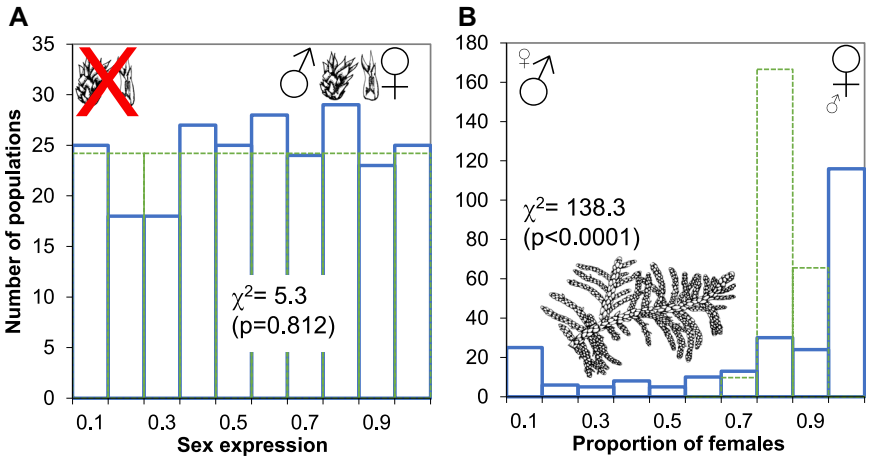

Fig. 2. **A**, Number of populations of *Pseudoscleropodium purum* with sex expression levels in each category (blue bars) and expected number of populations under the uniform distribution (green dotted line). **B**, Number of populations with observed female phenotypic sex ratios in each category (blue bars) and expected number of populations under the binomial distribution with the observed overall proportion of females—0.78—(green dotted line). Values of the chi-square statistic and their probability are shown within each graph.

were equally likely within the distributional range of this species. Accordingly, we did not find evidence for spatial autocorrelation of this trait, that is, the semivariograms were not significant (Fig. S1a; but see Section 3.2).

The overall phenotypic sex ratio of *P. purum* was strongly female-biased, that is, 78% of all expressed plants were females. The distribution of the observed female sex ratio across populations differed significantly from the one expected under a binomial distribution with no sex bias, that is, probability of females = 0.5 ($\chi^2 = 247$, $p < 0.0001$), and from the one expected under a binomial distribution in which the probability of females = 0.78, that is, the observed overall female ratio ($\chi^2 = 138.3$, $p < 0.0001$, Fig. 2B). Considering only populations with high sex expression (≥75% of expressed plants) and biased population sex ratio (≥25% deviation from the theoretical 1:1 expectation), 75% and 15% of the populations were female- and male-biased, respectively. These proportions were maintained when considering all populations (74% and 14% for females and males respectively). We found evidence for spatial autocorrelation, that is, the spatial structure of phenotypic sex ratio in the study area at distances lower than ca. 2 geographical degrees, mainly due to E-W and SW-NE variation (Fig. S1b; but see Section 3.2). This structure was reflected in the strongly male-biased populations clustered in very specific areas, at least within the Iberian Peninsula (Fig. 1H).

Sex expression and female sex ratio were not significantly correlated (Spearman rho = −0.045; *p*-value = 0.49), showing a lack of dependency between the number of sex-expressed shoots and the phenotypic sex ratio of *P. purum*.

Finally, 13% of our populations showed sporophytic females. The chi-square test showed a significant association between the female sex ratio and the presence of sporophytes for categories 3 and 4. Thus, populations with female sex ratio between 0.51 and 0.95 (category 3) showed more sporophytes than expected by chance ($\chi^2 = 16.3$, $p < 0.001$), whereas populations with female sex ratio between 0.96 and 1 (category 4) showed fewer sporophytes than expected by chance ($\chi^2 = 11.3$, $p < 0.001$). This effect is shown in Fig. 3, where sites with sporophytic females are mostly spread above the no sex bias line (i.e., probability of females = 0.5), and close to the observed overall female sex ratio line (i.e., probability of females = 0.78), regardless of the sex expression level. Additionally, the few populations showing >5 sporophytic females were close to the $p = 0.78$-line ratio. The few male-biased populations showing

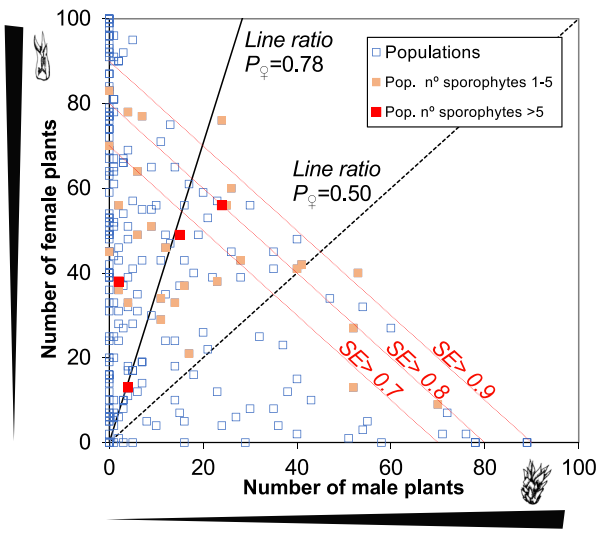

Fig. 3. Binomial plot showing the number of *Pseudoscleropodium purum* shoots of each sex within each population. The number of sporophytes is also shown. Solid line: overall proportion of female shoots (0.78); dotted line: expected proportion of females under no sex bias. Red dotted lines separate toward the right samples with sex expression (SE) levels higher than 0.7, 0.8, and 0.9.

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Table 1 Results of the generalized linear model (GLM) assessing the relationship between environmental variation (summarized as principal components—PCs) and female phenotypic sex ratio ($n = 235$ populations) and sex expression ($n = 242$ populations). Estimate: values of the model coefficients for each predictor; Std. error: standard error associated to the model coefficients; *z* value: value of the test statistic; $P(>|z|)$: significance value; ***$p < 0.001$

Variable	Predictor	Estimate	Std. error	*z* value	$P(>\|z\|)$
Fem. sex ratio	Intercept	1.291	0.022	58.44	$<2.0e^{-16}$***
	PC1	0.008	0.001	9.243	$<2.0e^{-16}$***
	PC2	−0.008	0.002	−4.809	$1.52e^{-6}$***
	PC3	0.022	0.002	14.34	$<2.0e^{-16}$***
	PC4	0.021	0.002	11.37	$<2.0e^{-16}$***
Sex expression	Intercept	0.092	0.013	7.197	$6.15e^{-13}$***
	PC2	−0.006	0.001	−10.24	$<2.0e^{-16}$***
	PC3	0.007	0.001	7.878	$3.32e^{-15}$***
	PC4	0.006	0.001	7.209	$5.62e^{-13}$***

sporophytic females had high sex expression levels (close to or above 70%).

3.2 Effect of the environment on sex expression and phenotypic sex ratio variation in *P. purum*

The first four components (PCs) of the PCA explained 94.6% of the total variation in the environmental variables′ dataset. These PCs were strongly and positively correlated (i.e., loading ≥ |0.9|) with 28 variables related to temperature and average monthly vapor pressure (PC1), 11 variables related to average monthly solar radiation (PC2), 10 variables related to average monthly wind speed (PC3), and 10 variables related with precipitation (PC4) (Table S3). The best GLM on the full female sex ratio dataset included all PCs (Table 1) and explained significantly more variation (9.2%) than the null model ($\chi^2 = 642.6$; $p < 2.2e^{-16}$). The model on the subset of populations with high sex expression (>75%) was also significant and explained 24.6% more variation than the null model ($\chi^2 = 899.7$; $p < 2.2e^{-16}$). The model on the reduced dataset with female sex ratios>0.05 and <0.95 was also significant and included all four PCs but explained less variation (7.5%; results not shown). The model on the sex expression level included PCs 2 to 4 and also explained more variance than the null model (3%) (Table 1).

The analysis of niche differentiation between males, females, and nonexpressed shoots showed considerable overlap in the climatic conditions under which each plant morph could be found in the field (between 78% and 94% depending on the pairwise comparison and the PC; Fig. S2). The probability density function showing the distribution of females under each of the PCs tended to be bimodal, with a second, smaller peak towards the higher end of the climatic conditions summarized by the PCs. The distribution of nonexpressed shoots showed a similar pattern, whereas that of males showed only one large peak (except in PC2; Fig. S2).

3.3 Differences in shoot morphological traits

The results of the linear mixed models searching for significant differences in shoot length, weight, and specific mass length between expressed and nonexpressed shoots, and between female and male shoots, showed that the values of all three variables were higher in expressed than nonexpressed shoots. Shoot weight and specific mass length (but not shoot length) were higher in females compared to males (Table 2; Fig. S3), that is, females were, on average, heavier but not longer than males.

On the other hand, the linear models used to test whether sex-specific differences in morphological traits differed depending on the environmental conditions, and on the female sex ratio, showed a significant interaction between sex and PC1 for specific mass length, between sex and female sex ratio for shoot weight, and between sex and PC1 and female sex ratio for shoot length (Table S4). Specific mass length decreased with the increase in PC1 (which was positively correlated with temperature and vapor pressure variables; Table S3) in both sexes but this decrease was more pronounced in males, leading to higher differences in specific mass length in sites with high values of PC1 (Fig. S4). Shoot length increased slightly with the increase in PC1. This effect was stronger in males, leading to longer males in sites with high values of PC1. Finally, females were heavier and longer in populations with a higher female sex ratio whereas this pattern was unclear for males (Fig. S4).

3.4 Historical signal in geographic patterns of phenotypic sex ratio variation in *P. purum*

We found evidence for a historical signature in the geographic variation of population sex ratio in *P. purum*. The proportion of expressed females was lower in areas potentially recolonized throughout the post-glacial expansion than in glacial refugia (Fig. 4), with the difference being statistically significant in three out of four paleoclimatic scenarios (IPSL-CM5A-LR, MIROC-ESM, and MPI-ESM-P with $p \leq 0.05$; NCAR-CCSM4 with $p = 0.07$).

4 Discussion

In this study, we present an unprecedented large-scale analysis of the geographic variation of sex expression and phenotypic sex ratio in a moss species and discuss its potential implications on the reproductive mode in unisexual bryophytes. Overall, we found high sex expression levels and predominantly female-biased populations but both traits exhibited high levels of variation across the entire

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Table 2 Results of the models testing for differences in specific mass length (SML; mg cm^{-1}), shoot weight (SW; mg), and shoot length (SL; cm) between expressed (E; $n = 11\,967$) and nonexpressed shoots (NE; $n = 10\,821$), and between females (F; $n = 9308$) and males (M; $n = 2659$) of *Pseudoscleropodium purum*. emmean: back-transformed estimated marginal means for each trait and sex level; LCL: lower confidence limit of the emmean; UCL: upper confidence limit of the emmean; Chi-square: value of the chi-square statistic for the significance of sex (E vs. NE and F vs. M); *p*-value: significance of the model

Trait	Sex	emmean	95% LCL	95% UCL	Chi-square	*p*-value
SML	E	4.46	4.28	4.62	156.2	$<2.2e^{-16}$
	NE	3.75	3.61	3.91		
	F	4.56	4.38	4.76	16.61	$4.59e^{-05}$
	M	4.17	3.97	4.41		
SW	E	39.8	38.0	41.7	200.1	$<2.2e^{-16}$
	NE	32.4	30.9	33.9		
	F	40.7	38.9	42.7	11.0	0.00091
	M	38.0	36.3	40.7		
SL	E	9.00	8.79	9.20	73.36	$<2.2e^{-16}$
	NE	8.55	8.35	8.74		
	F	9.01	8.81	9.25	0.461	0.4972
	M	9.10	8.83	9.35		

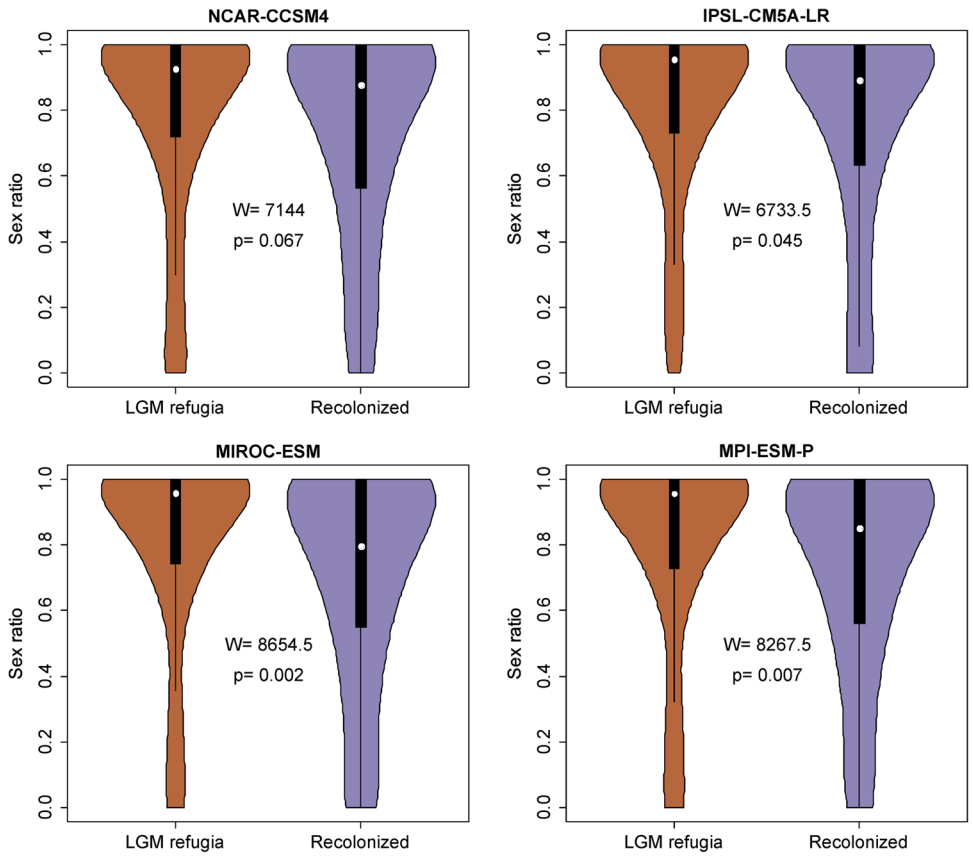

Fig. 4. Phenotypic sex ratio distribution across localities in glacial refugia (brown) versus postglacial recolonized areas (lilac), as estimated from four paleoclimatic scenarios (NCAR-CCSM4, IPSL-CM5A-LR, MIROC-ESM, and MPI-ESM-P). The violin plots illustrate kernel probability density, white dots the median, black bars the quartiles, and vertical lines 1.5 times the interquartile range. W statistic and *p*-values are provided for each scenario.

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distributional range of the species. Macroscale environmental factors explained part of this variation suggesting that sex-specific differences in environmental tolerance contributed to shaping the large-scale spatial patterns of phenotypic sex ratio variation in *Pseudoscleropodium purum*. The morphological differences observed between expressed and nonexpressed morphs of this species suggest that shoots need to reach a minimum size to express sex. In addition, expressed males were smaller than females suggesting that, before fertilization, males of *P. purum* might invest more resources in sexual reproduction than vegetative growth compared to females. Finally, strongly female-biased populations showed larger females and a higher probability of sexual reproduction, suggesting that females in female-dominated populations could allocate enough resources to grow and sustain sporophytes. In this context, the presence of a few scattered males would suffice to maintain sexual reproduction in the population.

Currently, there is contrasting evidence as to whether phenotypic sex ratios accurately represent the real population-level genotypic sex ratios. For example, Baughman et al. (2017) observed discrepancies between genotypic and phenotypic sex ratios in two populations of the desert moss *Syntrichia caninervis*. In turn, Bisang & Hedenäs (2013) found no differences between phenotypic and genotypic sex ratios in herbarium samples of *Drepanocladus lycopodioides* spanning a wide geographical range. Also, Stark et al. (2010) found very similar field phenotypic sex ratios (between 40.7% and 99.2% female) and laboratory phenotypic sex ratios of plants of the moss *Bryum argenteum* that had not expressed sex in the field (between 43.7% and 93.2% female). Differences between genotypic and phenotypic sex ratios would lead to an overestimation of the sex ratio bias, especially in populations with very low sex expression levels. Nonetheless, the lack of relationship between sex expression and female sex ratio, which refutes the "*shy male hypothesis*" (Stark et al., 2010) in this species, and the overall high levels of sex expression found across its distributional range, suggest that our phenotypic sex ratios constitute a reliable estimate of the actual population sex ratios in the bulk of our dataset.

Contrary to our initial expectation (*H*1), sex expression in *P. purum* was at the higher end compared to other perennial unisexual bryophytes, which often rely heavily on asexual propagation (Newton & Mishler, 1994; During, 1979; Longton, 1997; Frahm, 2007, but see Crawford et al., 2009). This finding, together with the considerable number of sporophytic populations across the whole study area (possibly underestimated because the sampling was not coordinated with the sporophyte development period and it is unknown how long the sporophytes might persist on the plants), suggests that sexual reproduction might be more frequent than *a priori* expected in this species—for which most floristic works report sporophytes infrequent (e.g., Smith, 2004). Population genetic studies carried out so far reached contrasting conclusions about the main mode of reproduction of this species. On the one hand, based on the low levels of genetic diversity and limited gene flow among four populations located within 2 km in the northwest of Spain, Boquete et al. (2016b) suggested prevailing asexual reproduction. On the other hand, based on the relatively higher genetic diversity levels observed among plants collected in 16 different sites spread across Germany, Fritz (2010) suggested that sexual reproduction could be relatively frequent in this species. Together with our findings, these results indicate that the relative frequency of sexual versus asexual reproduction likely varies widely among populations and possibly between years. Further population genetic studies combined with mid-to-long-term population monitoring should improve our understanding of the causes and the extent of this variation, and its implications in population demographic processes.

Interestingly, the probability of sexual reproduction in *P. purum* was higher in female-dominated populations with few scattered males. This finding concurs with the results of Eppley et al. (2018), that reported a negative relationship between male sex ratio and sporophyte formation in the model moss *Ceratodon purpureus*, with sporophytic females found only in populations with a male sex ratio <40%. In light of these results, the authors proposed that females might delay sporophyte production until they have outcompeted males in terms of clonal growth and, maybe, reached a size threshold for sporophyte production. Our data showed a positive association between female size (shoot length and weight) and female sex ratio indicating that slightly to strongly female-biased populations were characterized by larger females. Taken together, these results suggest that lower levels of intersexual competition, that is, stronger female-biased sex ratios, could lead to greater female vegetative growth and a higher probability of successful sexual reproduction events. Other authors, on the other hand, reported the opposite results, that is, fewer sporophyte production in strongly female-biased populations (Bisang et al., 2014; Blackstock, 2015; Bisang et al., 2020). Given that the reproductive capacity of bryophytes seems to be species-specific, and depends on historical factors (i.e., phylogenetic relatedness; Bisang et al., 2014) as well as on environmental conditions (Blackstock, 2015; Bisang et al., 2020), further studies are needed to confirm/refute the generality of this pattern.

Despite the predominantly female-biased population phenotypic sex ratios found in this study, we detected a substantial proportion of male-biased populations (14%). In line with *H*2, part of this variation in the female- to male-biased population sex ratios continuum is driven by environmental conditions. Overall, the female bias increased in warmer and wetter environments, which conforms with previous findings for this species (Boquete et al., 2016a) and for the wetland moss *Drepanocladus lycopodioides* (Bisang et al., 2020). This pattern was pronounced in the Iberian Peninsula where males were more common in areas with Sub Mediterranean and continental climates (e.g., northwest Iberian Peninsula and southern Pyrenees; Sánchez de Dios et al., 2009). We cannot determine whether females were favored, or males were unable to develop, or develop poorly, in wetter environments. Yet, regardless of the underlying mechanism, our GLM and the lack of a second peak in the male distribution at the higher end of the environmental gradients defined by PCs1, 3, and 4, support the idea that male and female plants of *P. purum* could have different macroscale environmental optima. Sexual dimorphism has been reported multiple times in bryophytes, mostly

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concerning morphology, desiccation, and heavy metal tolerance (e.g., Shaw & Gaughan, 1993; Bowker et al., 2000; Rosenstiel et al., 2012; Stieha et al., 2014; Balkan, 2016; Marks et al., 2016; Moore et al., 2016; Slate et al., 2017; Boquete et al., 2021; Kollar et al., 2021). Yet, the available evidence is still inconclusive about the generality of this pattern (Cameron & Wyatt, 1990, Bowker et al., 2000; Groen et al., 2010; Blackstock, 2015 vs. Fuselier & McLetchie, 2004; Bisang et al., 2015). Our results suggest that the environment could have a significant role in determining sex ratio variation at the macroscale, although the amount of variance explained is rather low. It is plausible that part of the variation unexplained here could be accounted for by microscale environmental variation (e.g., microtopography, orientation, or shading), which can be extremely large as pointed out by Stoutjesdijk & Barkman (1992). As this relationship is probably species- and environment-specific, more detailed studies are needed in this regard.

Congruent with previous findings for other moss species (Stark et al., 1998), our morphological data provided support for the "*size threshold*" hypothesis that states that shoot would need to reach a minimum pool of resources before being able to express sex (H3). On the other hand, potential, nonmutually exclusive explanations for the larger sizes of expressed *P. purum* females compared to expressed males (H4), would include that: (a) females may have a higher size threshold for sex expression because they require more resources to support sporophyte development, (b) males may have comparatively lower vegetative growth rates due to higher pre-fertilization reproductive investment, and that (c) females' more frequent occurrence in milder environments may positively contribute to higher vegetative growth rates compared to males. Regardless of the specific mechanism(s), our results suggest that sexual dimorphism vegetative growth in *P. purum* could contribute to sex ratio bias over time. This effect would be more pronounced in long-term persistent populations which would exhibit more biased sex ratios than recently established populations (H5). Indeed, we found statistical support for this hypothesis, which is congruent with the fact that the climatic oscillations of the Quaternary shaped the distribution of bryophytes in Europe, promoting the process of expansion, retraction, and refuge areas (Kyrkjeeide et al., 2014).

Despite some alternative processes that could also contribute to the tendency above (e.g., current climatic differences between long- and short-term persistent populations, or differences in their phylogeographic history), this result is remarkable and has been suggested for other bryophytes based on genetic data and considering the glacial periods of the Pleistocene (Alonso-García et al., 2020). Since our "young populations" have been potentially established since the LGM (from 19 000 years BP onwards), the persistence of such a historical signal suggests that shifts in sex ratios could operate at the scale of thousands of years (Cronberg, 2000; Alonso-García et al., 2020; Bisang et al., 2020; Blackstock, 2020). Recent evidence by Hedenäs et al. (2021), supports the hypothesis of a gradual shift in population sex ratio from balanced toward skewed sex ratios. These authors proposed that changes in microscale environmental conditions and competitive interactions could negatively affect one sex in the long run leading to this pattern. We call for further analyses on the evolution of the reproductive performance (e.g., sex expression, sex ratios, sporophyte frequency) through time involving colonization dates recovered from historical records, species distribution modeling, and/or spatial genetic demographic reconstructions (Patiño et al., 2015).

Altogether, our results show that both current environmental and historical factors contribute to intraspecific variation in reproductive traits in the unisexual moss *P. purum* across its large distributional range. The observed patterns were explained by sex-specific differences in environmental optima as well as vegetative growth which would both contribute to sex ratio bias over time, constraining the chances of sexual reproduction, especially in long-term persistent populations. Finally, we demonstrated that the probability of sexual reproduction in *P. purum* is highest in populations with larger females and only a few scattered males, which suggests that male availability is not a limiting factor for reproduction.

Author Contributions

JAF, MTB, RR, and JRA conceived the ideas; MTB, JAF, JP, and JRA conceived the methodology; ZV, CA, JAF, JC, AC, NC, BEP, JMGM, SL, JMA, NGM, ENO, JP, AV, HGZ, and JRA collected field samples; MTB, VFG, BEP, and NGM processed the samples and collected the data; MTB, AB, CGR, NGM, AVA, and JRA analyzed the data; MTB, JAF, JRA, AB, CGR, NGM, and AVA wrote the original draft; MTB, JRA, ZV, JAF, JMA, ENO, NGM, AVA, JAC, NC, AB, CGR, JP, AV, and HGZ revised and edited the manuscript; CB, RCC, JAF, JRA, JC, NC, BEP, JMGM, SL, JMA, NGM, ENO, JP, AV, and HGZ obtained funding for this project.

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Data Availability Statement

All data are available in the supplementary materials.

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Supplementary Material

The following supplementary material is available online for this article at http://onlinelibrary.wiley.com/doi/10.1111/jse.12897/suppinfo:

SM1. Selection of number of shoots collected. Box-and-whisker-plots (whiskers represent 2.5 and 97.5% percentiles) of the proportions of *Pseudoscleropodium purum* female, male and non-expressed shoots resulting from the random sampling (bootstrapping, 1000 repetitions) of n = 100 moss shoots from a population of n = 200 for each of the seven selected populations.

SM2. Location and permanence of gametangia on moss shoots. (A) Illustration of a *Pseudoscleropodium purum* shoot, and of the reproductive structures found in females (perichaetium: structure containing the female gametangia, i.e. the archegonia) and in males (perigonium: structure containing the male gametangia, i.e. antheridia). The color scale represents the approximate physiological state of the tissues along the shoot, from the most active living tissues in the apex (green to yellow-green), senescent tissues (yellow to brown), and dead tissues (dark brown). Red arrows show the potential location of gametangia on the main stem and on the lateral branch (first order and second order branches respectively). (B) photo of archegonia of *P. purum* (©MT Boquete). (C) photo antheridia of *P. purum* (©MT Boquete).

SM3. Spatial structure for sex expression and phenotypic sex ratio within the study area.

SM4. Evidence of niche differentiation between male, female and non-expressed morphs.

Table S1. Location of *Pseudoscleropodium purum* populations (Lon.: longitude; Lat.: latitude, WGS84). For each site (ID), the sampling date (SD), number of shoots (N; ♂: male; ♀: female; NE: non-expressed; T: total; SP: female shoots bearing sporophytes), and the corresponding proportions (P) are shown. Sex expression (SE), and phenotypic sex ratio (PSR: for ♂: males; and ♀: females) are also shown.

Table S2. Description, minimum, and maximum values of environmental and climatic variables used in this study. Values for prec, srad, tavg, tmax, tmin, vapr, and wind are summarized as maximums and minimums across the 12 months of the year (01 to 12).

Table S3. Principal components 1 to 4 (PC1 to PC4) from the principal component analysis on the environmental variables' dataset showing only variables with loadings ≥ |0.9| for each PC. Descriptions of the variables are available in Table S2

Table S4. Results of the linear models testing whether the magnitude of the differences in log10-transformed shoot mass length (SML), shoot weight (SW), and shoot length (SL) between females (F; n = 2957) and males (M; n = 1741) of *Pseudoscleropodium purum* depends on the environmental conditions (summarized as Principal Components - PCs) and on the female phenotypic sex ratio (FSR). DF: degrees of freedom; Sum Sq: Sum of squares; Mean Sq: Mean squares; F value: value of the test statistic; P(>F): significance value. ***: $p \leq 0.001$; **: $p \leq 0.01$; ns: not significant.

Fig. S1. Omnidirectional and directional semivariograms of sexual expression (a) and female phenotypic sex ratio (b). Line with dots: observed semivariogram; thin lines: $\alpha = 0.05$ confidence envelope.

Fig. S2. Pairwise overlap between the probability density functions of the frequency of presences along the first four axes of the PCA (PC1 to PC4) for each shoot. Overlap values and the probabilities that each observed overlap between each pair of density functions differed from the expected overlap under the null model (i.e. random assignment of each shoot to a sexual expression morph) are shown for each axis.

Fig. S3. Distribution of log transformed specific mass length (a, d), shoot length (b, e), and shoot weight (c, f) in

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expressed vs. non-expressed (a to c) and female vs. male (d to f) shoots of Pseudoscleropodium purum. Vertical dashed bars represent the mean value of each distribution.

Fig. S4. Log10-transformed shoot mass length (SML; A), shoot weight (SW; B), and shoot length (SL; C, D) plotted against the principal components (PCs) or the female phenotypic sex ratio that showed a significant interaction with Sex in the linear models testing whether the magnitude of the morphological differences between females (F; n = 2957) and males (M; n = 1741) of Pseudoscleropodium purum depended on the environmental conditions (summarized as Principal Components – PCs; Table S4) and the female phenotypic sex ratio. Red dots: females; Green dots: males; red line: linear regression line for females; green line: linear regression line for males; grey area surrounding the regression lines: 95% confidence interval.

Fig. S5. Map showing the current distribution of *Pseudoscleropodium purum* according to all European presence records (n = 111476) from GBIF.org (24 March 2021, GBIF Occurrence Download https://doi.org/10.15468/dl.2m4zak) and our own dataset (blue dots), superimposed on the current mean annual temperatures (extracted from the CHELSA bioclim dataset) truncated 5.4°C. This map shows how well the 5.4°C isotherm describes the northern range limit of *P. purum*.

Fig. S6. Maps showing the populations of *Pseudoscleropodium purum* studied in this work (blue dots), superimposed on the projected mean annual temperatures under 4 different scenarios (CHELSA LGM climatic conditions using CCSM4, IPSL, MIROC and PMI climatic models) truncated 5.4°C. Populations outside the refugia (i.e. localities with <5.4°C during the LGM) are assumed to be recolonized throughout the post-glacial expansion (i.e. less than 21K years ago).

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