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

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Female biased *Syntrichia caninervis* genotypic sex ratio

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ABSTRACT

- *Premise of research:* Natural populations of many mosses appear highly female-biased based on the presence of reproductive structures. This bias could be caused by increased male mortality, lower male growth rate, or a higher threshold for achieving sexual maturity in males. Here we test these hypotheses using samples from two populations of the Mojave Desert moss *Syntrichia caninervis.*
- *Methods:* We used double digest restriction-site associated DNA (RAD) sequencing to identify candidate sex-associated loci in a panel of sex-expressing plants. Next, we used putative sex-associated markers to identify the sex of individuals without sex structures.
- *Key results:* We found an 18:1 phenotypic female: male sex ratio in the higher elevation site (Wrightwood), and no sex expression at the low elevation site (Phelan). In contrast, based on genetic data we found a 2:1 female bias in the Wrightwood site and only females in the Phelan site. The area occupied by male and female genets was indistinguishable.
- *Conclusions:* These data suggest that both differential mortality and sexual dimorphism in thresholds for sex expression likely contribute to population genetic and phenotypic sex ratio biases, and that phenotypic sex expression alone fails to capture the extent of actual sex ratio bias present in natural populations of *S. caninervis.*

Key words: bryophyte; Mojave Desert; moss; Pottiaceae; reproductive strategy; sex expression; sex ratio; *Syntrichia caninervis*

INTRODUCTION

The ratio of males to females in sexually reproducing, dioecious species is generally predicted to be close to 1:1, given the assumption of equal parental investment in offspring of both sexes (Fisher, 1930). Analyses of consistent sex ratio biases therefore have provided important insights into the genetic basis of sex determination or the importance of variation in the roles of males and females in the life cycle (Charnov, 1982; West, 2009). Sex allocation theory predicts that skewed sex ratios can evolve as a result of both competitive and facilitative interactions among relatives (Hamilton, 1967), such that competition for resources among related individuals can bias sex ratios toward the more dispersive sex (Clark, 1978; Gowaty, 1993; Hewison and Gaillard, 1996), while mating among siblings can bias the sex ratio toward females (Herre, 1985; West and Herre, 1998; Reece et al., 2008). The fact that the cytoplasm is generally maternally inherited means that male-killing cyto-nuclear interactions often generate female biases (Schnable and Wise, 1998; Hurst et al., 1999). Other forms of sex-biased mortality also may cause sex ratio biases in specific demographic classes. Thus, generating a clear understanding of consistent sex ratio biases can provide insights into the natural history of a group of organisms, or the factors that govern genetic transmission from one generation to the next. Moreover, because biased sex ratios can profoundly influence the patterns of genetic variation within populations, such biases can have major implications for developing conservation strategies and estimating long term population persistence.

Female biased population sex ratios are a common yet unexplained phenomenon in bryophyte populations (Bisang and Hedenäs, 2005). In dioicous bryophytes, sex is determined at meiosis by a UV chromosomal system (Bachtrog et al., 2011); a spore

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 Hedenäs et al., 2010; Bisang and Hedenäs, 2013; Bisang et al., 2014; Bisang et al., 2015;
104 Hedenäs et al., 2016). A female bias in the production of sexually mature ramets in a
105 natural population could be caused by at least three distinct processes. First, an
106 apparently female biased population sex ratio could simply be a product of faster female
107 growth, as has been found in several species (Shaw and Beer, 1999; Stark et al., 2005a;
108 McDaniel et al., 2008). In this case, a population would contain more female ramets than
109 male ramets, and individual female genets (i.e., unique haplotypes) would on average
110 would occupy larger areas than male genets, although the genetic diversity within males
111 and females would be similar (but see Bengtsson and Cronberg, 2009). Second, males

112 might reach sexual maturity less frequently than females. In other words, fewer male
113 haplotypes might produce gametangia than female haplotypes. If this were true, males
114 would constitute a disproportionately large fraction of the sterile plants in a population
115 (termed 'the shy male hypothesis'; Mishler and Oliver, 1991; Stark et al., 2005). In this
116 case we would expect a genotypic sex ratio that is closer to 1:1 than the sex ratio
117 observed from counting fertile males and females, although again the genetic diversity
118 within males and females would be similar. Finally, a female bias could be caused by
119 elevated male mortality during spore production (McDaniel et al., 2007; Norrell et al.,
120 2014), establishment, or at some later point in the life-cycle. This hypothesis predicts a
121 genotypic female bias at both the ramet and genet level. Regardless of the proximate
122 cause, elevated male mortality would decrease the amount of genetic diversity in males
123 relative to females.

124 One of the most extreme cases of phenotypic sex ratio variation in mosses is in
125 Mojave Desert populations of *Syntrichia caninervis.* Previous studies based on counts of
126 sexually mature plants indicate that female ramets of *S. caninervis* outnumber males by
127 as much as 7:1 (Paasch et al., 2015) or 14:1 (Bowker et al., 2000; Bisang and Hedenäs,
128 2005), and that some populations lack sexually mature males entirely (Stark et al., 2001).
129 Mojave Desert *S. caninervis* is extremely desiccation tolerant and spends much of its life
130 in an air-dried state, limiting all biological functions to infrequent post-rainfall periods,
131 primarily in cool winter months (Stark, 1997; Stark et al., 1998). Differences in the
132 timing and duration of this biologically active period, such as precipitation differences
133 along elevation gradients, appear to affect frequency of sex expression (phenotypic sex)
134 in a population. A survey of 890 *S. caninervis* individuals from a 10 hectare elevation

135 gradient (Bowker et al., 2000) found that total percentage of sexually mature individuals
136 increased with elevation. Male sex expression occurred only at the higher elevations,
137 while lower elevation populations contained only a few females with archegonia. In
138 parallel with low levels of sex expression, sexual fertilization and sporophyte production
139 is relatively rare. Established desert *S. caninervis* populations apparently persist through
140 vegetative propagation (Paasch et al., 2015).

141 Here we expand upon this work using restriction site-associated DNA genome
142 sequencing (ddRADseq) to identify the sex of sterile ramets and study the patterns of
143 genetic variation in males and females in two Mojave Desert populations of *S. caninervis*.
144 We used these data to address four main questions. First, to test whether the phenotypic
145 female bias was caused by higher rates of gametangial production by females, we
146 estimated the phenotypic and genotypic sex ratio in two populations. Second, we
147 estimated genet size to test whether sexual dimorphism in growth rate was sufficient to
148 explain the female biased phenotypic sex ratios in this species. Third, to evaluate whether
149 a genetic sex ratio bias was explained by elevated male mortality, we calculated levels of
150 genetic diversity in male and female haplotypes in both populations. Finally, to evaluate
151 the potential for inbreeding or sex-specific dispersal patterns to generate conditions
152 favorable to local mate competition or local resource competition, we generated several
153 estimates of population differentiation. Collectively these data point to differential sex
154 expression and elevated male mortality as key causes of female-biased sex ratios in
155 Mojave Desert populations of *S. caninervis*.

156

157 MATERIALS AND METHODS

158 ***Sample Collection***— In the Mojave Desert, *S. caninervis* grows in a semi-
159 continuous carpet in both shaded and exposed microsites. We identified collections in the
160 field by the characteristic leaf morphology, color, and hair points, although all
161 identifications were confirmed with leaf cross-sections in the laboratory (Mishler, 2007).
162 We collected male, female, and sterile *S. caninervis* samples at two sites from Sheep
163 Creek Wash. The first site ('Wrightwood') is located at an elevation of 1800 m in Sheep
164 Creek Wash near Wrightwood, CA (34° 22' 33.85" N, 117° 36' 34.59" W), at the west
165 edge of the Mojave Desert and the northern base of the San Gabriel Mountains. The
166 average high and low annual temperature is 16.8 °C and 1.7 °C, with an average annual
167 precipitation of 49.4 cm (2007-2011, Wrightwood Weather Station, NOAA National
168 Climatic Data Center). This site experiences little foot traffic. The second site in the
169 lower portion of Sheep Creek Wash ('Phelan') is at an elevation of 1257 m near Phelan,
170 CA (34° 25' 29.80" N, 117° 36' 30.91" W), about nine miles northeast of the Wrightwood
171 site. The average high and low annual temperature is 27 °C and 10 °C, while average
172 annual precipitation is 28.2 cm (2005-2009, Phelan, CA, NOAA National Weather
173 Service). This site is also highly disturbed by foot traffic and erosion.
174 To establish a panel of plants of known sex to use to identify sex-linked molecular
175 markers, we isolated 11 *S. caninervis* female and 10 male ramets from the Wrightwood
176 site in March and April of 2013. In the laboratory under a dissecting microscope, patches
177 were hydrated and screened for presence of current or past antheridia and archegonia.
178 To estimate the phenotypic sex ratio of the Wrightwood population, in May 2014
179 we collected in 3-5 cm patches in three parallel 20 m linear north-south (N-S) transects,
180 10 m apart from one another, collecting one patch every 2 m from a variety of shaded and

181 exposed microhabitats. Collecting at regular intervals along a transect was important for
182 calculating spatial extent of haplotypes and sexes, while collection from different habitat
183 types increased our chances of finding both sexes. In June 2014 we made collections
184 from the Phelan site in a similar manner in two parallel 20 m N-S transects. Additionally,
185 due to the highly irregular distribution of the species in the Phelan site, a third mini-
186 transect was sampled (beginning at about the 23 m mark of transect 2 and extending
187 approximately 2 m), more densely (ca. every 0.3 m) selecting samples in a northwest-
188 southeast (NW-SE) direction from a variety of microhabitats. To estimate the genetic sex
189 ratio and perform diversity analyses, a maximum of three individual sterile ramets were
190 isolated from each patch using a dissecting microscope, resulting in a total of 99 ramets
191 from the Wrightwood site and, due to lower frequency of this species at this site, 42
192 ramets from the Phelan site, for a total of n = 141 individual ramets.

193 ***DNA Extraction and RADseq library preparation***— Total genomic DNA was
194 extracted and isolated from 162 total ramets (21 of known sex, and 141 of unknown sex)
195 using a modified cetyl trimethyl ammonium bromide-beta mercaptoethanol (CTAB)
196 procedure (McDaniel et al., 2007). Prior to extraction, samples were ground dry to a fine
197 powder using a GenoGrinder 2010 bead shaker (SPEX CertiPrep, Metuchen, NJ). DNA
198 quality was evaluated for each sample by electrophoresis and quantified using a Qubit
199 Fluorometer (Invitrogen, Carlsbad, CA, USA), samples' DNAs were normalized prior to
200 library preparation.

201 Illumina libraries were prepared for sequencing using a modified version of the
202 Peterson et al. (2012) protocol using the endonucleases EcoRI and MseI (New England
203 Biolabs, Ipswich, MA). Following double enzyme digestion, unique barcoded adaptors

204 were ligated to the resulting EcoRI cut sites and a non-barcoded universal adaptor to the
205 MseI cut site. Variable length barcodes of 8, 9, and 10 base pairs (bp) were used with
206 each barcode, differing by at least 4 bp. Illumina flow cell binding and sequencing primer
207 sites were added to the adaptor ligated fragments throughout 10 cycles of PCR using
208 NEB Q5 PCR master mix (New England Biolabs, Ipswich, MA). Success of library
209 construction was evaluated through agarose gel electrophoresis, after which 5 µL of each
210 sample's final library was pooled into a single tube for the 141 sterile samples and a
211 separate tube for the 21 samples of known sex. Size selection and sequencing were
212 performed at the University of Florida's Interdisciplinary Center for Biotechnology
213 Research. Pooled libraries were fractioned using Pippin ELF precision electrophoresis
214 (Sage Science, Beverly, MA) with the resulting 250-500 bp fraction being used for
215 Illumina sequencing. The sterile ramet sample library was sequenced using an Illumina
216 NextSeq 500 at mid throughput, producing 150 bp single-end reads. The library
217 consisting of the 21 known-sex ramets was pooled with libraries constructed in an
218 identical manner for a non-related study and sequenced on one lane of a HiSeq 3000
219 producing 100 bp single end reads.

220 ***Data analysis***— Raw ddRAD sequence reads were assessed for quality with
221 fastQC (Gordon and Hannon, 2010). All manipulation of raw sequence reads was
222 performed using tools from the FASTX-Toolkit (Gordon and Hannon, 2010)
223 implemented in Galaxy on the University of Florida's Research Computing Cluster. To
224 eliminate low quality bases at the 3' end of reads, all sequences were trimmed to 100 bp.
225 Reads were filtered for a minimum Phred quality score of 20 on at least 90% of the read.
226 All reads were then demultiplexed followed by the removal of the 5' barcode and EcoRI

227 enzyme cut site.

228 RAD loci were assembled de novo using STACKS, version 1.24, (Catchen et al.,
229 2011; Catchen et al., 2013). Pertinent parameters for the STACKS pipeline were as
230 follows: a minimum of 2 reads required for each allele within an individual (-m option
231 ustacks), a maximum allowable distance of 2 nucleotides between alleles within a locus (-
232 M option ustacks), a maximum of 2 mismatches allowed when aligning secondary reads
233 to primary stacks (-N option ustacks), and a maximum nucleotide distance of 2 allowed
234 between loci of different individuals (-n option cstacks). A subset of 30 samples, 15 from
235 each site, was used to create the master catalog of loci. Using the STACKS 'populations'
236 program, loci containing any SNP categorized as heterozygous for an individual were
237 identified. Since all individuals sampled were haploid gametophytes, all loci were
238 expected to be homozygous; therefore, those loci identified as heterozygous were
239 discarded as they likely represented paralogous or over-merged loci. Approximately 23%
240 of loci had at least one individual with a heterozygous SNP call. Although removing only
241 the heterozygous allele may be sufficient for removing errors, to be conservative we
242 remove the entire locus from the dataset. Sequence data were further filtered to retain
243 only bi-allelic loci with 1 or fewer SNPs, found in a minimum of 74% of samples, with a
244 minor allele frequency lower limit of 0.1, resulting in 2,234 loci used for subsequent
245 genetic analyses.

246 Twenty-one *S. caninervis* ramets of known sex (11 females expressing archegonia
247 and 10 males expressing antheridia) were used to identify potential sex-associated loci
248 (fragments) that would allow us to infer sex of sterile ramets. Any 80 bp locus that was
249 present in most individuals of one sex and absent in all individuals of the opposite sex

250 was considered a potential sex-associated locus. Using these criteria, we identified over
251 5,000 candidate sex-associated loci. Although one sex-linked locus would suffice for our
252 purposes, we selected the 100 male-associated and 100 female-associated loci with the
253 best coverage across all 21 individuals for further analyses. This is because a single
254 autosomal locus may show a spurious association with sex in a sample of that size, due to
255 chance or because it has a sex-biased allele frequency; the region of suppressed
256 recombination on the UV sex chromosomes, however, makes it likely that many sex-
257 linked markers will segregate in complete linkage disequilibrium, providing a signature
258 of true sex linkage. We used a custom Perl script to search for the candidate sex-linked
259 sequences in the alignments from the final 131 sterile ramets used in our analyses (see
260 Results). We then used the loci that were in complete linkage with one another in the 131
261 sterile ramet samples to determine the sex of each haplotype and tested for deviations
262 from a 1:1 sex ratio with a Chi-square test and a 2-tailed p-value.

263 In order to determine the number of unique haplotypes among the 131 sterile
264 ramets, clonal assignments were made using genetic distance under the infinite allele
265 model with a pairwise distance threshold of 0 between individuals. Higher mismatch
266 thresholds, up to 8, were evaluated but all resulted in the same clonal assignments. Figure
267 1 shows the clonal decay distribution of number of unique genets identified at different
268 genetic distance threshold levels. Furthermore, all ramets that were identified as clones of
269 the same genet were also genetically assigned to the same sex. Clonal assignments were
270 used to fill in missing data, when possible, by finding a consensus sequence among
271 ramets per haplotype using GENEIOUS version 8.1.4 (Kearse et al., 2012). To test
272 whether the female biased sex ratio was generated by greater female growth rates, we

273 compared the mean number of sites occupied by female and male haplotypes, as well as
274 the mean number of ramets per male and female haplotypes, testing for significance using
275 a one-tailed t-test.

276 To compare levels of genetic variation between the two study populations, and
277 between males and females in the Wrightwood population, we calculated five measures
278 of clonal diversity using the software GENODIVE version 2.0b23 (Meirmans and Van
279 Tienderen, 2004). Clonal diversity (P_d) is a measure of unique genets relative to (divided
280 by) the number of ramets sampled (Ellstrand and Roose, 1987). The effective number of
281 genotypes (N_{eff}) is an index that accounts for frequencies of each genet and describes the
282 number of haplotypes that have equal frequencies while minimizing low frequency
283 haplotypes. This index is analogous to effective number of alleles but instead counts
284 whole haplotypes. Clonal evenness (effective number of genotypes divided by number of
285 genotypes) indicates how evenly the haplotypes are divided over the population and
286 would be equal to 1.00 if all haplotypes were represented equally. We also calculated
287 Simpson's diversity index, corrected for sample size, and the corrected Shannon's index
288 (Chao and Shen, 2003). The latter is a measure of clonal variation that accounts for
289 singletons (genets or haplotypes sampled just once) in the population for sample sizes
290 greater than approximately 50 sampling units (Arnaud-Haond et al., 2007). We used
291 bootstrap tests with 1,000 permutations and subsampling to match population sizes to test
292 for differences in the latter three measures of clonal diversity.

293 To evaluate the degree to which differences between the two populations could be
294 due to allele frequency differences, we first calculated the population differentiation
295 statistic F_{ST} among the 131 sterile ramets from the Phelan and Wrightwood sites using

296 GENODIVE. Next, we estimated the population structure using the fastSTRUCTURE
297 (Raj et al., 2014) inference algorithm with a simple logistic prior and K=1 through K=4.
298 The dataset used for this contained 2,234 SNPs from 131 sterile ramets from the Phelan
299 and Wrightwood sites, where missing data were filled in with data from identical clonal
300 haplotypes, when possible. Membership coefficients for K=2 were plotted using
301 DISTRUCT version 2.2 from the fastSTRUCTURE software package.
302 For another means to visualize the patterns of genetic distance among genets in
303 the two populations, we constructed a midpoint-rooted neighbor joining genetic distance
304 tree using CLEARCUT. The dataset used for this contained 2,234 SNPs from 51 genets
305 from the Phelan and Wrightwood sites, where missing data were again filled in with
306 identical haplotypes, when possible. Two genets with greater than 80% missing data were
307 excluded. Because the RAD loci are not completely linked, this tree does not represent
308 the genealogical relationships among these individuals but rather genetic similarity.
309
310 RESULTS
311 ***Phenotypic sex ratios***— Of the 49 patches collected in the Wrightwood site, 31
312 contained no sex structures, 17 contained ramets expressing archegonia and were
313 classified as female, one contained ramets of both sexes, and no patches contained ramets
314 with only male gametangia. The resulting phenotypic sex ratio of 18 F:1 M differs
315 significantly from 1:1 meiotic expectations (Chi-squared test, two-tailed p-value <
316 0.0001).
317 ***Sequencing statistics***—Approximately 150 million total raw sequencing reads
318 were generated for the 141 ramet samples sequenced resulting in roughly 1.06 million

319 reads per barcode. About 40% of the reads were discarded due to low quality, resulting in
320 88 million reads that passed initial quality filters. Ten ramets with fewer than 4.5
321 thousand reads were discarded, leaving 98 sterile ramets in the Wrightwood site, 33
322 sterile ramets in the Phelan site, and 21 ramets of known sex from the Wrightwood site,
323 ranging from 51 thousand to 2.6 million reads per ramet. The data matrix of 2,234 SNPs
324 from 131 sterile ramet samples was 80% complete when using original reads but
325 increased to 94% complete when missing data were filled in with data from identical
326 clonal haplotypes. The data matrix of concatenated loci used for the RAxML analysis
327 was 83.3% complete with a mean depth of about 5.6 reads per locus.

328 ***Identifying sex linked loci and genetic sex ratio***— We used the 20 individuals of
329 known sex to identify 100 candidate male and 100 candidate female diagnostic markers.
330 We chose this approach because our sample size was necessarily small, meaning that any
331 single locus could show a spurious or population specific linkage to sex. Indeed,
332 approximately 25% of the loci from our trial set (52 loci between males and females)
333 were absent from our individuals of unknown sex, and 10% of the loci were apparently
334 misclassified as sex-linked because they failed to show complete linkage disequilibrium
335 with the other candidate loci. It is possible that the missing loci were lost due to subtle
336 differences in the library size selection procedure, or due to stochastic sampling during
337 the sequencing process. The remaining 65% of the candidate sex-linked loci were in
338 complete linkage with one another, as expected for a UV sex chromosome system. The
339 63 putatively male-linked loci had an average read depth of 6.3 and on average 33.5
340 male-associated loci were found within each newly identified genotypic male ramet. The
341 65 putatively female-linked loci had a mean read depth of 5.7 and an average of 25.3

342 female-associated loci were detected in each newly identified genotypic female ramet.
343 Thus, although some of these loci may later be shown to be only sex-correlated, or
344 limited to these populations, and not truly sex-linked, these data give us high confidence
345 that collectively these loci are diagnostic markers for sex in this sample of individuals.

346 Using these putative sex-linked markers, we found 65 female ramets and 33 male
347 ramets in samples from the Wrightwood site, equating to an approximate 2:1 female-
348 biased genetic sex ratio. This represents a significant deviation from both the 1:1
349 expectation (Chi-squared test, two-tailed p-value = 0.0012) and from the observed 18:1
350 phenotypic sex expression based on counts of sexually mature ramets (Chi-squared test,
351 two-tailed p-value < 0.0001). All 33 of the ramets from the Phelan site were female.

352 Including all of the 2,234 biallelic SNPs, we found 53 distinct genetic individuals,
353 or haplotypes, among the 131 sterile ramets. The Wrightwood site contained 45 unique
354 haplotypes and the Phelan site had eight haplotypes. A total of 53 haplotypes were
355 distinguishable at genetic distance thresholds of 0 through 7, after which the number of
356 haplotypes began to drop incrementally without distinct breaks, indicating that the
357 number of genotypes is unlikely to be inflated as a result of genotyping errors (Figure 1).
358 The plants in the Wrightwood site were assignable to 31 female haplotypes and 14 male
359 haplotypes, corresponding to an approximately 2:1 female-biased sex ratio.

360 ***Distribution of genotypic variation***— To test whether males and females
361 exhibited different patterns of vegetative expansion, we counted the number of sites that
362 each haplotype occupied. Most haplotypes in the Wrightwood site were restricted to a
363 single 3-5 cm patch. Of the 45 haplotypes in the Wrightwood site, three were found
364 within adjacent patches (approximately 2 m apart) and only one haplotype spanned three

365 patches across about 4 m (Figure 2). Female and male haplotypes did not occupy
366 significantly different numbers of patches (mean female patches = 1.097 ± 0.301, mean
367 male patches = 1.143 ± 0.535, one-tailed t-test p-value = 0.3565) nor did they contain
368 significantly different numbers of ramets per haplotype (mean female ramets = 2.097 ±
369 1.044, mean male ramets = 2.357 ± 1.151, one-tailed t-test p-value = 0.2286).

370 To test whether the sexes harbored different amounts of genetic diversity, we
371 calculated several indices. The Simpson's diversity and evenness values were not
372 significantly different between the two sexes in Wrightwood, but the corrected Shannon
373 Index was significantly higher for females than for males (Table 1, p-value = 0.001).
374 Both the Simpson's diversity and the corrected Shannon indices were significantly higher
375 in Wrightwood than in Phelan (p-value = 0.001). Evenness was also slightly greater in
376 the Wrightwood site, but not significantly so (Table 2).

377 ***Population differentiation and structure***— To evaluate the potential for sex
378 biased dispersal or inbreeding to drive sex ratio bias, we examined several measures of
379 population structure. The F_{ST} between the Phelan and Wrightwood sites was 0.102 when
380 all ramets were included. When estimated with just unique haplotypes, however, F_{ST}
381 dropped to 0.028, indicating that the patterns of haplotype expansion differ in the two
382 populations. The neighbor joining tree recovered haplotypes collected from Phelan nested
383 within Wrightwood haplotypes (Figure 3). Population structure analysis in
384 fastSTRUCTURE estimated a minimum of one model component to explain structure in
385 the data. However, this estimate was not supported by the corresponding marginal
386 likelihoods, which increased with the K parameter with no apparent maximum.

387 Membership coefficients for K =2, visualized on Figure 4 show some support for
388 structure among the two sites, consistent with inferences based on F_{ST}.
389
390 DISCUSSION
391 *S. caninervis* has long been a model for investigations regarding the evolution and
392 ecology of sex ratio variation in mosses (Stark et al., 1998; Stark et al., 2001; Stark et al.,
393 2005a; Stark et al., 2005b; Stark and McLetchie, 2006; Paasch et al., 2015). However,
394 our understanding of sex ratio variation in this species, as well as other bryophytes, has
395 been limited by the large number of sterile plants in most bryophyte populations. In
396 principle, this limitation is overcome by the use of sex-linked molecular markers, which
397 provide a simple means of determining the sex of sterile plants. Identifying reliable sex-
398 linked markers is not trivial, though, requiring screening large numbers of polymorphic
399 loci in a large pedigree or mapping population. In addition, sex linkage ideally should be
400 confirmed using a physical map, or molecular evolutionary analyses, for example to test
401 for complete linkage disequilibrium (LD) among sex linked loci, as predicted based on
402 the observation that recombination does not occur on UV sex chromosomes (Bachtrog et
403 al., 2011). To date, within mosses this has only been accomplished in the model system
404 *Ceratodon purpureus* (McDaniel et al., 2007; McDaniel et al., 2013). A less rigorous
405 approach involves testing for an association between a molecular marker and sex in a
406 large panel of unrelated individuals (as has been done in three *Drepanocladus* species;
407 (Bisang et al., 2010; Bisang and Hedenäs, 2013; Bisang et al., 2015; Hedenäs et al.,
408 2016). Here, we have used the latter approach, and screened several thousand restriction-
409 site associated sequenced (RADseq) loci in a small number of individuals of known sex.

410 Although the number of individuals was small, the large number of loci gave us
411 additional confidence because we could retain only those that exhibited complete LD
412 among all loci in a larger sample. Beyond the large numbers of polymorphisms, RADseq
413 loci have the advantage of being defined by a unique DNA sequence, which both allows
414 us to be confident in the homology of our loci (unlike gel band-length approaches), and
415 ultimately provides a means of identifying sex-linked genes in published transcriptomes
416 (Gao et al., 2014; Wickett et al., 2014).

417 We use these putative sex-linked markers to show that the genotypic sex ratios in
418 two Mojave Desert populations populations of *S. caninervis* are female biased (2 F:1 M
419 in the Wrightwood site, and entirely female in the Phelan site). Importantly, however, the
420 phenotypic sex ratios in this sample were far more biased (18 F:1 M in Wrightwood).
421 While it is certainly possible that we have over-estimated the long-term female sex
422 expression bias, due to drought in the study area in the years prior to collection (2013-
423 2014), collections from seasons with more typical winter weather patterns reported
424 phenotypic sex ratios of 7 F:1 M (Paasch et al., 2015), approximately three times more
425 female biased than the genotypic sex ratio we found. These data indicate that males
426 constitute a disproportionately large fraction of the sterile plants, providing unequivocal
427 support for the shy male hypothesis in these Mojave Desert populations of *S. caninervis*.

428 The greater frequency of female ramets, however, indicates that factors beyond
429 thresholds for sex expression must also contribute to the population sex ratio variation in
430 *S. caninervis*. Experimental manipulations show that females regenerate more readily
431 from plant fragments than males do under both cool conditions and desiccation stress
432 (Stark et al., 2004; Stark et al., 2005b; Stark and McLetchie, 2006), and potentially may

433 accumulate more biomass. However, the fact that we observed no difference in number
434 of sites occupied between female and male haplotypes indicates that the genotypic female
435 bias in these populations is unlikely to be due to faster female 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
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 et al., 2005a) and increased male mortality (Stark et al.,
485 2000). These results suggest the importance of interactions between environmental
486 conditions and demographic factors for shaping sex ratios in this species, and may have
487 important consequences for the persistence of local populations in the presence of long-
488 term shifts in climate. Importantly, both mechanisms are grounded in the disproportionate
489 pre-zygotic energetic allocation to sexual reproduction by males relative to females
490 (Mishler and Oliver, 1991; Stark et al., 2000; Stark et al., 2005a), in essence representing
491 a trade-off between natural and sexual selection. Our data suggest that local mate
492 competition and local resource competition are less likely to be major drivers of sex ratio
493 variation in Mojave Desert populations of *S. caninervis*, but we should caution that
494 studies of inbreeding and sex-biased dispersal are needed in bryophytes. We suspect that
495 the wider use of genomic approaches like those we have used here is likely to uncover
496 cases of sex ratio variation where other processes may predominate (Cronberg, 2003;
497 Bisang and Hedenäs, 2005; Stark et al., 2010; Horsley et al., 2011; Bisang et al., 2014;
498 Norrell et al., 2014). A future challenge is to determine the contributions of migration and
499 environmental factors like water availability, through its effects on determining sex
500 expression and mortality, on the trajectory of population sex ratio change through time.
501

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590 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*

591

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

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

594 1,000 permutations with subsampling to match population sizes. N = number of ramets

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

596 genotypes.

597

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*

598

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

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

601 1,000 permutations with subsampling to match population sizes. N = number of ramets

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

603 genotypes.

604

APPENDIX 1

Specimen vouchers were deposited in the California State University, Los Angeles Herbarium (CSLA).

FIGURE LEGENDS

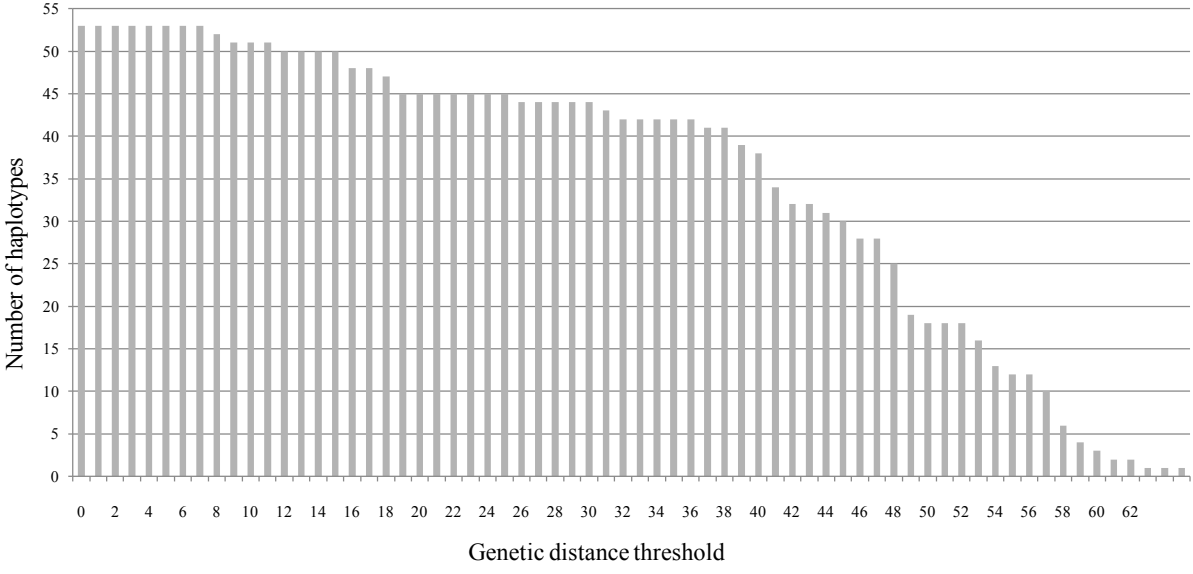
Figure 1. Clonal lineage decay at different genetic distance thresholds. This figure shows the number of genets that would be identified at each genetic distance parameter setting.

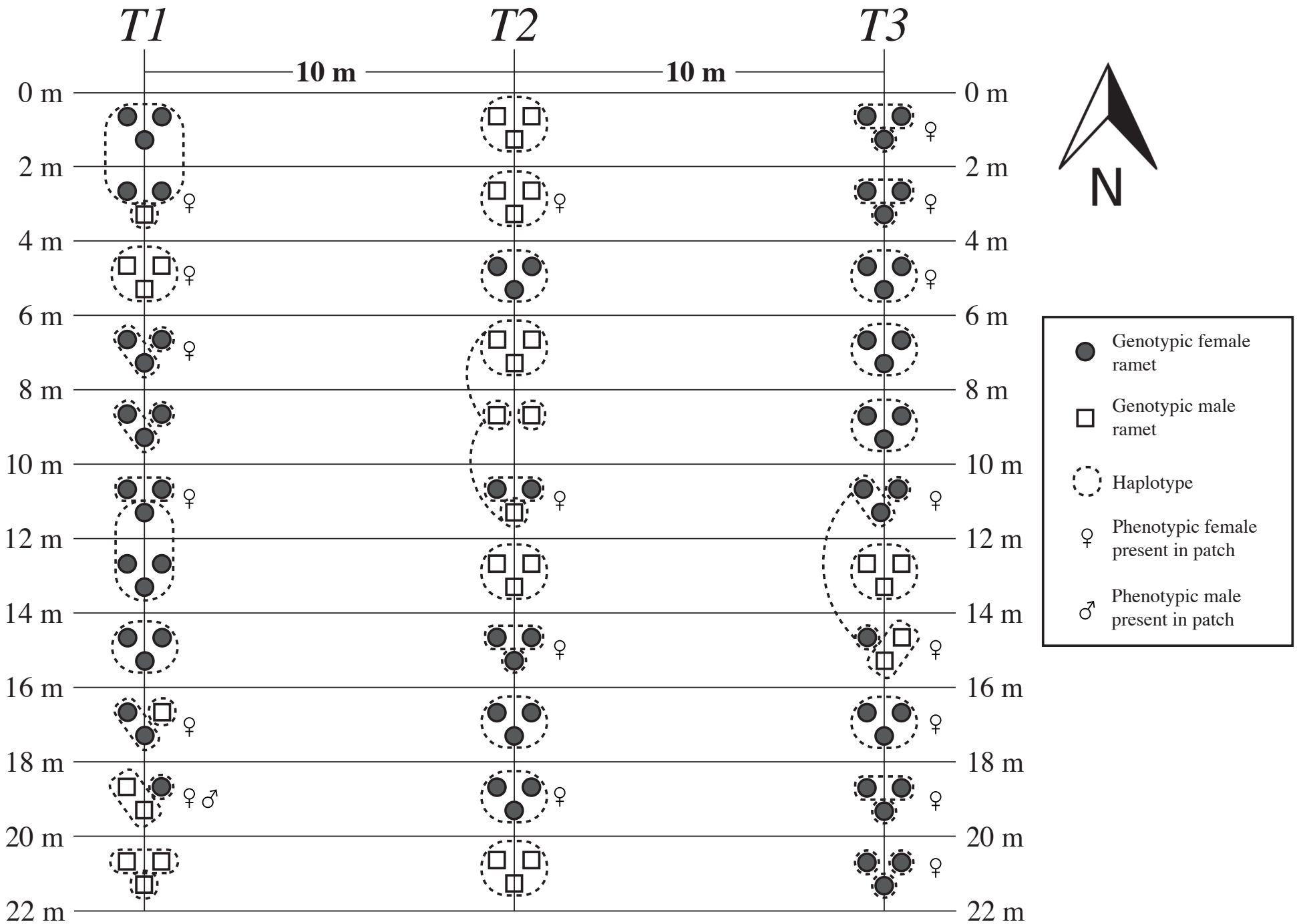
Figure 2. Spatial distribution of genets. Wrightwood contained 53 unique genets in 98 ramets from 33 patches along three transects (T1, T2, and T3). Genotypic females are represented as circles and genotypic males as squares. Genets are outlined in dashed lines. In cases that genets are in different patches, a dashed line connects different ramets of the same MLG. Presence of phenotypic sex in each patch is indicated with ♀ for phenotypic females and ♂ for phenotypic males.

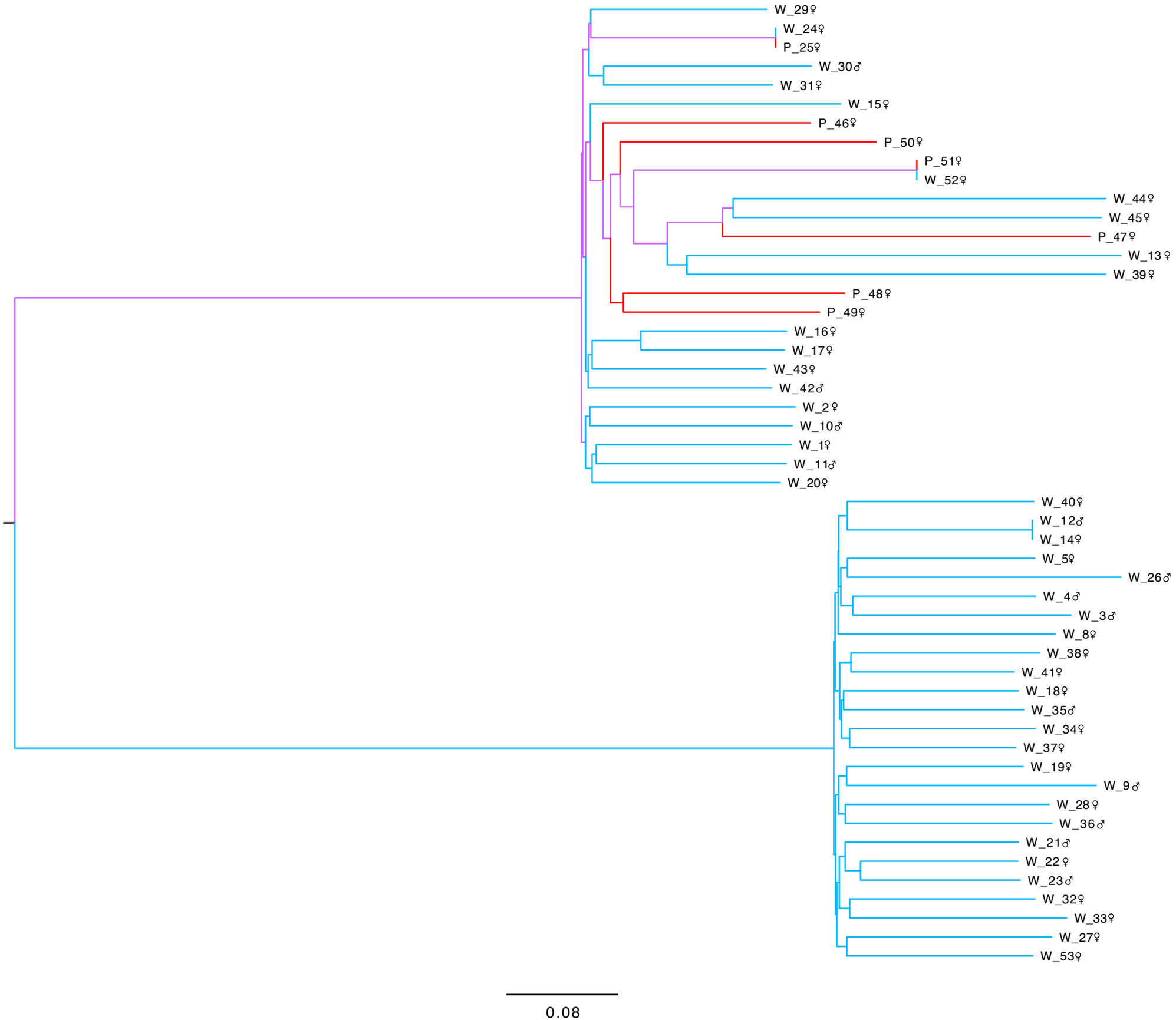
Figure 3. CLEARCUT neighbor joining genetic distance tree of ramets from the Wrightwood and Phelan sites. Branches that join samples from the Wrightwood site ('W') site are blue and those that join the Phelan site ('P') are red. Branches that support mixed-population groups are purple. Symbols indicate inferred sex.

Figure 4. fastSTRUCTURE membership coefficients for K = 2. Vertical bars represent individual ramets while colors represent genetic clusters detected (K = 2). Bars on the left

represent ramets from the Phelan site (n = 33) and bars on the right represent ramets from the Wrightwood site (n = 98). Membership coefficients are plotted for each ramet and are colored corresponding to the proportion of each ramet MLG that most closely aligns with each of the two clusters. The dataset used for this analysis contained 2,234 SNPs from 131 sterile ramets from Wrightwood and Phelan where missing data were filled in with clones, when possible.


Number of haplotypes
55
50
45
40
35
30
25
20
15
10
5
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62
Genetic distance threshold


T1
T2
T3
10 m
10 m
0 m
2 m
4 m
6 m
8 m
10 m
12 m
14 m
16 m
18 m
20 m
22 m
N
Genotypic female ramet
Genotypic male ramet
Haplotype
Phenotypic female present in patch
Phenotypic male present in patch


W_29♀
W_24♀
P_25♀
W_30♂
W_31♀
W_15♀
P_46♀
P_50♀
P_51♀
W_52♀
W_44♀
W_45♀
P_47♀
W_13♀
W_39♀
P_48♀
P_49♀
W_16♀
W_17♀
W_43♀
W_42♂
W_2♀
W_10♂
W_1♀
W_11♂
W_20♀
W_40♀
W_12♂
W_14♀
W_5♀
W_26♂
W_4♂
W_3♂
W_8♀
W_38♀
W_41♀
W_18♀
W_35♂
W_34♀
W_37♀
W_19♀
W_9♂
W_28♀
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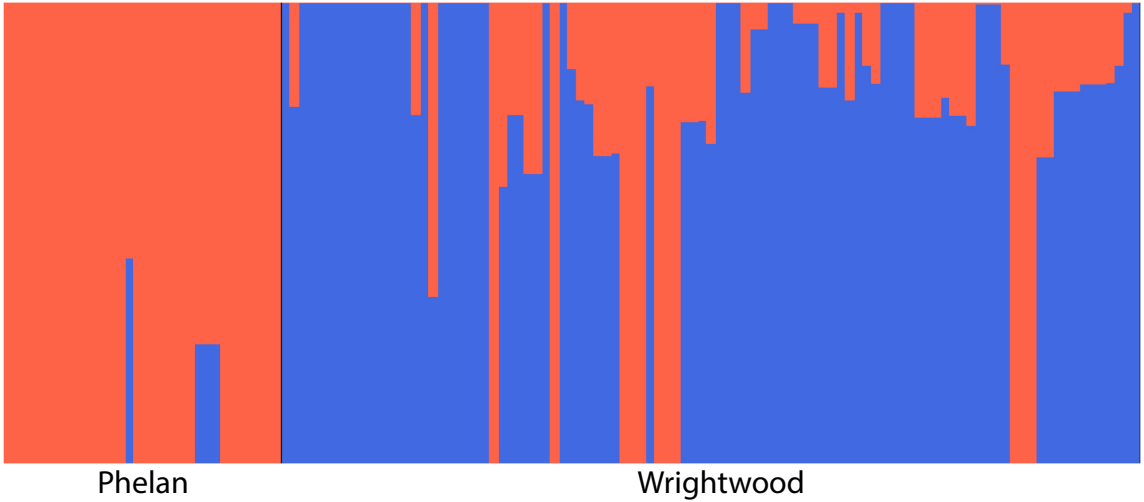
W_53♀
0.08


Phelan
Wrightwood