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APPLICATION ARTICLE

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Genotypic confirmation of a biased phenotypic sex ratio in a dryland moss using restriction fragment length polymorphisms

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Abstract

Premise: In dioicous mosses, sex is determined by a single U (female, ♀) or V (male, ♂) chromosome. Although a 1 : 1 sex ratio is expected following meiosis, phenotypic sex ratios based on the production of gametangia are often female-biased. The dryland moss *Syntrichia caninervis* (Pottiaceae) is notable for its low frequency of sex expression and strong phenotypic female bias. Here we present a technique to determine genotypic sex in a single shoot of *S. caninervis*, and report results of a case study examining genotypic and phenotypic sex ratios.

Methods: We reanalyzed 271 non-expressing gametophyte shoots from a previous study on *S. caninervis* sex expression across microhabitats using a restriction fragment length polymorphism (RFLP) method.

Results: We recovered a genotypic sex ratio in non-expressing shoots of 18.4♀ : 1♂, which exceeds the female bias of the phenotypic ratio (5.3♀ : 1♂; $P = 0.013$). We also found that the distribution of male and female genotypes across microsites with different levels of sun exposure was not predicted by patterns of sex expression in these microsites.

Discussion: These findings contribute to our understanding of how the environment may modulate sex ratios in *S. caninervis*, either through its direct influence on sex expression or through selection on genotypes with particular sex expression phenotypes.

KEYWORDS

bryophyte, desert, dryland, restriction fragment length polymorphism (RFLP), sex chromosome, sex ratio, *Syntrichia*

Like all land plants, the bryophyte life cycle oscillates between multicellular haploid and diploid stages (Haig, 2016). However, unlike tracheophytes, bryophytes are gametophyte dominant, where the haploid stage is characterized by a leafy or thalloid gametophyte that carries a single set of chromosomes. In dioicous mosses (where the term "dioicy" refers to the separate sex of *haploid* individuals, as opposed to the term "dioecy," which refers to separate sexes in *diploid* individuals), sex is determined by a single U (female, ♀) or V (male, ♂) chromosome in the gametophyte life stage; spores that carry a single U chromosome will produce a female gametophyte, while spores that possess a single V chromosome will form a male gametophyte (Bachtrog et al., 2011; Haig, 2016). After fertilization, the diploid sporophyte of mosses contains both the U and V chromosome and does not express sex (Bachtrog et al., 2011; Figure 1).

In sexually reproducing dioecious seed plants with genetic sex determination, a 1 : 1 female to male sex ratio is expected after syngamy, although a male-biased population is often observed (Field et al., 2013). In dioicous mosses, the expected sex ratio of haploid spores after meiosis is also 1 : 1. However, contrary to seed plants, female biases are commonly observed in bryophytes, especially in mosses

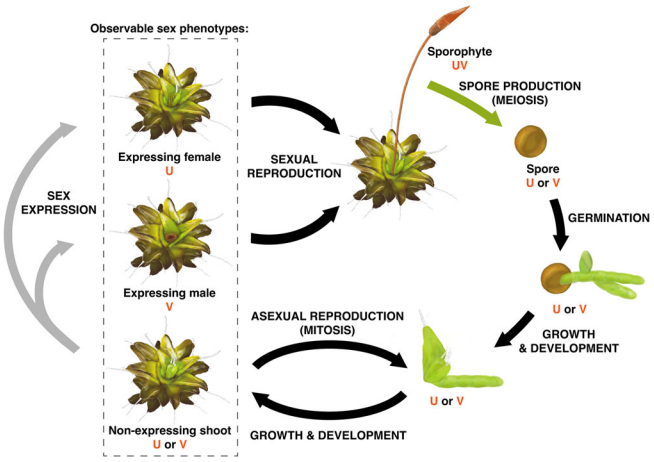

FIGURE 1 Life cycle diagram of *Syntrichia caninervis*. Orange text indicates the sex chromosome(s) associated with that stage. Shoots inside the dashed box are the three sex phenotypes observable in nature: expressing female (♀), expressing male (♂), and non-expressing. Gray arrows represent possible sex expression, depending on sex chromosome and expression rates. Gray and black arrows represent transitions that occur by mitosis while the green arrow represents a transition that occurs by meiosis. All life stages are haploid (*n*) except for the sporophyte, which is diploid (2*n*) and contains both sex chromosomes. Drawings are not to scale.

(Bisang and Hedenäs, 2005). Yet, most of these reported biased sex ratios in mosses are based on phenotype (gametangia production or sex expression), which may or may not reflect actual genotypic sex ratios, depending on the sex of non-expressing (sterile) plants. Understanding variation in phenotypic sex ratios provides insights into the conditions in which sex expression occurs, but phenotypic sex ratios fail to capture the sex of shoots (ramets) that do not express sex. Most studies focus on sex-expressing shoots and, in some species, the majority of shoots are non-expressing (reviewed in Bisang and Hedenäs, 2005); thus, understanding the underlying sex ratio of these non-expressing plants helps to determine what processes cause observed phenotypic sex ratio biases. Such information can lead to a better understanding of the relationship between ramet and genet sex ratios (McLetchie and García-Ramos, 2017).

A variety of hypotheses have been proposed to explain sex ratio biases in mosses, although these cannot be fully tested without a way to determine the genotypic sex of non-expressing shoots. A surplus of phenotypic females in a population has been attributed to multiple factors, which are outlined briefly here. First, the differential survival of male and female genotypes could contribute to this pattern, either at the meiotic stage, through differential viability of male and female spores, or at some point following germination. The former process of meiotic sex ratio bias has been well documented in *Ceratodon purpureus* (Hedw.) Brid., in spores resulting from an inter-population cross (McDaniel et al., 2007) and in spores from a panel of sporophytes from multiple natural populations (Norrell et al., 2014). In *Ceratodon* Brid., the overall low viability of spores from some sporophytes can be attributed to the incompatibility of certain combinations of maternal and paternal autosomal loci, whereas meiotic sex ratio bias could reflect lethal combinations of sex-linked and autosomal genes (Norrell et al., 2014). The "realized cost of sexual reproduction hypothesis" (Stark et al., 2000; Ekwealor et al., 2017) suggests that the low frequency of observed male sex expression mirrors actual genotypic male rarity through higher mortality at some stage of development or a clonal advantage by females. In mosses, in contrast to seed plants, female gametophytes expend fewer resources during gametangia production than males, based on the relative mass of antheridia and a high content of energetically costly oils of associated paraphyses in the male inflorescence (Barrett and Hough, 2013). Under this hypothesis, there might be an increased rate of male mortality due to a higher pre-zygotic reproductive investment by males, leaving less energy for allocation to stress tolerance and survival. Similarly, the sex that allocates more energy to sex expression tends to exhibit lower gametophyte clonal fitness, which can lead to female mosses outcompeting male mosses (Haig, 2016). Thus, females may clonally spread faster than males, contributing to a female sex ratio bias (Stark et al., 2005). Recent mathematical models have shown that the lack of offspring production can lead to a higher investment in clonal expansion in females relative to males (McLetchie et al., 2001; Rydgren et al., 2010; Stieha et al., 2017).

In contrast, the "shy male hypothesis" (Stark et al., 2010) suggests that the genotypic sex ratio is nearly equal, but male sex expression is less frequent than female expression (perhaps due to a higher threshold of a particular environmental cue or physiological need), resulting in lower rates of observed male sexual maturity and sex expression. In accordance with this hypothesis, genotypic males would be expected to make up a disproportionately large portion of non-expressing shoots within a given population (Glime and Bisang, 2017). None of these explanations for biased phenotypic sex ratios are mutually exclusive, so some combination of the above may account for individual cases of sex ratio bias observed in mosses.

An example of strongly female-biased sex expression is found in western North American populations of the dryland moss *Syntrichia caninervis* Mitt., where phenotypic sex ratios range from 7♀ : 1♂ to as high as 17♀ : 1♂ (Bowker et al., 2000; Paasch et al., 2015; Ekwealor et al., 2017). In many populations of *S. caninervis*, male sex expression and sporophytes may not be found at all (Stark et al., 2001; Ekwealor et al., 2017), and when they are, they are often limited to shaded microhabitats (Bowker et al., 2000; Stark et al., 2005). For successful fertilization to occur in bryophytes, an external source of water and close proximity of the male and female gametophytes are generally required to enable flagellated sperm dispersal (Mishler, 1988; Haig, 2016; Glime and Bisang, 2017). However, researchers may be underestimating the distance that such sperm can travel,

unassisted or assisted, following release from an antheridium (Granzow, 1989; Bisang et al., 2004; Rosenstiel et al., 2012; Pressel and Duckett, 2019; Shortlidge et al., 2021). In environments where moisture is not readily available, this requirement for free water in the environment forms a limitation for fertilization. Indeed, many dryland bryophytes reproduce primarily by asexual reproduction, limiting the energetic costs and uncertainty of sexual reproduction (Mishler, 1988; Paasch et al., 2015).

As non-expressing plants make up the majority of many moss populations, sex-associated genetic markers have recently been used as tools to accurately determine genotypic sex and population sex ratio. However, genotypic sex can sometimes be deduced from non-molecular methods, such as by cultivating non-expressing shoots until they produce gametangia, which is possible for some species (Stark et al., 2010), or by observing sex chromosomes in a chromosome squash (Newton, 1971, 1988). McDaniel et al. (2007) used 121 amplified fragment length polymorphism (AFLP) markers and three gene regions from male and female *C. purpureus* to create a linkage map and identify sex-determining linkage groups. Korpelainen et al. (2008) developed a sex-specific moss PCR marker that amplifies in female gametophytes of *Drepanocladus trifarius* (F. Weber & D. Mohr) Broth. (=*Pseudocalliergon trifarium* (F. Weber & D. Mohr) Loeske) but not in males. This marker has also been successfully applied in *D. lycopodioides* (Brid.) Warnst. (Bisang et al., 2010, 2017, 2020) and modified for use in *D. turgescens* (T. Jensen) Broth., where it amplifies in both sexes but can be used to distinguish them by sequencing of the PCR product (Hedenäs et al., 2016). Norrell et al. (2014) developed a PCR-based restriction fragment length polymorphism (RFLP) method and applied it to spores in capsules of *C. purpureus*. Additionally, 200 sex-segregating double-digest restriction-site-associated DNA (ddRAD) sequencing single nucleotide polymorphism (SNP) markers were used to characterize genotypic sex ratios in two Mojave Desert *S. caninervis* populations, and revealed an extreme female bias in both genotypic and phenotypic sex ratios, with lower sex expression rates in genotypic males (Ekwealor et al., 2017). However, while next-generation sequencing has been becoming increasingly more accessible, the quantity of tissue, laboratory expertise, as well as the bioinformatic and financial requirements for these studies may be out of reach for many researchers. To better understand the patterns and drivers of sex ratio variation in mosses, there is a need for easy, affordable, and reliable methods to determine genotypic sex in small tissue samples.

Here we present a PCR-based RFLP technique, based on a method developed for the moss *C. purpureus* (Norrell et al., 2014) and taking advantage of previously identified sex-associated SNPs in *S. caninervis* (Ekwealor et al., 2017), to determine genotypic sex in single gametophytic shoots of *S. caninervis*. We present sex chromosome PCR primer sequences that are highly conserved across the genus *Syntrichia* Brid. and use them to amplify a locus that is polymorphic between the sexes in *S. caninervis*. Importantly, this gene region contains an *Eco*RI cut site in *S. caninervis* females that is not present in males. Thus, PCR products can be digested with standard *Eco*RI enzyme and digested PCR product visualized via gel electrophoresis. The result is that males and females each have different banding patterns that can be used to determine genotypic sex, each functioning as a control for the performance of the *Eco*RI digest.

We applied this technique to a case study reanalyzing non-expressing gametophyte shoots preserved from a previous field study of *S. caninervis* (Bowker et al., 2000) to test whether the genotypic sex ratio of non-expressing shoots is consistent with the phenotypic sex ratio observed in that study. If the genotypic sex ratio of non-expressing shoots is equal to the phenotypic sex ratio, then actual male rarity would lend support to the realized cost of sexual reproduction hypothesis. On the other hand, a genotypic sex ratio of non-expressing shoots that is more balanced in the proportion of males and females than the phenotypic sex ratio would suggest a lower frequency of sex expression in males than females and support the shy male hypothesis.

Furthermore, we tested the hypothesis that occurrence of the genotypic sexes in microsites of different solar exposure is predicted by patterns of male and female sex expression observed in the study by Bowker et al. (2000), and assessed whether the presence of sex-expressing shoots is predictive of genotypic sex of nearby non-expressing shoots. These findings contribute to our understanding of how differential survival of males and females may interact with sex-specific environmental requirements for sex expression in *S. caninervis*, which has important implications for the mechanisms underlying observed patterns of sex ratio bias in dioicous mosses and sheds light on underlying processes leading to sex ratio variation in general. Our approach can serve as a model for the development of similar tools for any group of interest, so long as there are sex-associated alleles, leading to a better understanding of sex ratio biases and their underlying determinants.

METHODS

Primer and method design

Using a panel of sex-associated ddRAD sequences (Ekwealor et al., 2017) and expressed sequence tags (ESTs; Gao et al., 2014), we developed PCR primers for amplification of a sex-specific polymorphic locus on the sex chromosomes in *S. caninervis* (Table 1). We first mapped previously identified male- and female-associated ddRAD sequences to *S. caninervis* ESTs to find candidate genes that were present in both sexes but differed in the presence of an *Eco*RI cut site. Ideal candidate genes would be those that could have primer sites placed on more conserved exons but whose *Eco*RI cut site was located in an intron, increasing the odds of polymorphisms with less conservation. Mapping to

TABLE 1 Primer pair (Sc_sexF and Sc_sexR) for amplification of a sex-specific polymorphic locus on the sex chromosome of *Syntrichia caninervis*.

Direction	Sequence (5′–3′)	Priming site (bp)
Forward	GTGGTGTTGTGGATGCTTCA	3558–3578
Reverse	CAGCTTCCTCTTATGCTCTTCA	3853–3875

a reference genome sequence of a female *S. caninervis* gametophyte (Silva et al., 2020) indicated that the primers are located on exons of the gene Sc_g00229: lysine-specific demethylase, *JMJ25*-like, with an intron between them, on chromosome 13, the putative sex chromosome. To test that the primers amplify and digest consistently in both males and females of this species, we validated the protocol on a panel of known males and females (those expressing sex). The resulting PCR product is ca. 317 bp long and contains two *Eco*RI cut sites (GAATTC) in females and one cut site in males (the homologous sequence of the sex-specific female cut site in males is AAACGC).

Sample collection

We obtained specimens used in this study from the Bowker et al. (2000) specimen vouchers (UNLV herbarium; Thiers, 2022). In brief, the samples were derived from 2-cm cores of *S. caninervis* collected in 1997 every 15 m (where occurring) along four parallel transects of ca. 850 m length and 39 m apart from a 10-ha site near White Rock Spring in the Spring Mountains of southern Nevada, for a total of 89 cores containing *S. caninervis*. Henceforth, each of these cores will be referred to as "cushions," although they were previously referred to as "populations" (Bowker et al., 2000). At the time of collection, solar exposure of each cushion was recorded in three categories: under-shrub, intermediate, and exposed. Bowker et al. (2000) checked for gametangia on each stem and separated out those that were expressing sex from those that were not, the latter accounting for 85% of shoots. For the present study, we systematically sampled cushions; however, samples began to degrade once out of storage, thus 40 of the 89 previously collected cushions were analyzed. For each cushion, we selected 10 non-expressing shoots at random from each of these 40 cushions for downstream molecular analyses. Next, we separated shoots from other shoots and confirmed species identification of each. We removed rhizoids, sand, and other debris before each shoot was placed into its own microcentrifuge tube for single-step DNA extraction and amplification.

Determining genotypic sex

We amplified the sex-associated locus with the Phire Plant Direct PCR Kit (F130WH; Thermo Fisher Scientific, Waltham, Massachusetts, USA). We placed each shoot into 20 μL of Dilution Buffer and manually disrupted tissue with a dissection probe, thoroughly cleaning the probe with 70% ethanol between samples. The PCR reaction recipe consisted of, per reaction, 5.6 μL nuclease-free water, 10 μL 2X Plant Tissue PCR Buffer, 0.4 μL Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific), 1 μL each forward and reverse primers (Table 1), with 2 μL of Dilution Buffer solution from the disrupted tissue. Next, we centrifuged reaction mixtures and placed them in a thermal cycler with the following protocol: initial denaturation at 98°C for 5 min; followed by 40 cycles of 98°C for 5 s, annealing at 59°C for 5 s, and 72°C for 20 s; with a final extension step of 72°C for 1 min. PCR products were stored at 4°C until further use. For trials with *S. ruralis* (Hedw.) F. Weber & D. Mohr, we used an annealing temperature of 58°C.

After amplification, we digested PCR products with the *Eco*RI-HF enzyme (NEB-R3101; New England BioLabs, Ipswich, Massachusetts, USA) with the following recipe: per reaction, 1.8 μL nuclease-free water, 0.2 μL *Eco*RI-HF enzyme, 2 μL 10X NEB CutSmart Buffer (New England Biolabs), and 12 μL PCR product. We placed digestion reactions in a thermal cycler at 37°C for 4 h for digestion followed by 65°C for 20 min to deactivate the enzyme and halt digestion. Finally, we ran digested PCR products on a 3% agarose gel with 1 : 500 Biotium 10,000X GelRed Nucleic Acid Stain in Water (Biotium Inc., Fremont, California, USA) diluted in dimethyl sulfoxide (DMSO) and Promega 6X Blue/Orange Loading Dye (Promega Corporation, Madison, Wisconsin, USA), and visualized gel bands in ultraviolet light. We scored banding patterns as female, male, or inconclusive (if PCR amplification, complete digestion, or separation of bands failed), and repeated the protocol for inconclusive samples as necessary. After complete digestion and separation, female gametophytes have three fragments (ca. 79 bp, 103 bp, and 136 bp) while males have two (ca. 102 bp and 215 bp).

Statistical analyses

We analyzed phenotypic sex and exposure data from Bowker et al. (2000) and inferred genotypic sex data from the current study in a Python version 3.71 Jupyter notebook (Kluyver et al., 2016) using the packages NumPy (Harris et al., 2020) and pandas (McKinney, 2010).

We cleaned existing phenotypic sex and exposure data so they could be merged with inferred genotypic sex data. Because only a subset of the previously sampled cushions were assayed for the present study (40 of 89), we reduced the original Bowker et al. (2000) phenotypic sex and solar exposure data to match: only the 40 cushions that were assayed for genotypic sex were used for all downstream analyses.

To create the final data set, we characterized the following indicator variables for each cushion: cushion phenotype (female, male, or non-expressing), shoot-level genotypes (female and male shoot counts), and cushion exposure category. Additionally, we counted the total number of shoots of each sex phenotype (female, male, or non-expressing) and

each genotype (female and male) in the population in order to calculate population-level phenotypic and genotypic sex ratios.

To test whether the inferred population-level genotypic sex ratio differs from the previously observed phenotypic sex ratio, we built a 2×2 contingency table of shoot counts in each sex category and performed a two-tailed chi-square test (χ^2; McHugh, 2013). In addition to shoot-level genotypic and phenotypic sex, we also calculated cushion-level phenotypic and genotypic sex. We classed cushions as either phenotypically female, phenotypically male, or phenotypically mixed. Similarly, we summarized the genotypic sex of the non-expressing shoots per cushion and categorized the shoots as entirely female, entirely male, or mixed sex. To assess whether the genotypic sex of non-expressing shoots can be predicted by the phenotypic sex of the cushion, we compared the inferred genotypic sex of non-expressing shoots in each cushion to the phenotypic sex(es) present in each cushion.

To test whether patterns of abundance among exposure microhabitats within each genotypic sex differed for expressing and non-expressing shoots, we built two 3×2 contingency tables containing expressing and non-expressing shoot counts in each exposure category for each genotypic sex. Ten shoots did not have microhabitat exposure data and were thus omitted from this analysis. We performed two-tailed Fisher's exact tests (Fisher, 1934) on the contingency tables in R through the Python module rpy2 (Gautier, 2008) with default parameters and simulated *P* values due to small sample sizes.

Extension to other *Syntrichia* species

To gauge the potential applicability of our methodology to other dioicous *Syntrichia* species, we applied the protocol described above to male and female shoots of *S. ruralis* and compared their RFLP patterns with those of *S. caninervis*.

RESULTS

Shoot-level phenotypic and genotypic sex ratios

We successfully inferred the genotypic sex of 271 of the 400 assayed non-expressing shoots and found that this differed significantly from the previously observed phenotypic sex composition ($P = 0.013$, Table 2). We note that the pooling of ramets from different cushions may have inflated the power of our test, compared to a test of fully independent ramets, but our use of rare legacy collections necessitated this approach. The shoot-level genotypic sex ratio of 18.4♀ : 1♂ exceeded the phenotypic sex ratio of 5.3♀ : 1♂.

Cushion-level sex of non-expressing shoots

The cushion-level genotypic sex of non-expressing shoots was poorly predicted by the presence of phenotypic sex in the cushion (Table 3).

Distribution of the sexes among microhabitats

The distribution of genotypic females among shaded, intermediate, and exposed microhabitats differed significantly from the distribution of expressing females ($P = 0.008$, Table 4). Intermediate habitats contained a higher proportion of the phenotypic females (those expressing sex; 79.3%) compared to the proportion of non-expressing genotypic females (55.9%). There was a much higher proportion of non-expressing females in exposed microhabitats (32.0%) than there were expressing females (6.9%).

The distribution of genotypic males among exposure microhabitats did not differ significantly overall from the distribution of phenotypic males (Table 4). However, 50.0% of non-expressing males occurred in exposed microhabitats, while none of the expressing males were found in the exposed sites. Most of the expressing males (83.3%) occurred in intermediate exposure microhabitats.

Extension to other species of *Syntrichia*

The amplification and digestion of *S. ruralis* male and female samples resulted in the same sex-specific RFLP patterns observed in *S. caninervis* (Appendix S1).

TABLE 2 Counts of phenotypic and genotypic females, males, and non-expressing shoots.

Sex	Phenotypic count[a]	Genotypic count	χ^2	df, *N*	*P* value
Female	32 (7.8%)	257 (94.8%)	6.2132	1, 309	0.013*
Male	6 (1.5%)	14 (5.2%)			
Shoot sex ratio	5.3♀ : 1♂	18.4♀ : 1♂	—	—	—
Non-expressing	372 (90.7%)	—	—	—	—
Total	410 (100%)	271 (100%)	—	—	—

Note: χ^2 = chi-square statistic; df = degrees of freedom; *N* = sample size; *P* value = *P* value from χ^2 test.

[a]Phenotypic count data obtained from Bowker et al. (2000).

*Significance at $P < 0.05$.

TABLE 3 Counts of cushions whose non-expressing shoots are genotypically female, male, or mixed sex in cushions that are entirely non-expressing or contain only phenotypically female or phenotypically male shoots.

	Genotype of non-expressing shoots in each cushion		
Cushion phenotype	**All female**	**All male**	**Mixed sex**
Entirely non-expressing	22	0	4
Phenotypically female	11	1	0
Phenotypically male	0	1	1
Phenotypically mixed sex	0	0	0

TABLE 4 Shoot sex expression rates per genotypic sex in shaded, intermediate, and exposed microhabitats.

Sex	Exposure	Expressing count (%)	Non-expressing count (%)	*P* value[a]
Female	Under shrub	4 (13.8%)	30 (12.1%)	0.009*
	Intermediate	23 (79.3%)	138 (55.9%)	
	Exposed	2 (6.9%)	79 (32.0%)	
Male	Under shrub	1 (16.7%)	2 (14.3%)	0.072
	Intermediate	5 (83.3%)	5 (35.7%)	
	Exposed	0 (0%)	7 (50.0%)	

[a]*P* value reported from Fisher's exact tests for each sex.

*Significance at $P < 0.05$.

DISCUSSION

Utility and scope of the RFLP protocol

The Sc_sex PCR primer pair and associated RFLP protocol is effective in determining genotypic sex of non-expressing shoots of *S. caninervis*. The primer pair also amplifies the target locus and identifies males and females via identical *Eco*RI RFLPs in the related *S. ruralis*. Broad conservation of primer sequences and restriction sites suggests the applicability of this protocol for other *Syntrichia* species. Our group's phylogenetic studies (currently unpublished) have shown that *S. caninervis* and *S. ruralis* are separated on either side of the basal split within a well-supported major clade of *Syntrichia* that contains a number of other closely related species, including *S. papillosissima* (Copp.) Loeske, *S. latifolia* (Bruch ex Hartm.) Huebener, *S. norvegica* F. Weber, *S. montana* Nees, *S. laevipila* Brid., *S. virescens* (De Not.) Ochyra, and several other common Northern Hemisphere taxa. Of course, sex-specific digestion and banding patterns need to be investigated and validated for males and females of individual species to confirm this. Nonetheless, these preliminary results give us confidence that this tool will be of wide use with minimal species-specific adjustments or optimization within *Syntrichia* and possibly its close relatives.

Shoot-level genotypic vs. phenotypic sex ratios

In our *S. caninervis* case study, the genotypic sex ratio of the population was much more female-biased than the observed phenotypic sex ratio (Table 2), confirming that male rarity exists and is not simply an artifact of differential sex expression (Stark et al., 2000; Ekwealor et al., 2017). While the male rarity we observed in this study is consistent with the realized cost of sexual reproduction hypothesis, our results do not exclude the possibility that sex ratio bias is established prior to gametophyte development through the differential viability of male and female spores (McDaniel et al., 2007; Norrell et al., 2014) or through other processes influencing male and female gametophyte survival prior to sexual maturity (Eppley et al., 2018).

The pattern observed in this study contrasts with comparisons of phenotypic and genotypic sex ratios in *S. caninervis* at another Mojave Desert population where the genotypic sex ratio, while still female-biased, was less extreme than that of the phenotypes (Ekwealor et al., 2017). Our results do not support the shy male hypothesis, which would predict a greater abundance of genotypic males than genotypic females in the non-expressing shoots of a population that is phenotypically female-biased (i.e., a genotypic sex ratio that is less female-biased than the phenotypic sex ratio; Stark et al., 2010). Studies in several other moss populations with female-biased sex expression have also found little support for the shy male hypothesis, including the cosmopolitan *Bryum argenteum* Hedw. and two wetland mosses, *D. lycopodioides* and *D. trifarius* (Stark et al., 2010; Bisang and Hedenäs, 2013). Together with earlier studies (Shaw and Gaughan, 1993), the results and findings mentioned here highlight the presence of population-specific variation in sex expression and genotypic sex ratio patterns. Comparing these population-level life history traits with environmental factors may reveal what is controlling and maintaining sex ratio biases and the events that lead to male rarity.

The presence of gametangia as a predictor of genotypic sex of nearby shoots

Prior to widespread access to genotyping technologies, bryologists had frequently assumed that a single, distinct cushion of moss is likely to be a single genetic individual. However, research on even highly clonal species, such as the present focal species *S. caninervis*, has found that a few to several distinct genetic individuals can occur within small cushions (Cronberg, 1996; Bisang et al., 2015; Paasch et al., 2015; Ekwealor et al., 2017). Thus, characterizing the sex of a cushion by observing presence of a sex organ within it is risky. The design of this study allowed us to ask whether the presence of a shoot expressing phenotypic sex can predict the genotypic sex of non-expressing shoots in the same cushion. We found that the presence of a phenotypic female is a good predictor of the genotype of non-expressing shoots

in the cushion, although not foolproof (Table 3). However, in one of the 12 phenotypically female cushions, all sampled non-expressing shoots in the cushion were genotypically male, despite the presence of at least one phenotypic female shoot in the cushion. Similarly, in studying small-scale genotypic sex distribution in the highly clonal wetland moss *D. trifarius*, Bisang et al. (2015) found that although shoots collected within 25 cm of each other were likely to be the same sex, the shortest distance between two shoots of different sexes was 3 cm.

In the female-biased moss *C. purpureus*, female shoots are more likely to produce sporophytes in female-dominated cushions, whereas genotypic females in artificially produced male-dominated cushions invested more energy into clonal growth than sexual reproduction (Eppley et al., 2018). Similarly, it is possible that in *S. caninervis* the presence of a phenotypic female is strongly associated with local genotypic female dominance. The presence of a phenotypic male in a cushion only predicted the genotype of non-expressing shoots in one of two cushions, although the rarity of males makes it hard to know whether this pattern would hold with increased sampling. One should be wary of assuming genotypic sex of entire cushions based on the presence of shoots expressing their sex, as these results show that even small cushions (≤2 cm) can be mixed sex.

Distribution of sexes among exposure microhabitats

The distribution of expressing sexes among solar exposure microhabitats did not predict the distribution of genotypic sexes. Although sex expression was most common in intermediate microsites (female) or shaded microsites (male) in the original study, we found non-expressing shoots of both genotypic sexes across all exposure microsites (Table 4). For instance, females can occur in exposed microsites (32.0% of all sampled non-expressing shoots), although they are unlikely to express sex there—only 6.9% of phenotypic females had been found in exposed microhabitats. Strikingly, half of all non-expressing genotypic males identified here occurred in exposed microsites, where no males were found to be expressing sex in the original study. These results demonstrate that restriction of sex expression to one type of microsite does not indicate that the sex in question only occurs in that type of microsite.

Non-expressing shoots of each sex also occupied exposure microsites in different proportions. In particular, the majority of non-expressing genotypic female shoots occurred in intermediate microsites while the majority of non-expressing male shoots occurred in exposed microsites. (Table 4). This is in contrast to patterns observed in *D. trifarius*, in which the distribution of genotypic sexes was not explained by a range of environmental parameters (Bisang et al., 2015). Environment did explain the distribution of genotypic sexes in *D. lycopodioides*, however: non-expressing genotypic males were found to occur in drier sites than genotypic females (Bisang et al., 2020). As hypothesized by Bisang et al. (2020), the association of genotypic females with wetter sites (and out-competition of males in these habitats) in wetland *D. lycopodioides* can be explained by the increased resource demands of sporophyte production. This hypothesis may also explain patterns of *S. caninervis* genotypic sex distribution. In drylands, microsites with high solar exposure dry out faster and, conversely, mosses in shaded sites stay hydrated longer, offering increased hydroperiods for energy-intensive sporophyte production.

The realized cost of sexual reproduction hypothesis posits that male sex expression (which is more energetically costly than female sex expression and trades off with clonal growth and/or results in a higher male mortality rate), combined with low fertilization rates and subsequent spore production (thus, males are not replenished in the population via spores), could lead to male rarity (Stark et al., 2000; Ekwealor et al., 2017). Overall, females of this species have a clonal growth advantage (in the form of faster clonal spread) over males, even under a variety of stressors (Stark et al., 2004; Stark and McLetchie, 2006). However, this advantage is diminished under thermal stress, where males regenerate faster post-stress than females (Stark and McLetchie, 2006). Non-expressing males may occupy exposed microsites due to decreased competition there with genotypic females, i.e., males have increased clonal fitness in microsites where costly sex expression is suppressed by environmental conditions (Farah, 2020). Alternatively, exposed microsites may select for male genotypes that have lower fertility or a very high threshold for initiating sex expression.

CONCLUSIONS

The RFLP protocol presented here is not only effective, it is also affordable and easy to use. Additionally, the protocol was successful with specimens that were collected more than 20 years earlier. Although this is a PCR-based method, by utilizing a direct-PCR protocol it makes the expensive and time-consuming DNA extraction and purification that normally precedes PCR reactions unnecessary. Based on its ability to discriminate male and female shoots in both *S. caninervis* and *S. ruralis*, we predict that this tool could have broad usefulness across the dioicous species of *Syntrichia*, facilitating research in this ecologically important group. Furthermore, this study and method can serve as a model for the development of similar tools in other bryophyte groups. Because the UV chromosome system is ancient (Carey et al., 2021), the workflow presented here may be applicable to other dioicous bryophytes, allowing for a better understanding of sex ratio variation patterns and the processes that create and maintain them.

AUTHOR CONTRIBUTIONS

L.R.S. conceived of the study and curated and provided moss tissue, while J.T.B.E. and K.M.F. designed the study.

J.T.B.E., J.Z.J., and S.D.B. performed laboratory analyses. J.Z.J. and J.T.B.E. performed statistical analyses. S.D.B. and J.T.B.E. wrote the initial draft of the manuscript and all authors contributed to data interpretation and manuscript revisions. All authors approved the final version of the manuscript.

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DATA AVAILABILITY STATEMENT

Data and all analysis code are openly available at the following repository: https://github.com/jzjomsky/URAP_Sex_Ratio.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Agarose gel electrophoresis image of restriction fragment length polymorphism products in *Syntrichia caninervis* and *S. ruralis*. Amplicons are from the gene Sc_g00229: lysine-specific demethylase, *JMJ25*-like, amplified with the Sc_sex PCR primers, and digested with the *Eco*RI enzyme. Lanes identified with M are molecular weight standards. Lanes 1 and 2 are *S. caninervis*; lanes 3 and 4 are *S. ruralis*. Lanes 1 and 3 are female; lanes 2 and 4 are male. The band at 317 bp represents the undigested PCR product. Gel is 2% agarose that was run in 1% TBE and was stained with ethidium bromide.

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