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*

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

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

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*.

155

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

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

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

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.
308
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

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

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

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.

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}.
388
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

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

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

effects on determining sex expression and mortality, on the trajectory of population sex ratio change through time.

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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

APPENDIX 1

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

FIGURE LEGENDS

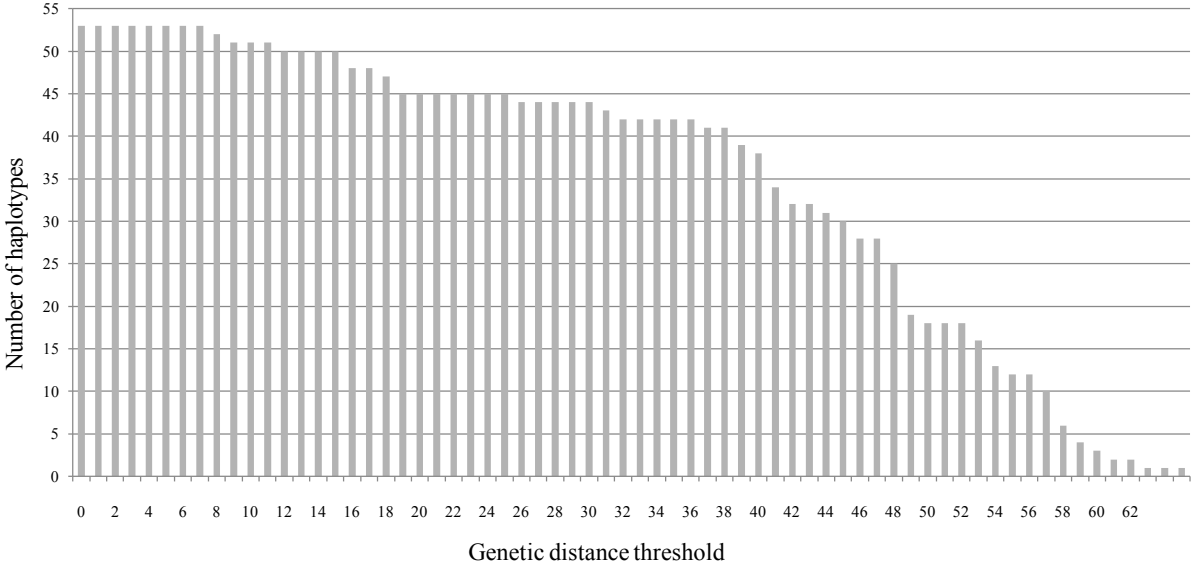
Figure 1. Clonal lineage decay at different genetic distance thresholds. This figure shows the number of genets that would be identified at each genetic distance parameter setting.

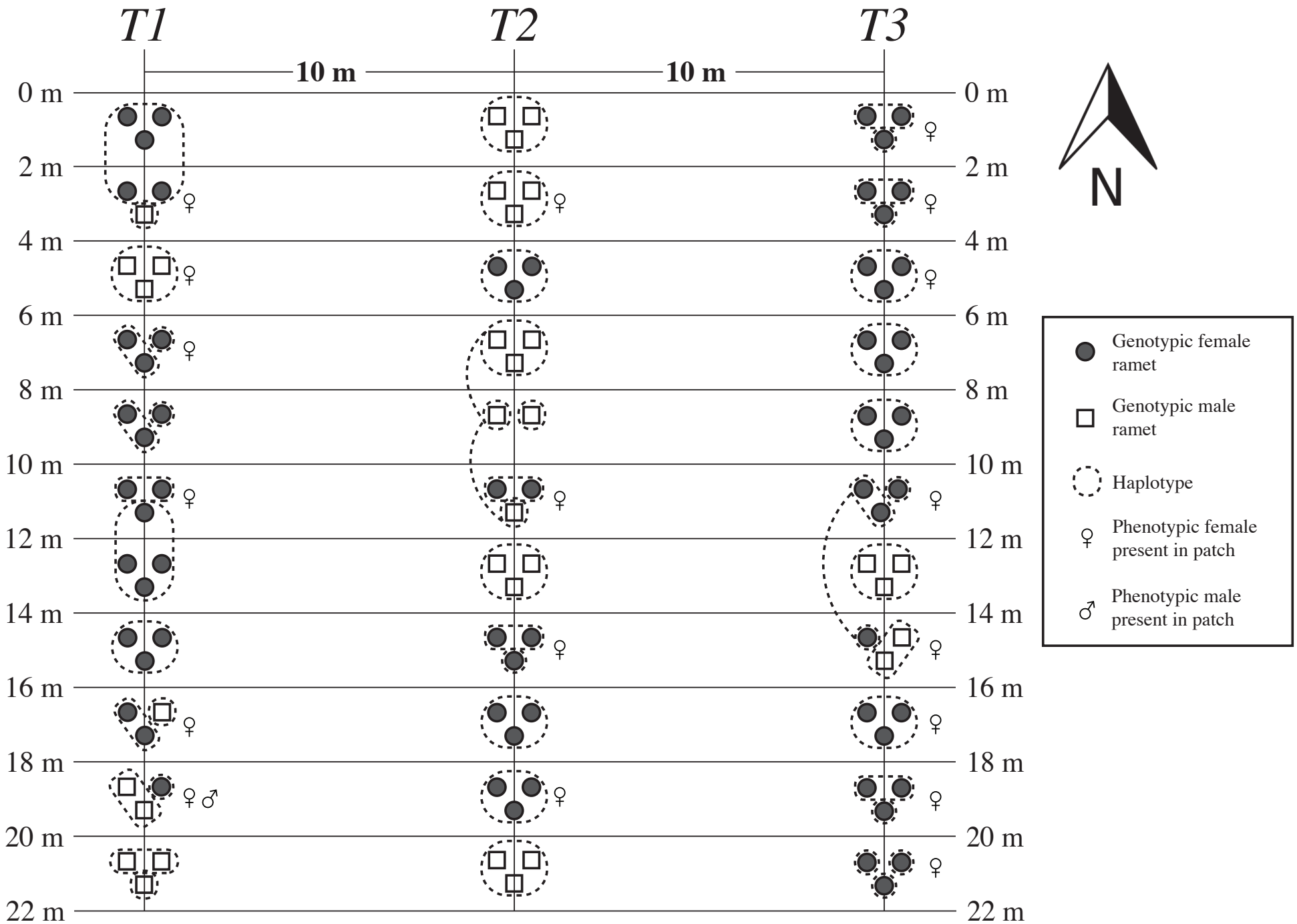
Figure 2. Spatial distribution of genets. Wrightwood contained 53 unique genets in 98 ramets from 33 patches along three transects (T1, T2, and T3). Genotypic females are represented as circles and genotypic males as squares. Genets are outlined in dashed lines. In cases that genets are in different 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.

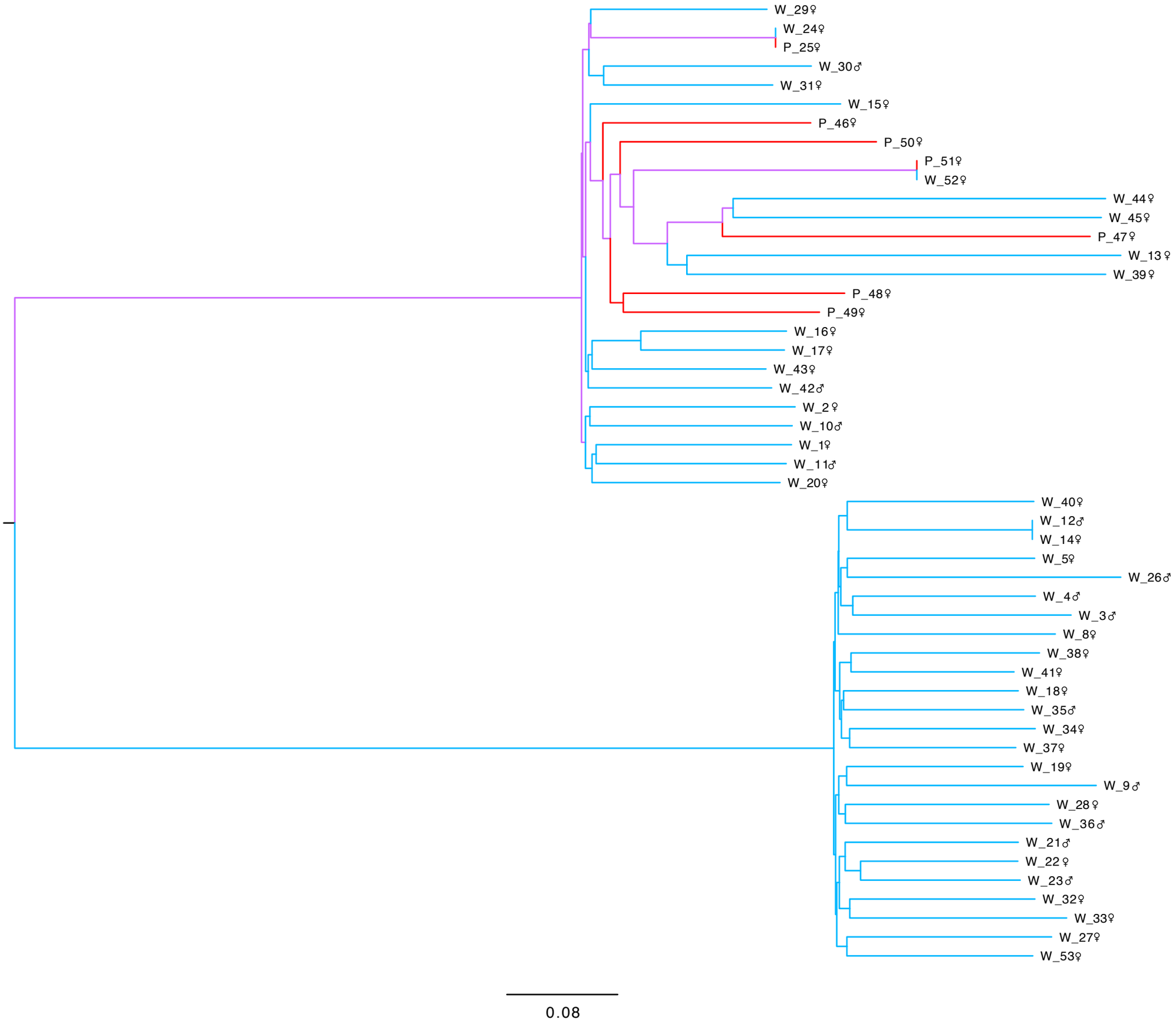
Figure 3. CLEARCUT neighbor joining genetic distance tree of ramets from the Wrightwood and Phelan sites. Branches that join samples from the Wrightwood site ('W') site are blue and those that join the Phelan site ('P') are red. Branches that support mixed-population groups are purple. Symbols indicate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

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.


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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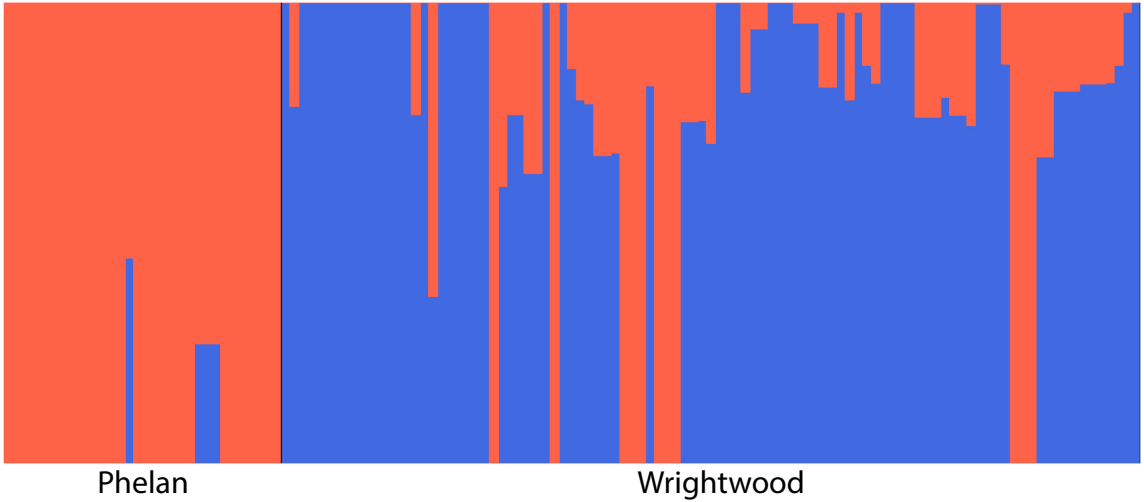
W_53♀
0.08


Phelan
Wrightwood