1   Multiple factors influence population sex ratios in the Mojave Desert moss *Syntrichia*

2   *caninervis*[1]

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15   Female biased *Syntrichia caninervis* genotypic sex ratio

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43   ABSTRACT

44   • *Premise of research:* Natural populations of many mosses appear highly female-

45   biased based on the presence of reproductive structures. This bias could be caused

46   by increased male mortality, lower male growth rate, or a higher threshold for

47   achieving sexual maturity in males. Here we test these hypotheses using samples

48   from two populations of the Mojave Desert moss *Syntrichia caninervis.*

49   • *Methods:* We used double digest restriction-site associated DNA (RAD)

50   sequencing to identify candidate sex-associated loci in a panel of sex-expressing

51   plants. Next, we used putative sex-associated markers to identify the sex of

52   individuals without sex structures.

53   • *Key results:* We found an 18:1 phenotypic female: male sex ratio in the higher

54   elevation site (Wrightwood), and no sex expression at the low elevation site

55   (Phelan). In contrast, based on genetic data we found a 2:1 female bias in the

56   Wrightwood site and only females in the Phelan site. The area occupied by male

57   and female genets was indistinguishable.

58   • *Conclusions:* These data suggest that both differential mortality and sexual

59   dimorphism in thresholds for sex expression likely contribute to population

60   genetic and phenotypic sex ratio biases, and that phenotypic sex expression alone

61   fails to capture the extent of actual sex ratio bias present in natural populations of

62   *S. caninervis.*

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64   Key words: bryophyte; Mojave Desert; moss; Pottiaceae; reproductive strategy; sex

65   expression; sex ratio; *Syntrichia caninervis*

66   INTRODUCTION

67   The ratio of males to females in sexually reproducing, dioecious species is

68   generally predicted to be close to 1:1, given the assumption of equal parental investment

69   in offspring of both sexes (Fisher, 1930). Analyses of consistent sex ratio biases therefore

70   have provided important insights into the genetic basis of sex determination or the

71   importance of variation in the roles of males and females in the life cycle (Charnov,

72   1982; West, 2009). Sex allocation theory predicts that skewed sex ratios can evolve as a

73   result of both competitive and facilitative interactions among relatives (Hamilton, 1967),

74   such that competition for resources among related individuals can bias sex ratios toward

75   the more dispersive sex (Clark, 1978; Gowaty, 1993; Hewison and Gaillard, 1996), while

76   mating among siblings can bias the sex ratio toward females (Herre, 1985; West and

77   Herre, 1998; Reece et al., 2008). The fact that the cytoplasm is generally maternally

78   inherited means that male-killing cyto-nuclear interactions often generate female biases

79   (Schnable and Wise, 1998; Hurst et al., 1999). Other forms of sex-biased mortality also

80   may cause sex ratio biases in specific demographic classes. Thus, generating a clear

81   understanding of consistent sex ratio biases can provide insights into the natural history

82   of a group of organisms, or the factors that govern genetic transmission from one

83   generation to the next. Moreover, because biased sex ratios can profoundly influence the

84   patterns of genetic variation within populations, such biases can have major implications

85   for developing conservation strategies and estimating long term population persistence.

86   Female biased population sex ratios are a common yet unexplained phenomenon

87   in bryophyte populations (Bisang and Hedenäs, 2005). In dioicous bryophytes, sex is

88   determined at meiosis by a UV chromosomal system (Bachtrog et al., 2011); a spore

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89   carrying the female sex-determining locus (U) will form a gametophyte that produces

90   archegonia and ultimately bears the offspring embryo (a sporophyte), while spores with

91   the male sex-determining locus (V) form gametophytes that produce antheridia. Because

92   the diploid sporophyte is produced by the union of a U-bearing egg with a V-bearing

93   sperm, the sporophyte is always heterozygous at the sex-determining chromosome (i.e.,

94   UV). As a consequence of meiotic segregation, therefore, the null expectation is a 1:1 sex

95   ratio. Understanding if the persistently female-biased phenotypic sex ratios in moss

96   populations represent a deviation from this genetic expectation is an essential first step

97   toward understanding the ultimate evolutionary causes (e.g., local resource competition,

98   genetic conflict) and consequences of sex ratio bias in this ecologically important group

99   of plants.

100   Most of the female biases documented in mosses to date have been based on

101   counting the number of sexually mature male and female ramets, or branches, in a

102   population (but see McLetchie et al., 2001; Korpelainen et al., 2008; Bisang et al., 2010,

103   2014, 2015, Hedenäs et al., 2010, 2016; Bisang and Hedenäs, 2013). A female bias in the

104   production of sexually mature ramets in a natural population could be caused by at least

105   three distinct processes. First, an apparently female biased population sex ratio could

106   simply be a product of faster female growth, as has been found in several species (Shaw

107   and Beer, 1999; Stark, Nichols, et al., 2005; McDaniel et al., 2008). In this case, a

108   population would contain more female ramets than male ramets, and individual female

109   genets (i.e., unique haplotypes) would on average would occupy larger areas than male

110   genets, although the genetic diversity within males and females would be similar (but see

111   Bengtsson and Cronberg, 2009). Second, males might reach sexual maturity less

112   frequently than females. In other words, fewer male haplotypes might produce

113   gametangia than female haplotypes. If this were true, males would constitute a

114   disproportionately large fraction of the sterile plants in a population (termed 'the shy male

115   hypothesis'; Mishler and Oliver, 1991; Stark et al., 2005). In this case we would expect a

116   genotypic sex ratio that is closer to 1:1 than the sex ratio observed from counting fertile

117   males and females, although again the genetic diversity within males and females would

118   be similar. Finally, a female bias could be caused by elevated male mortality during spore

119   production (McDaniel et al., 2007; Norrell et al., 2014), establishment, or at some later

120   point in the life-cycle. This hypothesis predicts a genotypic female bias at both the ramet

121   and genet level. Regardless of the proximate cause, elevated male mortality would

122   decrease the amount of genetic diversity in males relative to females.

123   One of the most extreme cases of phenotypic sex ratio variation in mosses is in

124   Mojave Desert populations of *Syntrichia caninervis*. Previous studies based on counts of

125   sexually mature plants indicate that female ramets of *S. caninervis* outnumber males by

126   as much as 7:1 (Paasch et al., 2015) or 14:1 (Bowker et al., 2000; Bisang and Hedenäs,

127   2005), and that some populations lack sexually mature males entirely (Stark et al., 2001).

128   Mojave Desert *S. caninervis* is extremely desiccation tolerant and spends much of its life

129   in an air-dried state, limiting all biological functions to infrequent post-rainfall periods,

130   primarily in cool winter months (Stark, 1997; Stark et al., 1998). Differences in the

131   timing and duration of this biologically active period, such as precipitation differences

132   along elevation gradients, appear to affect frequency of sex expression (phenotypic sex)

133   in a population. A survey of 890 *S. caninervis* individuals from a 10 hectare elevation

134   gradient (Bowker et al., 2000) found that total percentage of sexually mature individuals

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135   increased with elevation. Male sex expression occurred only at the higher elevations,

136   while lower elevation populations contained only a few females with archegonia. In

137   parallel with low levels of sex expression, sexual fertilization and sporophyte production

138   is relatively rare. Established desert *S. caninervis* populations apparently persist through

139   vegetative propagation (Paasch et al., 2015).

140   Here we expand upon this work using restriction site-associated DNA genome

141   sequencing (ddRADseq) to identify the sex of sterile ramets and study the patterns of

142   genetic variation in males and females in two Mojave Desert populations of *S. caninervis*.

143   We used these data to address four main questions. First, to test whether the phenotypic

144   female bias was caused by higher rates of gametangial production by females, we

145   estimated the phenotypic and genotypic sex ratio in two populations. Second, we

146   estimated genet size to test whether sexual dimorphism in growth rate was sufficient to

147   explain the female biased phenotypic sex ratios in this species. Third, to evaluate whether

148   a genetic sex ratio bias was explained by elevated male mortality, we calculated levels of

149   genetic diversity in male and female haplotypes in both populations. Finally, to evaluate

150   the potential for inbreeding or sex-specific dispersal patterns to generate conditions

151   favorable to local mate competition or local resource competition, we generated several

152   estimates of population differentiation. Collectively these data point to differential sex

153   expression and elevated male mortality as key causes of female-biased sex ratios in

154   Mojave Desert populations of *S. caninervis*.

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156   MATERIALS AND METHODS

157   ***Sample Collection***— In the Mojave Desert, *S. caninervis* grows in a semi-

158   continuous carpet in both shaded and exposed microsites. We identified collections in the

159   field by the characteristic leaf morphology, color, and hair points, although all

160   identifications were confirmed with leaf cross-sections in the laboratory (Mishler, 2007).

161   We collected male, female, and sterile *S. caninervis* samples at two sites from Sheep

162   Creek Wash. The first site ('Wrightwood') is located at an elevation of 1800 m in Sheep

163   Creek Wash near Wrightwood, CA (34° 22' 33.85" N, 117° 36' 34.59" W), at the west

164   edge of the Mojave Desert and the northern base of the San Gabriel Mountains. The

165   average high and low annual temperature is 16.8 °C and 1.7 °C, with an average annual

166   precipitation of 49.4 cm (2007-2011, Wrightwood Weather Station, NOAA National

167   Climatic Data Center). This site experiences little foot traffic. The second site in the

168   lower portion of Sheep Creek Wash ('Phelan') is at an elevation of 1257 m near Phelan,

169   CA (34° 25' 29.80" N, 117° 36' 30.91" W), about nine miles northeast of the Wrightwood

170   site. The average high and low annual temperature is 27 °C and 10 °C, while average

171   annual precipitation is 28.2 cm (2005-2009, Phelan, CA, NOAA National Weather

172   Service). This site is also highly disturbed by foot traffic and erosion.

173   To establish a panel of plants of known sex to use to identify sex-linked molecular

174   markers, we isolated 11 *S. caninervis* female and 10 male ramets from the Wrightwood

175   site in March and April of 2013. In the laboratory under a dissecting microscope, patches

176   were hydrated and screened for presence of current or past antheridia and archegonia.

177   To estimate the phenotypic sex ratio of the Wrightwood population, in May 2014

178   we collected in 3-5 cm patches in three parallel 20 m linear north-south (N-S) transects,

179   10 m apart from one another, collecting one patch every 2 m from a variety of shaded and

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180   exposed microhabitats. Collecting at regular intervals along a transect was important for

181   calculating spatial extent of haplotypes and sexes, while collection from different habitat

182   types increased our chances of finding both sexes. In June 2014 we made collections

183   from the Phelan site in a similar manner in two parallel 20 m N-S transects. Additionally,

184   due to the highly irregular distribution of the species in the Phelan site, a third mini-

185   transect was sampled (beginning at about the 23 m mark of transect 2 and extending

186   approximately 2 m), more densely (ca. every 0.3 m) selecting samples in a northwest-

187   southeast (NW-SE) direction from a variety of microhabitats. To estimate the genetic sex

188   ratio and perform diversity analyses, a maximum of three individual sterile ramets were

189   isolated from each patch using a dissecting microscope, resulting in a total of 99 ramets

190   from the Wrightwood site and, due to lower frequency of this species at this site, 42

191   ramets from the Phelan site, for a total of n = 141 individual ramets.

192   ***DNA Extraction and RADseq library preparation***— Total genomic DNA was

193   extracted and isolated from 162 total ramets (21 of known sex, and 141 of unknown sex)

194   using a modified cetyl trimethyl ammonium bromide-beta mercaptoethanol (CTAB)

195   procedure (McDaniel et al., 2007). Prior to extraction, samples were ground dry to a fine

196   powder using a GenoGrinder 2010 bead shaker (SPEX CertiPrep, Metuchen, NJ). DNA

197   quality was evaluated for each sample by electrophoresis and quantified using a Qubit

198   Fluorometer (Invitrogen, Carlsbad, CA, USA), samples' DNAs were normalized prior to

199   library preparation.

200   Illumina libraries were prepared for sequencing using a modified version of the

201   Peterson et al. (2012) protocol using the endonucleases EcoRI and MseI (New England

202   Biolabs, Ipswich, MA). Following double enzyme digestion, unique barcoded adaptors

203   were ligated to the resulting EcoRI cut sites and a non-barcoded universal adaptor to the

204   MseI cut site. Variable length barcodes of 8, 9, and 10 base pairs (bp) were used with

205   each barcode, differing by at least 4 bp. Illumina flow cell binding and sequencing primer

206   sites were added to the adaptor ligated fragments throughout 10 cycles of PCR using

207   NEB Q5 PCR master mix (New England Biolabs, Ipswich, MA). Success of library

208   construction was evaluated through agarose gel electrophoresis, after which 5 μL of each

209   sample's final library was pooled into a single tube for the 141 sterile samples and a

210   separate tube for the 21 samples of known sex. Size selection and sequencing were

211   performed at the University of Florida's Interdisciplinary Center for Biotechnology

212   Research. Pooled libraries were fractioned using Pippin ELF precision electrophoresis

213   (Sage Science, Beverly, MA) with the resulting 250-500 bp fraction being used for

214   Illumina sequencing. The sterile ramet sample library was sequenced using an Illumina

215   NextSeq 500 at mid throughput, producing 150 bp single-end reads. The library

216   consisting of the 21 known-sex ramets was pooled with libraries constructed in an

217   identical manner for a non-related study and sequenced on one lane of a HiSeq 3000

218   producing 100 bp single end reads.

219   ***Data analysis***— Raw ddRAD sequence reads were assessed for quality with

220   fastQC (Gordon and Hannon, 2010). All manipulation of raw sequence reads was

221   performed using tools from the FASTX-Toolkit (Gordon and Hannon, 2010)

222   implemented in Galaxy on the University of Florida's Research Computing Cluster. To

223   eliminate low quality bases at the 3' end of reads, all sequences were trimmed to 100 bp.

224   Reads were filtered for a minimum Phred quality score of 20 on at least 90% of the read.

225   All reads were then demultiplexed followed by the removal of the 5' barcode and EcoRI

226   enzyme cut site.

227   RAD loci were assembled de novo using STACKS, version 1.24, (Catchen et al.,

228   2011, 2013). Pertinent parameters for the STACKS pipeline were as follows: a minimum

229   of 2 reads required for each allele within an individual (-m option ustacks), a maximum

230   allowable distance of 2 nucleotides between alleles within a locus (-M option ustacks), a

231   maximum of 2 mismatches allowed when aligning secondary reads to primary stacks (-N

232   option ustacks), and a maximum nucleotide distance of 2 allowed between loci of

233   different individuals (-n option cstacks). A subset of 30 samples, 15 from each site, was

234   used to create the master catalog of loci. Using the STACKS 'populations' program, loci

235   containing any SNP categorized as heterozygous for an individual were identified. Since

236   all individuals sampled were haploid gametophytes, all loci were expected to be

237   homozygous; therefore, those loci identified as heterozygous were discarded as they

238   likely represented paralogous or over-merged loci. Approximately 23% of loci had at

239   least one individual with a heterozygous SNP call. Although removing only the

240   heterozygous allele may be sufficient for removing errors, to be conservative we remove

241   the entire locus from the dataset. Sequence data were further filtered to retain only bi-

242   allelic loci with 1 or fewer SNPs, found in a minimum of 74% of samples, with a minor

243   allele frequency lower limit of 0.1, resulting in 2,234 loci used for subsequent genetic

244   analyses.

245   Twenty-one *S. caninervis* ramets of known sex (11 females expressing archegonia

246   and 10 males expressing antheridia) were used to identify potential sex-associated loci

247   (fragments) that would allow us to infer sex of sterile ramets. Any 80 bp locus that was

248   present in most individuals of one sex and absent in all individuals of the opposite sex

249   was considered a potential sex-associated locus. Using these criteria, we identified over

250   5,000 candidate sex-associated loci. Although one sex-linked locus would suffice for our

251   purposes, we selected the 100 male-associated and 100 female-associated loci with the

252   best coverage across all 21 individuals for further analyses. This is because a single

253   autosomal locus may show a spurious association with sex in a sample of that size, due to

254   chance or because it has a sex-biased allele frequency; the region of suppressed

255   recombination on the UV sex chromosomes, however, makes it likely that many sex-

256   linked markers will segregate in complete linkage disequilibrium, providing a signature

257   of true sex linkage. We used a custom Perl script to search for the candidate sex-linked

258   sequences in the alignments from the final 131 sterile ramets used in our analyses (see

259   Results). We then used the loci that were in complete linkage with one another in the 131

260   sterile ramet samples to determine the sex of each haplotype and tested for deviations

261   from a 1:1 sex ratio with a Chi-square test and a 2-tailed p-value.

262   In order to determine the number of unique haplotypes among the 131 sterile

263   ramets, clonal assignments were made using genetic distance under the infinite allele

264   model with a pairwise distance threshold of 0 between individuals. Higher mismatch

265   thresholds, up to 8, were evaluated but all resulted in the same clonal assignments. Figure

266   1 shows the clonal decay distribution of number of unique genets identified at different

267   genetic distance threshold levels. Furthermore, all ramets that were identified as clones of

268   the same genet were also genetically assigned to the same sex. Clonal assignments were

269   used to fill in missing data, when possible, by finding a consensus sequence among

270   ramets per haplotype using GENEIOUS version 8.1.4 (Kearse et al., 2012). To test

271   whether the female biased sex ratio was generated by greater female growth rates, we

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272   compared the mean number of sites occupied by female and male haplotypes, as well as

273   the mean number of ramets per male and female haplotypes, testing for significance using

274   a one-tailed t-test.

275   To compare levels of genetic variation between the two study populations, and

276   between males and females in the Wrightwood population, we calculated five measures

277   of clonal diversity using the software GENODIVE version 2.0b23 (Meirmans and

278   Tienderen, 2004). Clonal diversity (P_d) is a measure of unique genets relative to (divided

279   by) the number of ramets sampled (Ellstrand and Roose, 1987). The effective number of

280   genotypes (N_{eff}) is an index that accounts for frequencies of each genet and describes the

281   number of haplotypes that have equal frequencies while minimizing low frequency

282   haplotypes. This index is analogous to effective number of alleles but instead counts

283   whole haplotypes. Clonal evenness (effective number of genotypes divided by number of

284   genotypes) indicates how evenly the haplotypes are divided over the population and

285   would be equal to 1.00 if all haplotypes were represented equally. We also calculated

286   Simpson's diversity index, corrected for sample size, and the corrected Shannon's index

287   (Chao and Shen, 2003). The latter is a measure of clonal variation that accounts for

288   singletons (genets or haplotypes sampled just once) in the population for sample sizes

289   greater than approximately 50 sampling units (Arnaud-Haond et al., 2007). We used

290   bootstrap tests with 1,000 permutations and subsampling to match population sizes to test

291   for differences in the latter three measures of clonal diversity.

292   To evaluate the degree to which differences between the two populations could be

293   due to allele frequency differences, we first calculated the population differentiation

294   statistic F_{ST} among the 131 sterile ramets from the Phelan and Wrightwood sites using

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295   GENODIVE. Next, we estimated the population structure using the fastSTRUCTURE

296   (Raj et al., 2014) inference algorithm with a simple logistic prior and K=1 through K=4.

297   The dataset used for this contained 2,234 SNPs from 131 sterile ramets from the Phelan

298   and Wrightwood sites, where missing data were filled in with data from identical clonal

299   haplotypes, when possible. Membership coefficients for K=2 were plotted using

300   DISTRUCT version 2.2 from the fastSTRUCTURE software package.

301   For another means to visualize the patterns of genetic distance among genets in

302   the two populations, we constructed a midpoint-rooted neighbor joining genetic distance

303   tree using CLEARCUT. The dataset used for this contained 2,234 SNPs from 51 genets

304   from the Phelan and Wrightwood sites, where missing data were again filled in with

305   identical haplotypes, when possible. Two genets with greater than 80% missing data were

306   excluded. Because the RAD loci are not completely linked, this tree does not represent

307   the genealogical relationships among these individuals but rather genetic similarity.

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309   RESULTS

310   ***Phenotypic sex ratios***— Of the 49 patches collected in the Wrightwood site, 31

311   contained no sex structures, 17 contained ramets expressing archegonia and were

312   classified as female, one contained ramets of both sexes, and no patches contained ramets

313   with only male gametangia. The resulting phenotypic sex ratio of 18 F:1 M differs

314   significantly from 1:1 meiotic expectations (Chi-squared test, two-tailed p-value <

315   0.0001).

316   ***Sequencing statistics***—Approximately 150 million total raw sequencing reads

317   were generated for the 141 ramet samples sequenced resulting in roughly 1.06 million

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318   reads per barcode. About 40% of the reads were discarded due to low quality, resulting in

319   88 million reads that passed initial quality filters. Ten ramets with fewer than 4.5

320   thousand reads were discarded, leaving 98 sterile ramets in the Wrightwood site, 33

321   sterile ramets in the Phelan site, and 21 ramets of known sex from the Wrightwood site,

322   ranging from 51 thousand to 2.6 million reads per ramet. The data matrix of 2,234 SNPs

323   from 131 sterile ramet samples was 80% complete when using original reads but

324   increased to 94% complete when missing data were filled in with data from identical

325   clonal haplotypes. The data matrix of concatenated loci used for the RAxML analysis

326   was 83.3% complete with a mean depth of about 5.6 reads per locus.

327   *Identifying sex linked loci and genetic sex ratio*— We used the 20 individuals of

328   known sex to identify 100 candidate male and 100 candidate female diagnostic markers.

329   We chose this approach because our sample size was necessarily small, meaning that any

330   single locus could show a spurious or population specific linkage to sex. Indeed,

331   approximately 25% of the loci from our trial set (52 loci between males and females)

332   were absent from our individuals of unknown sex, and 10% of the loci were apparently

333   misclassified as sex-linked because they failed to show complete linkage disequilibrium

334   with the other candidate loci. It is possible that the missing loci were lost due to subtle

335   differences in the library size selection procedure, or due to stochastic sampling during

336   the sequencing process. The remaining 65% of the candidate sex-linked loci were in

337   complete linkage with one another, as expected for a UV sex chromosome system. The

338   63 putatively male-linked loci had an average read depth of 6.3 and on average 33.5

339   male-associated loci were found within each newly identified genotypic male ramet. The

340   65 putatively female-linked loci had a mean read depth of 5.7 and an average of 25.3

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341   female-associated loci were detected in each newly identified genotypic female ramet.

342   Thus, although some of these loci may later be shown to be only sex-correlated, or

343   limited to these populations, and not truly sex-linked, these data give us high confidence

344   that collectively these loci are diagnostic markers for sex in this sample of individuals.

345   Using these putative sex-linked markers, we found 65 female ramets and 33 male

346   ramets in samples from the Wrightwood site, equating to an approximate 2:1 female-

347   biased genetic sex ratio. This represents a significant deviation from both the 1:1

348   expectation (Chi-squared test, two-tailed p-value = 0.0012) and from the observed 18:1

349   phenotypic sex expression based on counts of sexually mature ramets (Chi-squared test,

350   two-tailed p-value < 0.0001). All 33 of the ramets from the Phelan site were female.

351   Including all of the 2,234 biallelic SNPs, we found 53 distinct genetic individuals,

352   or haplotypes, among the 131 sterile ramets. The Wrightwood site contained 45 unique

353   haplotypes and the Phelan site had eight haplotypes. A total of 53 haplotypes were

354   distinguishable at genetic distance thresholds of 0 through 7, after which the number of

355   haplotypes began to drop incrementally without distinct breaks, indicating that the

356   number of genotypes is unlikely to be inflated as a result of genotyping errors (Figure 1).

357   The plants in the Wrightwood site were assignable to 31 female haplotypes and 14 male

358   haplotypes, corresponding to an approximately 2:1 female-biased sex ratio.

359   ***Distribution of genotypic variation***— To test whether males and females

360   exhibited different patterns of vegetative expansion, we counted the number of sites that

361   each haplotype occupied. Most haplotypes in the Wrightwood site were restricted to a

362   single 3-5 cm patch. Of the 45 haplotypes in the Wrightwood site, three were found

363   within adjacent patches (approximately 2 m apart) and only one haplotype spanned three

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364   patches across about 4 m (Figure 2). Female and male haplotypes did not occupy

365   significantly different numbers of patches (mean female patches = 1.097 ± 0.301, mean

366   male patches = 1.143 ± 0.535, one-tailed t-test p-value = 0.3565) nor did they contain

367   significantly different numbers of ramets per haplotype (mean female ramets = 2.097 ±

368   1.044, mean male ramets = 2.357 ± 1.151, one-tailed t-test p-value = 0.2286).

369   To test whether the sexes harbored different amounts of genetic diversity, we

370   calculated several indices. The Simpson's diversity and evenness values were not

371   significantly different between the two sexes in Wrightwood, but the corrected Shannon

372   Index was significantly higher for females than for males (Table 1, p-value = 0.001).

373   Both the Simpson's diversity and the corrected Shannon indices were significantly higher

374   in Wrightwood than in Phelan (p-value = 0.001). Evenness was also slightly greater in

375   the Wrightwood site, but not significantly so (Table 2).

376   ***Population differentiation and structure***— To evaluate the potential for sex

377   biased dispersal or inbreeding to drive sex ratio bias, we examined several measures of

378   population structure. The F_{ST} between the Phelan and Wrightwood sites was 0.102 when

379   all ramets were included. When estimated with just unique haplotypes, however, F_{ST}

380   dropped to 0.028, indicating that the patterns of haplotype expansion differ in the two

381   populations. The neighbor joining tree recovered haplotypes collected from Phelan nested

382   within Wrightwood haplotypes (Figure 3). Population structure analysis in

383   fastSTRUCTURE estimated a minimum of one model component to explain structure in

384   the data. However, this estimate was not supported by the corresponding marginal

385   likelihoods, which increased with the K parameter with no apparent maximum.

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386   Membership coefficients for K = 2, visualized on Figure 4 show some support for

387   structure among the two sites, consistent with inferences based on F_{ST}.

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389   DISCUSSION

390   *S. caninervis* has long been a model for investigations regarding the evolution and

391   ecology of sex ratio variation in mosses (Stark et al., 1998, 2001; Stark, McLetchie, et al.,

392   2005; Stark, Nichols, et al., 2005; Stark and McLetchie, 2006; Paasch et al., 2015).

393   However, our understanding of sex ratio variation in this species, as well as other

394   bryophytes, has been limited by the large number of sterile plants in most bryophyte

395   populations. In principle, this limitation is overcome by the use of sex-linked molecular

396   markers, which provide a simple means of determining the sex of sterile plants.

397   Identifying reliable sex-linked markers is not trivial, though, requiring screening large

398   numbers of polymorphic loci in a large pedigree or mapping population. In addition, sex

399   linkage ideally should be confirmed using a physical map, or molecular evolutionary

400   analyses, for example to test for complete linkage disequilibrium (LD) among sex linked

401   loci, as predicted based on the observation that recombination does not occur on UV sex

402   chromosomes (Bachtrog et al., 2011). To date, within mosses this has only been

403   accomplished in the model system *Ceratodon purpureus* (McDaniel et al., 2007, 2013). A

404   less rigorous approach involves testing for an association between a molecular marker

405   and sex in a large panel of unrelated individuals (as has been done in three

406   *Drepanocladus* species; (Bisang et al., 2010, 2015; Bisang and Hedenäs, 2013; Hedenäs

407   et al., 2016). Here, we have used the latter approach, and screened several thousand

408   restriction-site associated sequenced (RADseq) loci in a small number of individuals of

409   known sex. Although the number of individuals was small, the large number of loci gave

410   us additional confidence because we could retain only those that exhibited complete LD

411   among all loci in a larger sample. Beyond the large numbers of polymorphisms, RADseq

412   loci have the advantage of being defined by a unique DNA sequence, which both allows

413   us to be confident in the homology of our loci (unlike gel band-length approaches), and

414   ultimately provides a means of identifying sex-linked genes in published transcriptomes

415   (Gao et al., 2014; Wickett et al., 2014).

416   We use these putative sex-linked markers to show that the genotypic sex ratios in

417   two Mojave Desert populations populations of *S. caninervis* are female biased (2 F:1 M

418   in the Wrightwood site, and entirely female in the Phelan site). Importantly, however, the

419   phenotypic sex ratios in this sample were far more biased (18 F:1 M in Wrightwood).

420   While it is certainly possible that we have over-estimated the long-term female sex

421   expression bias, due to drought in the study area in the years prior to collection (2013-

422   2014), collections from seasons with more typical winter weather patterns reported

423   phenotypic sex ratios of 7 F:1 M (Paasch et al., 2015), approximately three times more

424   female biased than the genotypic sex ratio we found. These data indicate that males

425   constitute a disproportionately large fraction of the sterile plants, providing unequivocal

426   support for the shy male hypothesis in these Mojave Desert populations of *S. caninervis*.

427   The greater frequency of female ramets, however, indicates that factors beyond

428   thresholds for sex expression must also contribute to the population sex ratio variation in

429   *S. caninervis*. Experimental manipulations show that females regenerate more readily

430   from plant fragments than males do under both cool conditions and desiccation stress

431   (Stark et al., 2004; Stark, Nichols, et al., 2005; Stark and McLetchie, 2006), and

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432   potentially may accumulate more biomass. However, the fact that we observed no

433   difference in number of sites occupied between female and male haplotypes indicates that

434   the genotypic female bias in these populations is unlikely to be due to faster female

435   growth.

436   We do, however, find some evidence that elevated male mortality contributes to

437   the overall female biased sex ratio. The male-mortality hypothesis uniquely predicts a

438   lower male haplotype diversity, relative to females, as we found (corrected Shannon

439   index, 1.596 in females, 1.208 in males, $p = 0.001$). When comparing the two

440   populations, the Phelan site is less clonally diverse (Simpson's and corrected Shannon

441   indices, $p = 0.001$) and has a greater genotypic female bias than the Wrightwood site, also

442   suggesting greater mortality. Importantly, the relatively low F_{ST} between these two

443   populations indicates that most surveyed polymorphisms are shared between Phelan and

444   Wrightwood, suggesting that sex ratio differences between them are unlikely to result

445   from long isolation.

446   With our current data it is not possible to isolate when in the life cycle male and

447   female demography differ, nor whether the two sexes exhibit different microhabitat

448   preferences. However, the observed pattern of lower male diversity (i.e., elevated male

449   mortality) suggests that the interaction between male physiology and extrinsic

450   environmental factors are more likely to govern population sex ratio than exclusively

451   intrinsic factors like sex ratio distorters (McDaniel et al., 2007; Norrell et al., 2014).

452   Indeed, the available evidence suggests that *S. caninervis* female biased phenotypic sex

453   ratios in Mojave Desert populations correlate with precipitation and temperature (Bowker

454   et al., 2000). One potential cause of the rarity of males observed in *S. caninervis* is the

455   difference in timing of resource allocation to reproduction between the sexes. Although

456   sexual reproduction is costly for both sexes, in the moss *Hylocomium splendens* males

457   make a higher initial investment in the production of antheridia, while fertilized females

458   only allocate energy to reproduction after fertilization through nurturing a sporophyte

459   (Rydgren et al., 2010). Although we observe sporophytes only rarely in *S. caninervis*, the

460   relatively high diversity evident in our sample, along with the limited population

461   structure and weak structure in the population genealogy all indicate that sexual

462   reproduction is or has been present in these populations. Thus, males may experience a

463   trade-off between sexual selection, to produce more reproductive tissue, and natural

464   selection, which may favor investment in the maintenance of vegetative tissues, as has

465   been seen in the moss *C. purpureus* (McDaniel, 2005).

466   Several bryophyte demographic models predict the eventual local extinction of

467   males, based on vegetative growth patterns similar to those found in *S. caninervis*

468   (McLetchie et al., 2001; Crowley et al., 2005; Rydgren et al., 2010), although these

469   models generally assume that migration is negligible. The lack of population structure

470   between our two study sites indicates that population sex ratios may be influenced by

471   migration of spores from other populations in addition to local population dynamics. The

472   mixing of genotypes in the distance tree suggests that both sites in Sheep Creek Wash

473   draw spores from the same metapopulation. Some populations, like the Wrightwood site,

474   both recruit and produce spores that contribute to subsequent generations, while other

475   sites, like Phelan, permit growth but no sexual reproduction and become genotype sinks.

476   Our sampling is insufficient to quantitatively evaluate the population structure of Mojave

477   Desert populations of *S. caninervis*, but these inferences are consistent with studies in

21

478   other mosses that report some population structure at the patch scale (Hutsemékers et al.,

479   2010; Leonardía et al., 2013; Rosengren et al., 2016) but limited structure at regional or

480   larger spatial scales (McDaniel and Shaw, 2005; Vanderpoorten et al., 2008; Korpelainen

481   et al., 2012; Shaw et al., 2014; Désamoré et al., 2016).

482   *Conclusions*—This study demonstrates that the highly female-biased sex ratios

483   observed in Mojave Desert *S. caninervis* are congruent with both the shy male hypothesis

484   (Mishler and Oliver, 1991; Stark, McLetchie, et al., 2005) and increased male mortality

485   (Stark et al., 2000). These results suggest the importance of interactions between

486   environmental conditions and demographic factors for shaping sex ratios in this species,

487   and may have important consequences for the persistence of local populations in the

488   presence of long-term shifts in climate. Importantly, both mechanisms are grounded in

489   the disproportionate pre-zygotic energetic allocation to sexual reproduction by males

490   relative to females (Mishler and Oliver, 1991; Stark et al., 2000; Stark, McLetchie, et al.,

491   2005), in essence representing a trade-off between natural and sexual selection. Our data

492   suggest that local mate competition and local resource competition are less likely to be

493   major drivers of sex ratio variation in Mojave Desert populations of *S. caninervis*, but we

494   should caution that studies of inbreeding and sex-biased dispersal are needed in

495   bryophytes. We suspect that the wider use of genomic approaches like those we have

496   used here is likely to uncover cases of sex ratio variation where other processes may

497   predominate (Cronberg, 2003; Bisang and Hedenäs, 2005; Stark et al., 2010; Horsley et

498   al., 2011; Bisang et al., 2014; Norrell et al., 2014). A future challenge is to determine the

499   contributions of migration and environmental factors like water availability, through its

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500   effects on determining sex expression and mortality, on the trajectory of population sex

501   ratio change through time.

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503

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698   TABLES

Sex	N	G	P_d	N_{eff}	Simpson's	Evenness	Shannon (corrected)
Female	65	31	0.477	25.000	0.975	0.806	1.596*
Male	33	14	0.424	11.463	0.941	0.819	1.208*

699

700   Table 1. Clonal diversity indices for the sexes in the Wrightwood site.

701   Note: *Statistical difference between the sexes in Wrightwood, p-value = 0.001, based on

702   1,000 permutations with subsampling to match population sizes. N = number of ramets

703   sampled, G = number of genets, P_d = clonal diversity, N_{eff} = effective number of

704   genotypes.

705

Site	N	G	P_d	N_{eff}	Simpson's	Evenness	Shannon (corrected)
Wrightwood	98	45	0.459	36.379	0.983*	0.808	1.753*
Phelan	33	8	0.242	6.368	0.869*	0.796	0.874*

706

707   Table 2. Clonal diversity indices for Wrightwood and Phelan sites.

708   Note: *Statistical difference between Wrightwood and Phelan, p-value = 0.001, based on

709   1,000 permutations with subsampling to match population sizes. N = number of ramets

710   sampled, G = number of genets, P_d = clonal diversity, N_{eff} = effective number of

711   genotypes.

712

713   APPENDIX 1

714   Specimen vouchers were deposited in the California State University, Los Angeles

715   Herbarium (CSLA).

716

717   FIGURE LEGENDS

718   Figure 1. Clonal lineage decay at different genetic distance thresholds. This figure shows

719   the number of genets that would be identified at each genetic distance parameter setting.
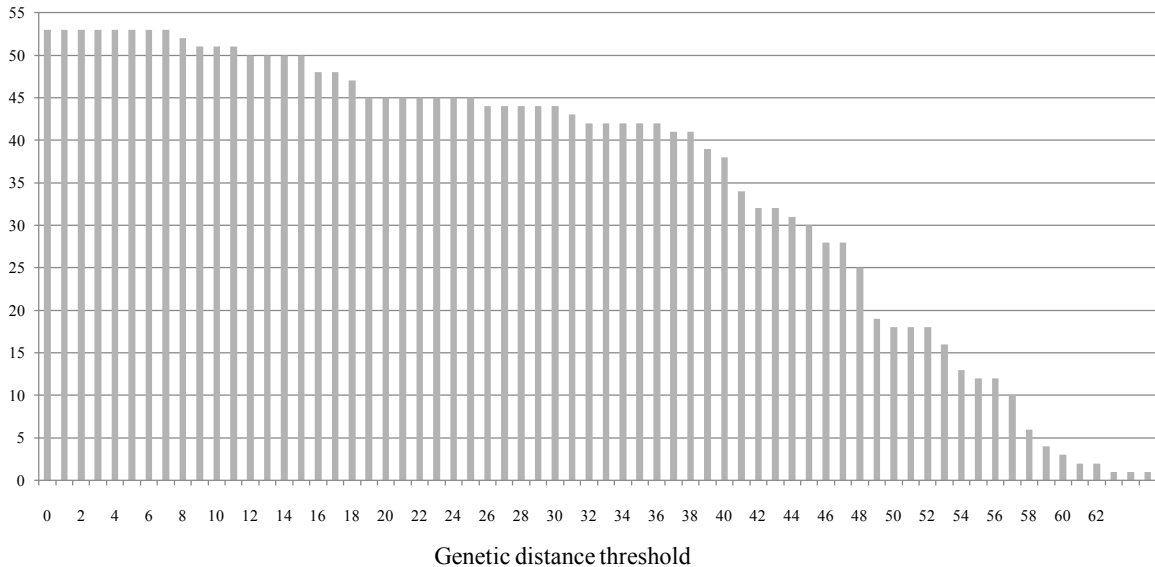
720

721   Figure 2. Spatial distribution of genets. Wrightwood contained 53 unique genets in 98

722   ramets from 33 patches along three transects (T1, T2, and T3). Genotypic females are

723   represented as circles and genotypic males as squares. Genets are outlined in dashed

724   lines. In cases that genets are in different patches, a dashed line connects different ramets

725   of the same MLG. Presence of phenotypic sex in each patch is indicated with ♀ for

726   phenotypic females and ♂ for phenotypic males.

727

728   Figure 3. CLEARCUT neighbor joining genetic distance tree of ramets from the

729   Wrightwood and Phelan sites. Branches that join samples from the Wrightwood site

730   ('W') site are blue and those that join the Phelan site ('P') are red. Branches that support

731   mixed-population groups are purple. Symbols indicate inferred sex.

732

733   Figure 4. fastSTRUCTURE membership coefficients for K = 2. Vertical bars represent

734   individual ramets while colors represent genetic clusters detected (K = 2). Bars on the left

735   represent ramets from the Phelan site (n = 33) and bars on the right represent ramets from

736   the Wrightwood site (n = 98). Membership coefficients are plotted for each ramet and are

737   colored corresponding to the proportion of each ramet MLG that most closely aligns with

738   each of the two clusters. The dataset used for this analysis contained 2,234 SNPs from

739   131 sterile ramets from Wrightwood and Phelan where missing data were filled in with

740   clones, when possible.

Phelan   Wrightwood