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1 Sex ratio and gamete size across eastern North America in *Dictyostelium discoideum*, a social
2 amoeba with three sexes

3

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14 **Running Title:** Sex ratio and gamete size in *D. discoideum*

15 Abstract

16 Theory indicates that numbers of mating types should tend towards infinity or remain at
17 two. The social amoeba, *Dictyostelium discoideum*, however, has three mating types. It is
18 therefore a mystery how this species has broken the threshold of two mating types, but has not
19 increased towards a much higher number. Frequency dependent selection on rare types in
20 combination with isogamy, a form of reproduction involving gametes similar in size, could
21 explain the evolution of multiple mating types in this system. Other factors, such as drift, may
22 be preventing the evolution of more than three. We first looked for evidence of isogamy by
23 measuring gamete size associated with each type. We found no evidence of size dissimilarities
24 between gametes. We then looked for evidence of balancing selection, by examining mating
25 type distributions in natural populations and comparing genetic differentiation at the mating type
26 locus to that at more neutral loci. We found that mating type frequency varied among the three
27 populations we examined, with only one of the three showing an even sex ratio, which does not
28 support balancing selection. However, we found more population structure at neutral loci than
29 the mating type locus, suggesting that the three mating types are indeed maintained at
30 intermediate frequencies by balancing selection. Overall, the data are consistent with balancing
31 selection acting on *D. discoideum* mating types, but with a sufficiently weak rare sex advantage
32 to allow for drift, a potential explanation for why these amoebae have only three mating types.

33

34 Key Words: social amoeba, *Dictyostelium discoideum*, sex ratio, mating type, balancing
35 selection

36

37 Introduction

38 Research on the evolution and maintenance of sex and sex ratios in eukaryotes has
39 historically focused heavily on those systems exhibiting two distinct mating types, one male and
40 one female. But more than two mating types occur in some species. Recently, researchers have
41 begun to explore the many natural systems that exhibit more diverse sexual strategies. In nature,
42 the observed numbers of mating types in systems that have evolved past two can range from low
43 numbers like those seen in many ciliates (3-15 mating types; Collins & Gorovsky, 2005; Phadke
44 & Zufall, 2009), and the acellular slime mold *Physarum polycephalum* (≥ 13 mating types;
45 Collins & Tang, 1977) to hundreds or even thousands of mating types like those seen in many
46 fungal species (Kothe, 1996; Billiard et al., 2011; 2012). The fungus *Schizophyllum commune* is
47 the most commonly recognized example of a high number of mating types due to its tetrapolar
48 mating type system, with over 20,000 allele combinations currently estimated (Raper, 1966;
49 Kothe, 1996). Variation in mating systems is also common in plants, where self-incompatibility
50 alleles can range from fewer than 10 to an estimated 200 (Lawrence, 2000; Castric & Vekemans,
51 2004; Busch et al., 2014).

52 With all this diversity, it is important to understand how differing numbers of mating
53 types can evolve and be maintained in natural systems. Theory predicts that the number of
54 mating types should tend towards infinity or remain at two (Iwasa & Sasaki, 1987). In their
55 model suggesting large numbers of mating types, Iwasa and Sasaki propose that a new mating
56 type that arises in the population should be favored by selection because it can mate with a larger
57 proportion of the population. This negative frequency-dependent selection theory assumes both
58 that there is a cost to not finding a mate and that all mating types are inter-compatible. Plant
59 theory for numbers of self-incompatibility alleles also centers on negative frequency-dependent

60 selection for explaining how new alleles arise in populations and why we see so many (Wright,
61 1939). Iwasa and Sasaki (1987) also constructed a model for why only two mating types might
62 remain. In this model, individuals or gametes can wait, without cost, for a suitable mate, and
63 populations tend to lose all but two mating types most likely due to drift. More recent theory
64 focuses on explaining more actively why we often only see two mating types (reviewed in
65 Billiard et al., 2011). The evolution of anisogamy, cytoplasmic conflict leading to uniparental
66 organellar inheritance, and high selfing rates that reduce the cost of finding a mate are just a few
67 of the hypothesized constraints on the evolution of more than two mating types.

68 *Dictyostelium discoideum* shows evidence of intermediate numbers of mating types.
69 These social amoebae and other members of the Dictyosteliidae produce a sexual structure called
70 a macrocyst, the diploid fusion product of two haploid cells of different mating types (Blaskovics
71 & Raper, 1957; Filosa & Dengler, 1972; Erdos et al., 1973a,b; O'Day, 1979; O'Day & Durston,
72 1979; Saga & Yanagisawa, 1983; O'Day & Keszei, 2012; Bloomfield, 2013). Dictyostelia
73 exhibit a variety of mating strategies with evidence of homothallic, or self-compatible species, as
74 well as systems of 2, 3 and 4 mating types (Erdos et al., 1973a, 1975; Clark et al., 1973; Francis,
75 1975; Cavender et al., 1981, 2005; Chang & Raper, 1981; Kawakami & Hagiwara, 1999). The
76 most commonly studied of these, *D. discoideum*, has three self-incompatible mating types
77 determined by a single locus with three alleles, which cannot mate with themselves but can mate
78 with either of the other two types (Erdos et al., 1973a; Clark et al., 1973; Bloomfield et al.,
79 2010). We know that sex is common in nature from evidence of rapid decay in linkage
80 disequilibrium with distance along the chromosome and recombinant genotypes in wild
81 populations (Flowers et al., 2010). However, direct evidence from hatching macrocysts in the
82 lab has been challenging to obtain. Though much of the process has been documented, many

83 aspects of the *D. discoideum* mating system are still yet to be understood. One such missing
84 element is a clearer understanding of how the number and distribution of its mating types fit in
85 with the theory that explains mating type evolution in the rest of the eukaryotes. What keeps *D.*
86 *discoideum* at three?

87 The possible selective pressures maintaining low numbers of mating types in microbial
88 eukaryotes are likely to vary across lineages, as indicated in ciliates (Phadke & Zufall, 2009).
89 However, even in ciliates, the forces driving patterns of mating type numbers and their
90 distributions remain unclear. Overall, this field is vastly understudied across microbial
91 eukaryotes. Since this question has never been addressed in *Dictyostelium*, we investigated how
92 three mating types are maintained in *D. discoideum*, considering two common characteristics of
93 mating systems, anisogamy and negative frequency dependent selection at the mating type locus.
94 First, physical differences between gametes, most notably size differences, have been associated
95 with the evolution and maintenance of two-sex systems (Randerson & Hurst, 2001; Bulmer &
96 Parker, 2002). This type of reproduction, labeled anisogamy, can result from disruptive selection
97 favoring increases in both the size and number of gametes. Once this happens, it removes the
98 frequency dependent advantages of a rare sex, as gametes are no longer universally compatible.
99 Small gametes only mate with large gametes and vice versa. While anisogamy is common in
100 multicellular organisms, the opposite, isogamy, is more often found in unicellular organisms
101 where vegetative structures are less complex and increased gamete size yields less of a
102 reproductive fitness gain (Parker et al., 1972; Knowlton, 1974; Bell, 1978). Size differences
103 between *D. discoideum* gametes could suggest differentiation and/or specialization of mating
104 types that would make intermediate mating types unfavorable and limit the evolution of more
105 mating types.

106 Second, we focused on two manifestations of negative frequency dependent selection at
107 the mating type locus. First, mate availability is extremely important for reproduction and can be
108 a limiting factor. Similar to the theory predicting the evolution of an infinite number of mating
109 types (Iwasa & Sasaki, 1987), equal sex ratios are predicted to be caused and maintained by a
110 frequency-dependent selection favoring the rarer sex (Fisher, 1930; Wright, 1939). Deviations,
111 though rare, can be caused by a variety of factors such as local mate competition, mate
112 attractiveness, maternal condition and environmental dynamics (Hamilton, 1967; Charnov, 1982;
113 West, 2009). Evenness is expected to persist even in systems with multiple mating types (Orias
114 & Rohlf, 1964; Iwasa & Sasaki, 1987). It is not known if all three of the *D. discoideum* mating
115 types persist in all natural populations or if they do, at what frequencies. Skewed mating type
116 distributions could indicate differential pressures on sex allocation suggesting that larger
117 numbers of some mating types may result from other sources of selection or drift.

118 Second, unlike neutral alleles, genes responsible for sex determination or mating
119 compatibility are generally under balancing selection. Evidence for this is fairly ubiquitous in
120 sexual species, most notably in self-incompatibility alleles in plants (Vekemans & Slatkin, 1994)
121 and mating compatibility genes in fungi (May et al., 1999). Balancing selection contributes to
122 both allelic diversification and the maintenance of ancient alleles. Allelic diversification, as
123 proposed by models for the evolution of high numbers of sex determination alleles in which rare
124 types are favored in the population, has been discussed previously (Wright, 1939; Iwasa &
125 Sasaki, 1987). But, balancing selection also tends to maintain alleles for mating compatibility in
126 a population over long periods of evolutionary time (reviewed in Delph & Kelly, 2014). In *D.*
127 *discoideum*, we know from the very divergent sequences of the alleles at the mating type locus,
128 that the mating types have been diverging in the species for a very long time (Bloomfield et al.

129 2010). This suggests that balancing selection is acting on the mating types. It is unknown if the
130 distributions of mating type alleles found in each population also show evidence of balancing
131 selection.

132 Here, we investigated two questions: Do *D. discoideum* gametes of each mating type
133 differ in size? What are the relative roles of balancing selection and drift on maintaining mating
134 type frequencies in natural populations? To answer these questions, we identified the mating
135 types of 170 individual clones from three well-sampled natural populations and measured the
136 gamete sizes from a representative subset of two of these populations. We show evidence of
137 isogamy, not anisogamy, and evidence that while balancing selection appears to be maintaining
138 the frequencies of the three mating types when compared to more neutral markers, sex allocation
139 varies across populations.

140

141 Materials and Methods

142 *Study Populations*

143 To look at mating type distributions, we identified the mating types of *Dictyostelium*
144 *discoideum* clones from frozen stocks originally isolated from soil samples. We analyzed 170
145 clones, collected from four geographic locations: 87 near the Mountain Lake Biological Station
146 in Virginia (Fortunato et al., 2003), 47 from the Houston Arboretum in Houston, Texas and 36
147 from two locations in North Carolina (Table S1). We analyzed a subset of the 170 clones,
148 focusing only on the Virginia and Texas populations, to measure gamete size. Before all
149 analyses, we grew the clones from clonal frozen stocks on nutrient agar plates with the bacterial
150 food source *Klebsiella pneumoniae*.

151 In choosing our clones, we accounted for the possibility of oversampling issues affecting
152 our results. Many more isolates were collected from the populations we focused on here than
153 were used in this study. We used information on soil sample, mating type and microsatellite
154 allele markers to make sure our list of clones was comprised of independent samples. Isolates
155 from different soil samples were assumed to be independent samples but duplicate isolates from
156 a single soil sample were excluded whenever they showed the same mating type and the same
157 genotypes at five microsatellite loci.

158 *Gamete Size Measurement*

159 To measure gamete size, we sampled multiple clones from each of the three self-
160 incompatible mating types from two populations. Because two haploid cells fuse to form the
161 reproductive zygote during the sexual cycle of *D. discoideum*, we measured the size of cells
162 prepared in the absence of a compatible mating partner but in conditions conducive for sexual
163 fusion, to get at their size right before fusion. These fusion-competent cells are considered at this
164 point to be gametes (Saga et al., 1983; O'Day et al., 1987; Urushihara & Muramoto, 2006).
165 Specifically, we plated 2×10^5 spores on LP agar plates (0.1% lactose, 0.1% peptone, 1.5% agar)
166 in an excess of Bonner's salt solution (SS: 0.06% NaCl, 0.03% CaCl₂, 0.075% KCl) with *K.*
167 *pneumoniae* and incubated the plates in the dark for 3 days at 22° C. We then collected the
168 resulting dark-grown cells and measured the cell diameters using a Nexcelom Cellometer Auto
169 1000 (Lawrence, MA). We used the default settings with the exception of a cell size minimum
170 set to 5 um and a maximum set to 15 um. In each population, we measured 160 cell diameters
171 from each of four to six clones per mating type.

172 For comparison, we also measured the size of cells grown in conditions conducive for
173 fruiting body formation in order to get at vegetative cell sizes when clones are not preparing for

174 sexual fusion. We plated 2×10^5 spores on SM/5 agar plates with *K. pneumoniae* and allowed the
175 plates to grow on a bench for ~36 hours. We collected pre-aggregate vegetative cells in buffer
176 and used the same methods as previous for measuring cell diameters.

177 *Mating Type Identification and Microsatellite Analysis*

178 We developed mating type specific primers (see Table S2) based on the published mating
179 type gene sequences identified by Bloomfield et al. (2010). Each mating type expresses a unique
180 set of genes (Type I: matA; Type II: matC, matB, matD; Type III: matS, matT), allowing for the
181 development of a gene presence/absence assay for mating type identification. We repeated
182 techniques described in Douglas et al. (2011) for DNA extraction, amplification and sequencing.
183 We extracted DNA from spores using a Chelex/Proteinase K protocol and amplified, by
184 polymerase chain reaction (PCR), regions of the mating type genes using the primers we
185 developed. We ran the PCR product on a 1% agarose gel to identify presence/absence of bands
186 as an indication of mating type. To verify the use of this method to identify mating types, we
187 also checked the accuracy of approximately 15% of our results using either Sanger sequencing
188 and/or mating compatibility tests. We used methods similar to those available on dictyBase for
189 the compatibility tests (http://dictybase.org/techniques/media/mating_types.html, Basu et al.,
190 2013). We plated spores from two *D. discoideum* clones together in an excess of SS buffer on
191 LP agar plates with *K. pneumoniae* and incubated the plates in the dark for at least one week.
192 Presence of macrocysts at this point indicated mating compatibility. Based on these assessments,
193 we found our methods to be an excellent technique for identifying the presence of mating type
194 genes.

195 To look for balancing selection on the three mating types, we compared F_{ST} at the mating
196 type locus to that at more neutral microsatellite loci. Lower F_{ST} at the mating type locus would

197 mean that its alleles were maintained at more even frequencies across populations than the
198 neutral loci, and thus represent evidence for balancing selection on that locus. We acquired data
199 for microsatellite allele sizes at 5 select loci for 168 *D. discoideum* clones from populations in
200 Virginia (104 clones), Texas (40 clones) and North Carolina (24 clones) from Smith (2004;
201 Table S1). Of those 168 clones, 139 overlapped with the clones we looked at in this study.

202 *Statistical Analyses*

203 Gamete Size: Unless otherwise indicated, all statistical analyses were performed using R
204 software (version 3.2.3.) (R Core Team, 2015). We implemented a Welch's two sample t-test to
205 compare the diameters of gametes to vegetative cells. To analyze the relationships between cell
206 diameter measurements and both geographic origin and mating type, we fitted separate linear
207 mixed-effects models to the gametic and vegetative datasets using the "lme" function from the R
208 package "nlme" (Pinheiro et al., 2014). We treated geographic origin and mating type as fixed
209 effects and clone identity as the random effect. Based on AIC and BIC scores, this model fit the
210 data better than a model including the interaction effects of geographic origin and mating type.
211 We used Type III tests to estimate the significance of the fixed effects. Though our data
212 appeared to have a normal distribution based on the kurtosis and skewness, they failed the
213 Shapiro-Wilk test of normality. Because of this, and because our errors were also not normally
214 distributed, we implemented techniques based on Anderson & ter Braak (2003) where we
215 applied permutation tests to the residuals under a reduced model. We used R code written for
216 Noh & Henry (2015) that permuted residuals from fitting a model of only the effect not being
217 tested. For example, the permutation test for mating type resampled residuals of a model that
218 included only population origin as the fixed effect. The permuted p-values we report reflect the

219 proportion of times the F-value of the resampled data were larger or equal to the F-value of the
220 real data.

221 Mating Type Frequency: To analyze the evenness of the frequencies of mating types
222 within populations, we performed chi-squared goodness-of-fit tests using R software. We
223 corrected for multiple comparisons by implementing the Benjamini-Hochberg procedure for
224 controlling false discovery rates (Benjamini & Hochberg, 1995). The reported significant results
225 remained significant after this correction. We examined the standardized residuals from
226 statistically significant tests to identify the mating types that were more or less prevalent than
227 expected.

228 Population Differentiation: We compared the differences between populations both in
229 mating type frequencies and microsatellite allele frequencies by calculating estimates of F_{ST}
230 using FSTAT version 2.9.3 (Goudet, 2001) and Hedrick's G'_{ST} (Hedrick, 2005) using the R
231 package "diveRsity" (Keenan, et al., 2013). The latter is a standardized measure of genetic
232 differentiation that can account for the high mutation rates and diversity of microsatellites,
233 addressing the underestimation of genetic structure observed using only F_{ST} (Meirmans &
234 Hedrick, 2011). Estimates of F_{ST} range from 0.0 to 1.0, but when there are large numbers of
235 alleles at a locus, a value of 1.0 can never be reached even with complete differentiation. This is
236 due to within-population diversity. Hedrick's G'_{ST} corrects for this by dividing the
237 differentiation estimate by the maximum value it could take given the numbers of populations
238 and alleles.

239

240 Results

241 *Gamete sizes do not differ by mating type, but Texas gametes are smaller*

242 We measured a total of 4640 gamete cells, representing 14 clones from Virginia (5 Type
243 I, 4 Type II, 5 Type III) and 15 clones from Texas (6 Type I, 4 Type II, 5 Type III). We also
244 measured 4800 vegetative cells, representing 15 clones from Virginia (5 Type I, 5 Type II, 5
245 Type III) and 15 clones from Texas (6 Type I, 4 Type II, 5 Type III). We did not detect evidence
246 of cell size differences between mating types in either cell type (gamete: $F_{2,25} = 0.38$, $P_{perm} =$
247 0.68 ; vegetative: $F_{2,26} = 0.43$, $P_{perm} = 0.64$; Fig. 1A-1B). Overall, we found that gametes were
248 significantly larger than vegetative cells (mean 9.99 and 9.32 microns, respectively; $t_{45} = 5.33$, p
249 < 0.0001 ; Fig. 1C). Gametes from Virginia, averaged 10.23 microns and were significantly
250 larger than gametes from Texas at an average of 9.77 microns ($F_{1,25} = 4.78$, $P_{perm} = 0.01$; Fig.
251 1D). We did not see this geographic difference between vegetative cells (Virginia = mean 9.37
252 microns, Texas = mean 9.24 microns; $F_{1,26} = 0.43$, $P_{perm} = 0.64$).

253 *Frequencies of mating types are unequal and vary between locations*

254 We identified the mating types of individual clones collected at well-sampled populations
255 from four distinct geographic regions. In total, we identified 77 Type I, 39 Type II and 55 Type
256 III individuals (Fig. 2, Table S1). Overall, the distribution of mating types differed from the
257 balancing selection expectation of equal frequencies ($\chi^2 = 12.8$, $df = 2$, $p = 0.01$). Examining the
258 standardized residuals from the chi-square test revealed that this departure is due to the
259 identification of significantly more than expected Type I individuals and significantly fewer than
260 expected Type II individuals (Table S3). Within individual populations, we found a range of
261 distributions. In the population near Mountain Lake Biological Station, Virginia, we found an
262 even distribution of mating types (34 Type I, 25 Type II, 28 Type III; $\chi^2 = 1.45$, $df = 2$, $p = 0.48$).
263 The population in Houston, Texas significantly differed from an even distribution, with
264 significantly fewer observed Type II individuals (22 Type I, 8 Type II, 18 Type III; $\chi^2 = 6.5$, $df =$

265 2, $p = 0.04$). Due to low sample numbers, we combined two populations in North Carolina. We
266 identified 10 Type I, 3 Type II, and 2 Type III individuals in Linville Falls, NC and 11 Type I, 3
267 Type II, and 7 Type III individuals in Little Butts Gap, NC. Overall, we again found an uneven
268 distribution of mating types when we combined these two populations, with significantly more
269 than expected Type I individuals but significantly fewer than expected Type II individuals ($\chi^2 =$
270 10.5, $df = 2$, $p = 0.005$).

271 *Balancing selection maintains mating type distributions across populations*

272 When we compared the three geographic populations to each other, we found no
273 significant genetic differentiation in mating type frequency by geographic location ($F_{ST} = 0.01$,
274 $G'_{ST} = 0.05$; Table 1). We found substantially higher levels of genetic differentiation at the
275 microsatellite loci (Mean: $F_{ST} = 0.10$, $G'_{ST} = 0.55$, Range: $F_{ST} = 0.10-0.13$, $G'_{ST} = 0.32-0.77$).
276 Both the F_{ST} and G'_{ST} estimates for the mating type locus fell well below all the respective 95%
277 confidence intervals for the microsatellite loci, suggesting strong evidence for balancing
278 selection.

279

280 Discussion

281 Here we give the first empirical evidence for isogamy in *D. discoideum*. Individuals of
282 each of the three mating types expressed in *D. discoideum* produce gametes that are
283 indistinguishable in size. Because *D. discoideum* has evolved multiple mating types and lives
284 primarily in a unicellular form, we were not surprised to find a lack of evidence for mating type-
285 specific gamete size differences. Unicellular species are commonly isogamous, with gametes
286 that are usually undifferentiated in form and sex-determination mechanisms that are regulated
287 only at the molecular level by a mating type locus (Billiard et al., 2011; Bachtrog et al., 2014).

288 This observation may be due to the relatively short incubation time in unicellular organisms
289 between fertilization and maturation of a zygote compared to the ultimately much larger
290 multicellular organisms, such that there is less of a fitness advantage for increased zygote size
291 and therefore no disruptive selection on gamete size (Knowlton, 1974). In anisogamous
292 organisms, where there is a pull between increasing the number of gametes and increasing the
293 size of the gametes in order to produce more and larger zygotes, two mating types result, one
294 small but abundant, one large but limited. In this case, any intermediate type is likely to be
295 disfavored. Since gametes in *D. discoideum* are identical in size, there would be no intermediate
296 type and new types could have the selective advantage described by Iwasa and Sasaki (1987).
297 This is consistent with the fact that we see more than two mating types in *D. discoideum*.

298 We also found evidence for balancing selection acting on the frequencies of the mating
299 types when we compared population genetic differentiation at the mating type locus to that at
300 presumably neutral microsatellite loci. Mating types and other self-incompatibility or self-
301 recognition genes tend to evolve under balancing selection (reviewed in Fijarczyk & Babik,
302 2015). In *D. discoideum*, we observed no evidence of population structure at the mating type
303 locus ($F_{ST} = 0.01$) but evidence of moderate genetic differentiation at the neutral microsatellite
304 loci ($F_{ST} = 0.10$), with the estimate at the mating type locus falling well below the 95%
305 confidence interval for the microsatellite loci. Though this in itself is strong evidence for
306 balancing selection at the mating type locus, we expected the F_{ST} values for the microsatellite
307 loci could be underestimated due to the tendency of microsatellites to have high mutation rates
308 and diversity (Balloux et al., 2000). Because of this, we used an alternative method to further
309 estimate genetic differentiation at these markers that addresses this problem. We calculated
310 estimates for Hedrick's G'_{ST} , a measure specifically designed to correct the underestimation of

311 microsatellite data, for both the microsatellite loci and the mating type locus. The new estimate
312 still showed about a ten-fold increase in population differentiation at the microsatellite loci
313 compared to the mating type locus (Microsatellite: $G'_{ST} = 0.55$; Mating: $G'_{ST} = 0.05$), further
314 strong evidence that mating types are maintained by balancing selection.

315 But, according to theory, isogamy and balancing selection allow for the evolution of an
316 infinite number of mating types, not just for the transition from 2 to 3 that we see in *D.*
317 *discoideum*. Though balancing selection may maintain the overall diversity of mating types
318 across populations, we also see evidence of drift acting on individual populations, suggesting
319 that the advantage of rare mating types may be weak. Microbial eukaryotes with multiple mating
320 types are expected to reach a stable equilibrium where all mating types are equal in a population.
321 The few known examples come from ciliates, where equal frequencies of multiple mating types
322 have been observed empirically and predicted theoretically (Orias & Rolf, 1964; Doerder et al.,
323 1995). These equal frequencies are also common for self-incompatibility alleles in plants
324 (reviewed in Castric & Vekemans, 2004). However, in *D. discoideum*, the overall frequencies of
325 the three mating types were not equal, with fewer observed Type II individuals. Between
326 locations, the frequencies of the three mating types also differed, with only one of the three
327 populations, Virginia, showing equal frequencies of the three sexes. Differences in mating type
328 frequencies between populations most likely reflect drift in the face of weak selection. Though
329 less common, this pattern of drift is not unusual to mating type systems, having also been
330 observed at self-incompatibility loci in plants (Campbell & Lawrence, 1981; Kato & Mukai,
331 2004). Thus the data are consistent with balancing selection but with a common sex
332 disadvantage that is so weak that it is unable to maintain allele frequencies that are even or

333 uniform across populations. Such a weak rare sex advantage might also explain why the number
334 of sexes has remained low.

335 *Conclusions and Implications for Future Research*

336 Since relatively little is known about macrocysts in *D. discoideum* compared to the more
337 commonly studied fruiting body, the intent of this study was to further characterize aspects of the
338 sexual cycle that could shed light on how low numbers of mating types are maintained. In doing
339 so, we found evidence of isogamy and balancing selection, both conducive for the evolution of
340 multiple mating types. However, we also found evidence for drift acting on the mating types that
341 could explain why we only see three mating types. Returning to the original models proposed by
342 Iwasa and Sasaki (1987), in which a common sex disadvantage promotes the evolution of many
343 mating types but drift can reduce that number to just two, we suspect that the missing piece to
344 this puzzle may be a more thorough understanding of the cost of mating (or not) in *D.*
345 *discoideum*. These models predict a very large number of mating types to evolve if common
346 mating types suffer a fitness cost for not having as many potential mating partners, but only two
347 if they do not. We know that mating in *D. discoideum* is a potentially costly event in itself.
348 Though not addressed here, macrocyst formation is a uniquely social process that differs from
349 the sexual cycles in other organisms. Upon formation, hundreds of amoebae are attracted to and
350 then cannibalized by the diploid zygote, a potentially altruistic act. Understanding the social
351 contract involved in sex and macrocyst formation in *D. discoideum* and the costs of not
352 participating could further our understanding of how the mating system is maintained.

353

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360

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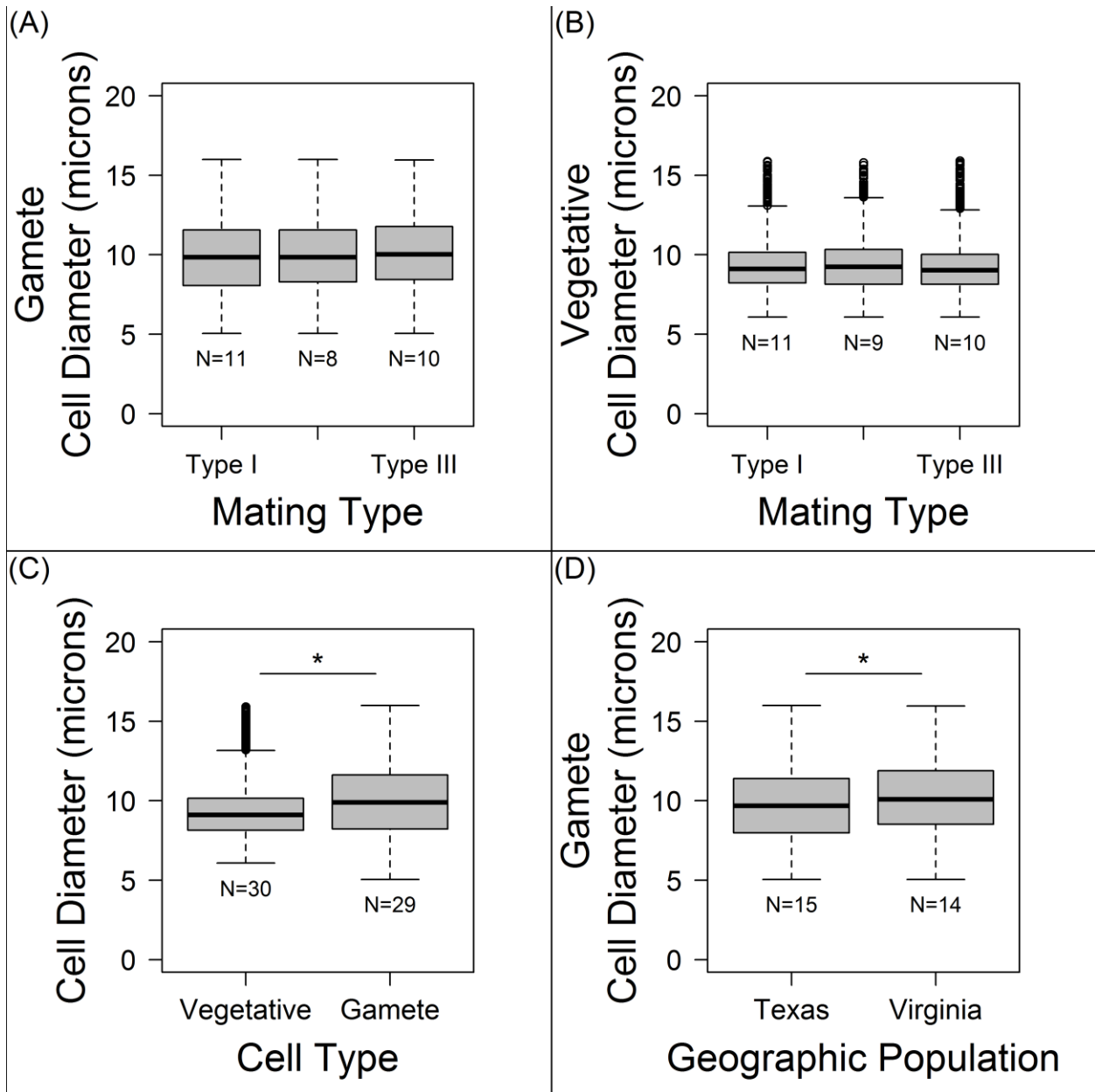
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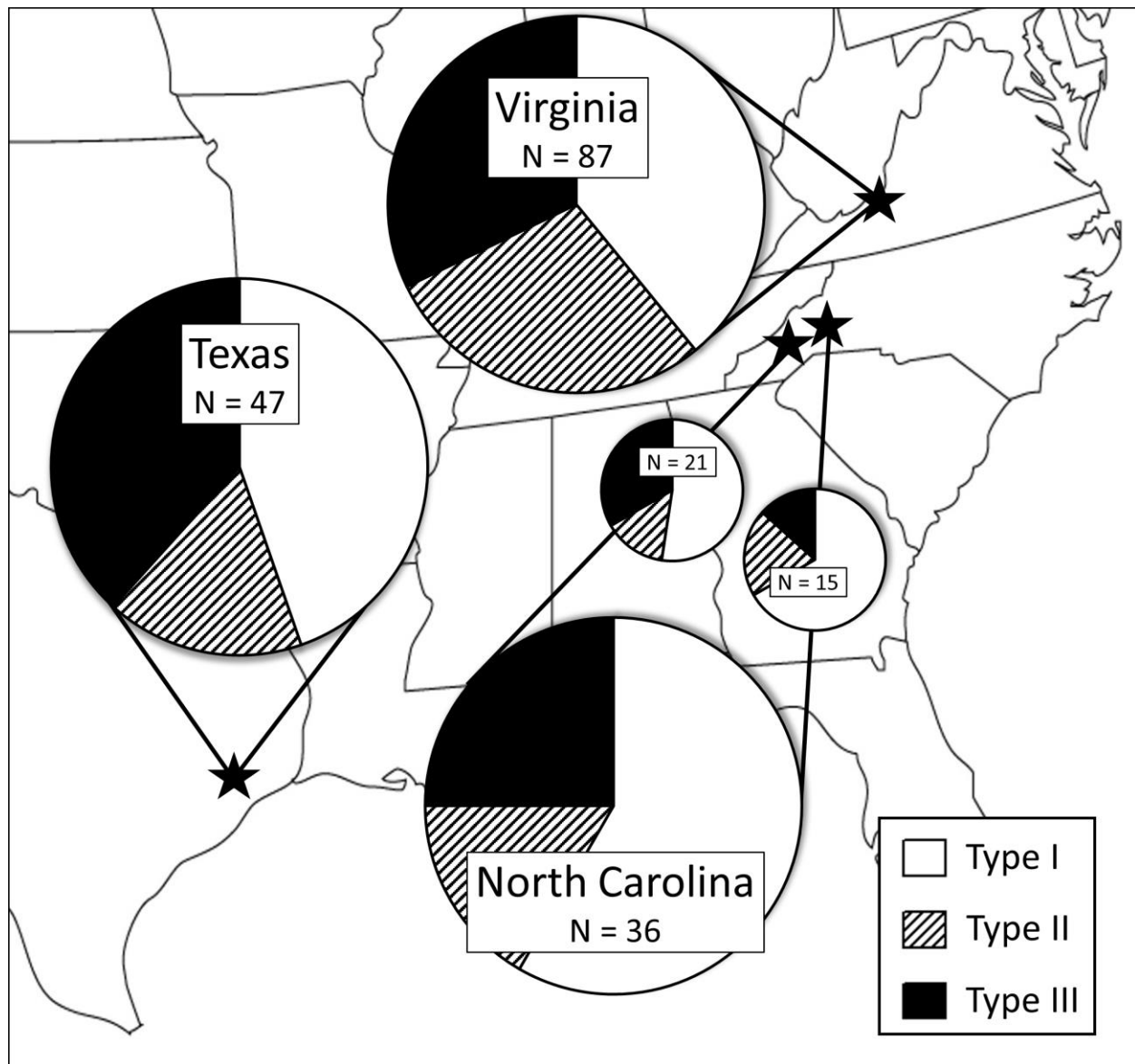
497 Table 1. F_{ST} and G'_{ST} values show differentiation in mating type frequencies and microsatellite
 498 allele frequencies between populations of *Dictyostelium discoideum*. We included the 95%
 499 confidence intervals for each of the overall microsatellite loci differentiation estimates.

| Locus | F_{ST} | G'_{ST} | # of alleles |
|----------------------------|----------------------------|-----------------------------|---------------------|
| Microsatellite Loci | | | |
| Dict5 | 0.097 | 0.592 | 15 |
| Dict13 | 0.128 | 0.770 | 17 |
| Dict19 | 0.104 | 0.315 | 7 |
| Dict23 | 0.086 | 0.672 | 22 |
| Dict25 | 0.097 | 0.668 | 21 |
| Average | 0.103 | 0.548 | 16.4 |
| 95% CI | 0.091-0.116 | 0.475-0.609 | |
| Mating Type Locus | | | |
| Mat | 0.009 | 0.051 | 3 |

501 Figure 1. Gametes are larger in Virginia, but are the same across mating types. Plots show cell
502 diameter for A) gametes of each mating type, B) vegetative cells of each mating type, C)
503 vegetative cells compared to gametes, and D) gamete cells divided by geographic population.
504 Asterisk represents statistical significance. N represents number of clones from which 160 cell
505 diameters were measured.



507 Figure 2. Mating type proportions vary by population. The pie charts show the distributions of
508 mating types within each of the four geographic populations, with the large pie for North
509 Carolina representing the combined totals from the two populations represented individually by
510 the smaller pies. Stars indicate approximate locations of sampling sites.



511

512

Table S1. *Dictyostelium discoideum* clones from the four populations used in this study (LF = Linville Falls [35°57.197' N, 81°56.516' W], LBG = Little Butts Gap [35°46' N, 82°20' W], H = Houston [29°46' N, 95°27' W], MLBS = Mountain Lake Biological Station [37°21' N, 80°31' W]) and their associated mating type genes and/or microsatellite allele sizes. X's denote confirmed presence of mating type genes. To confirm types, we required evidence of at least one mating type gene associated with that type (Type 1: matA; Type 2: matB, matC, matD; Type 3: matS, matT). Microsatellite allele sizes are from Smith (2004).

| Clone Name | Population | Type | Confirmed Mating Type Genes | | | | | | Microsatellite Allele Size (bp) | | | | | |
|------------|-------------------|------|-----------------------------|------|------|------|------|------|---------------------------------|-----------|-----------|-----------|-----------|-----|
| | | | matA | matB | matC | matD | matS | matT | Dict5AAC | Dict13CAT | Dict19AAC | Dict23AAC | Dict25AAC | |
| NC21B1 | N. Carolina (LF) | 1 | X | | | | | | | 234 | 187 | 158 | 182 | 226 |
| NC21C1C | N. Carolina (LF) | 2 | | X | | X | | | | - | - | - | - | - |
| NC21D1 | N. Carolina (LF) | 1 | X | | | | | | | 240 | 187 | 161 | 206 | 253 |
| NC21H1A | N. Carolina (LF) | 3 | | | | | X | X | | 240 | 160 | 176 | 185 | 205 |
| NC22J1 | N. Carolina (LF) | 1 | X | | | | | | | - | - | - | - | - |
| NC26D1 | N. Carolina (LF) | 1 | X | | | | | | | 234 | 187 | 158 | 182 | 226 |
| NC26L1 | N. Carolina (LF) | 1 | X | | | | | | | 210 | 199 | 161 | 161 | 262 |
| NC28A1 | N. Carolina (LF) | 3 | | | | | | X | | - | - | - | - | - |
| NC28B1 | N. Carolina (LF) | 1 | X | | | | | | | 234 | 187 | 158 | 182 | 226 |
| NC28C1 | N. Carolina (LF) | 1 | X | | | | | | | 240 | 187 | 158 | 188 | 262 |
| NC28D1 | N. Carolina (LF) | 2 | | X | | X | | | | 237 | 187 | 173 | 188 | 220 |
| NC29B1 | N. Carolina (LF) | 1 | X | | | | | | | 294 | 250 | 161 | 188 | 247 |
| NC29E1 | N. Carolina (LF) | 1 | X | | | | | | | 252 | 265 | 161 | 188 | 247 |
| NC29R1 | N. Carolina (LF) | 1 | X | | | | | | | 294 | 250 | 161 | 212 | 172 |
| NC32B1 | N. Carolina (LF) | 2 | | X | X | X | | | | 210 | 238 | 170 | 200 | 259 |
| NC105.1 | N. Carolina (LBG) | 3 | | | | | X | X | | - | - | - | - | - |
| NC28.1 | N. Carolina (LBG) | 1 | X | | | | | | | - | - | - | - | - |
| NC34 | N. Carolina (LBG) | 2 | | X | X | X | | | | - | - | - | - | - |
| NC34.1 | N. Carolina (LBG) | 3 | | | | | X | X | | - | - | - | - | - |
| NC39.1 | N. Carolina (LBG) | 1 | X | | | | | | | - | - | - | - | - |
| NC41.2 | N. Carolina (LBG) | 1 | X | | | | | | | - | - | - | - | - |
| NC43.1 | N. Carolina (LBG) | 3 | | | | | X | X | | - | - | - | - | - |
| NC47.2 | N. Carolina (LBG) | - | | | | | | | | 237 | 187 | 158 | 197 | 223 |
| NC4B | N. Carolina (LBG) | 3 | | | | | X | X | | - | - | - | - | - |
| NC4C | N. Carolina (LBG) | 1 | | X | | | | | | - | - | - | - | - |
| NC52.3 | N. Carolina (LBG) | 1 | X | | | | | | | - | - | - | - | - |
| NC58.1 | N. Carolina (LBG) | - | | | | | | | | 210 | 160 | 173 | 182 | 244 |
| NC59.2 | N. Carolina (LBG) | - | | | | | | | | 237 | 160 | 173 | 161 | 256 |
| NC60.1 | N. Carolina (LBG) | - | | | | | | | | 237 | 160 | 173 | 182 | 244 |
| NC60.2 | N. Carolina (LBG) | - | | | | | | | | 210 | 184 | 173 | 182 | 205 |
| NC61.1 | N. Carolina (LBG) | - | | | | | | | | 240 | 160 | 161 | 239 | 220 |

| | | | | | | | | | | | | | |
|---------|-------------------|---|---|---|---|---|---|---|-----|-----|-----|-----|-----|
| NC63.2 | N. Carolina (LBG) | 3 | | | | | X | X | 240 | 160 | 176 | 185 | 205 |
| NC66.2 | N. Carolina (LBG) | - | | | | | | | 234 | 160 | 173 | 182 | 253 |
| NC67.2 | N. Carolina (LBG) | - | | | | | | | 237 | 187 | 176 | 230 | 205 |
| NC69.1 | N. Carolina (LBG) | - | | | | | | | 213 | 238 | 161 | 173 | 271 |
| NC70.1 | N. Carolina (LBG) | 2 | | X | X | X | | | - | - | - | - | - |
| NC74.1 | N. Carolina (LBG) | - | | | | | | | 231 | 187 | 173 | 194 | 223 |
| NC75.2 | N. Carolina (LBG) | 1 | X | | | | | | 240 | 160 | 161 | 239 | 220 |
| NC76.1A | N. Carolina (LBG) | 1 | X | | | | | | - | - | - | - | - |
| NC76.1B | N. Carolina (LBG) | 3 | | | | | X | | - | - | - | - | - |
| NC78.2 | N. Carolina (LBG) | 1 | X | | | | | | - | - | - | - | - |
| NC80.1 | N. Carolina (LBG) | 1 | X | | | | | | - | - | - | - | - |
| NC85.1 | N. Carolina (LBG) | 2 | | X | X | X | | | - | - | - | - | - |
| NC85.2 | N. Carolina (LBG) | 3 | | | | | X | X | - | - | - | - | - |
| NC98.1 | N. Carolina (LBG) | 1 | X | | | | | | - | - | - | - | - |
| NC99.1 | N. Carolina (LBG) | 1 | X | | | | | | - | - | - | - | - |
| H10C | Texas (H) | 1 | X | | | | | | - | - | - | - | - |
| H15B | Texas (H) | 3 | | | | | X | X | - | - | - | - | - |
| H3 | Texas (H) | 3 | | | | | X | X | - | - | - | - | - |
| H3B | Texas (H) | 1 | X | | | | | | - | - | - | - | - |
| HD12C | Texas (H) | 1 | X | | | | | | - | - | - | - | - |
| HD13A1 | Texas (H) | 2 | | X | X | X | | | 255 | 211 | 161 | 158 | 256 |
| HD1D1 | Texas (H) | 1 | X | | | | | | 255 | 211 | 161 | 158 | 256 |
| HD20B2b | Texas (H) | 3 | | | | | X | X | - | - | - | - | - |
| HD24A | Texas (H) | 3 | | | | | X | X | - | - | - | - | - |
| HD24B1 | Texas (H) | 2 | | X | X | X | | | 228 | 205 | 176 | 227 | 184 |
| HD24C1 | Texas (H) | 2 | | X | X | X | | | 234 | 208 | 182 | 167 | 172 |
| HD24D1 | Texas (H) | 1 | X | | | | | | 225 | 205 | 161 | 158 | 256 |
| HD25A1 | Texas (H) | 2 | | X | X | X | | | 228 | 205 | 176 | 227 | 184 |
| HD2D1 | Texas (H) | 1 | X | | | | | | 255 | 211 | 161 | 158 | 256 |
| HD30A1 | Texas (H) | 3 | | | | | X | X | 282 | 181 | 161 | 230 | 250 |
| HD31B1 | Texas (H) | 1 | X | | | | | | 225 | 205 | 161 | 158 | 256 |
| HD31C1 | Texas (H) | 1 | X | | | | | | 255 | 211 | 161 | 158 | 256 |
| HD32C1 | Texas (H) | 2 | | X | X | X | | | 234 | 208 | 182 | 167 | 172 |
| HD35D1 | Texas (H) | 1 | X | | | | | | 255 | 211 | 161 | 158 | 256 |
| HD37D1 | Texas (H) | 1 | X | | | | | | 255 | 211 | 161 | 158 | 256 |
| HD38A1 | Texas (H) | - | | | | | | | 255 | 208 | 161 | 158 | 256 |
| HD38B1 | Texas (H) | 1 | X | | | | | | 282 | 181 | 161 | 230 | 250 |
| HD38C1 | Texas (H) | 2 | | X | X | X | | | 234 | 166 | 161 | 161 | 253 |

| | | | | | | | | | | | | | |
|---------|-----------------|---|---|---|---|----|----|----|-----|-----|-----|-----|-----|
| HD40D1 | Texas (H) | 1 | X | | | | | | 225 | 205 | 161 | 158 | 256 |
| HD41B1 | Texas (H) | 3 | | | | | X | X | 225 | 205 | 161 | 158 | 250 |
| HD41C1 | Texas (H) | 3 | | | | | X | X | 282 | 181 | 161 | 230 | 250 |
| HD42A1 | Texas (H) | 3 | | | | | X | X | 282 | 181 | 161 | 230 | 250 |
| HD43C1 | Texas (H) | 3 | | | | | X | X | 282 | 181 | 161 | 230 | 250 |
| HD44A1 | Texas (H) | 1 | X | | | | | | 282 | 181 | 161 | 230 | 250 |
| HD44B1 | Texas (H) | 3 | | | | | X | X | 282 | 181 | 161 | 230 | 250 |
| HD45A1 | Texas (H) | 1 | X | | | | | | 234 | 166 | 161 | 140 | 250 |
| HD45B1 | Texas (H) | 1 | X | | | | | | 225 | 205 | 161 | 158 | 256 |
| HD45C1 | Texas (H) | 2 | | X | | | | | 228 | 205 | 176 | - | 184 |
| HD45D1 | Texas (H) | 3 | | | | | X | X | 234 | 187 | 161 | 197 | 220 |
| HD47B | Texas (H) | 1 | X | | | | | | - | - | - | - | - |
| HD48B1 | Texas (H) | 3 | | | | | X | X | 231 | 187 | 173 | 188 | 220 |
| HD48C1 | Texas (H) | 3 | | | | | X | X | - | 181 | 161 | 230 | 250 |
| HD48D1 | Texas (H) | 1 | X | | | | | | 225 | 205 | 161 | 158 | 256 |
| HD49A1 | Texas (H) | 3 | | | | | X | X | 282 | 181 | 161 | 230 | 250 |
| HD49B1 | Texas (H) | 3 | | | | | X | | 234 | 187 | 161 | 197 | 220 |
| HD49C1 | Texas (H) | 1 | X | | | | | | 255 | 211 | 161 | 158 | 256 |
| HD4A1 | Texas (H) | 1 | X | | | | | | 234 | 205 | 161 | 146 | 250 |
| HD4B1 | Texas (H) | 3 | | | | | X | X | 234 | 205 | 161 | 146 | 250 |
| HD50A1 | Texas (H) | 1 | X | | | | | | 225 | 205 | 161 | 158 | 256 |
| HD50C1 | Texas (H) | 3 | | | | | X | X | 234 | 166 | 158 | 185 | 175 |
| HD54C1 | Texas (H) | 1 | X | | | | | | - | - | - | - | - |
| HD5A1 | Texas (H) | 2 | | X | X | X | | | 234 | 205 | 161 | 146 | 250 |
| HD5B1 | Texas (H) | 1 | X | | | | | | 234 | 205 | 161 | 146 | 250 |
| HD5C1 | Texas (H) | 3 | | | | | X | X | 234 | 205 | 161 | 146 | 250 |
| V301B1 | Virginia (MLBS) | 2 | | X | X | X | | | 234 | 163 | 161 | 161 | 253 |
| V301B2 | Virginia (MLBS) | 2 | | X | X | X | | | 234 | 163 | 161 | 152 | 253 |
| V303A1 | Virginia (MLBS) | 3 | | | | | X* | X* | 234 | 205 | 176 | 179 | 172 |
| V303A2a | Virginia (MLBS) | 2 | | X | X | X | | | 228 | 205 | 176 | 227 | 184 |
| V303A2b | Virginia (MLBS) | - | | | | | | | 228 | 166 | 158 | 227 | 184 |
| V303C1a | Virginia (MLBS) | 3 | | | | | X | X | 234 | 205 | 176 | 185 | 172 |
| V303C1b | Virginia (MLBS) | - | | | | | | | 234 | 166 | 158 | 185 | 172 |
| V303D1 | Virginia (MLBS) | 1 | X | | | | | | 234 | 205 | 176 | 185 | 172 |
| V304A1 | Virginia (MLBS) | 1 | | | | X* | | | 234 | 205 | 176 | 179 | 172 |
| V304A2b | Virginia (MLBS) | 3 | | | | | X | X | 234 | 163 | 158 | 179 | 172 |
| V304B1 | Virginia (MLBS) | 1 | X | | | | | | 234 | 163 | 176 | 179 | 172 |
| V304B4 | Virginia (MLBS) | - | | | | | | | 234 | 163 | 158 | 185 | 172 |

| | | | | | | | | | | | | | |
|---------|-----------------|---|----------------|---|---|----|----|----|-----|-----|-----|-----|-----|
| V304C1a | Virginia (MLBS) | 3 | | | | | X | X | 234 | 205 | 176 | 179 | 172 |
| V304C1b | Virginia (MLBS) | - | | | | | | | 234 | 166 | 176 | 179 | 172 |
| V304D1 | Virginia (MLBS) | 3 | | | | | X* | X* | 234 | 163 | 158 | 185 | 175 |
| V305B1 | Virginia (MLBS) | 3 | | | | | | X* | 234 | 163 | 158 | 185 | 172 |
| V305B4 | Virginia (MLBS) | 3 | | | | | X | X | 234 | 163 | 161 | 158 | 256 |
| V306D1 | Virginia (MLBS) | 2 | | X | X | X | | | - | - | - | - | - |
| V315B1 | Virginia (MLBS) | 1 | X | | | | | | 255 | 211 | 161 | 158 | 256 |
| V315D1 | Virginia (MLBS) | 1 | X | | | | | | 228 | 205 | 176 | 227 | 184 |
| V315D2 | Virginia (MLBS) | 2 | | X | X | X | | | 228 | 205 | 176 | 227 | 184 |
| V316A1 | Virginia (MLBS) | 3 | | | | | X | X | 264 | 226 | 161 | 158 | 169 |
| V317A1 | Virginia (MLBS) | 2 | | | | X* | | | 228 | 205 | 176 | 227 | 184 |
| V317D | Virginia (MLBS) | 1 | X | | | | | | 228 | 205 | 176 | 227 | 184 |
| V318A1 | Virginia (MLBS) | 2 | | | X | X | | | 228 | 205 | 176 | 227 | 184 |
| V319A | Virginia (MLBS) | 3 | | | | | X | X | 264 | 205 | 161 | 158 | 172 |
| V319B1 | Virginia (MLBS) | 3 | | | | | X | X | 255 | 214 | 161 | 158 | 256 |
| V319B3 | Virginia (MLBS) | 3 | | | | | X | X | 234 | 163 | 158 | 185 | 175 |
| V319C1 | Virginia (MLBS) | 1 | X | | | | | | 234 | 163 | 161 | 161 | 253 |
| V319D2 | Virginia (MLBS) | 3 | | | | | X | X | 234 | 163 | 158 | 185 | 277 |
| V320C1 | Virginia (MLBS) | 2 | | | | X | | | 234 | 163 | 161 | 161 | 253 |
| V321B1 | Virginia (MLBS) | 3 | | | | | | X | 234 | 208 | 158 | 167 | 172 |
| V321C1 | Virginia (MLBS) | - | | | | | | | 234 | 166 | 161 | 161 | 253 |
| V321D1 | Virginia (MLBS) | 1 | X | | | | | | 225 | 205 | 161 | 158 | 259 |
| V322A1a | Virginia (MLBS) | 1 | X | | | | | | 255 | 211 | 161 | 158 | 259 |
| V322A1b | Virginia (MLBS) | - | | | | | | | 255 | 166 | 161 | 158 | 175 |
| V322B1 | Virginia (MLBS) | 1 | X | | | | | | 225 | 205 | 161 | 158 | 259 |
| V322C3a | Virginia (MLBS) | 1 | X | | | | | | 225 | 205 | 161 | 158 | 256 |
| V322C3b | Virginia (MLBS) | - | | | | | | | 225 | 205 | 161 | 158 | 172 |
| V322D1a | Virginia (MLBS) | - | | | | | | | 225 | 205 | 161 | 167 | 172 |
| V322D1b | Virginia (MLBS) | - | | | | | | | 234 | 205 | 182 | 167 | 172 |
| V323A1 | Virginia (MLBS) | - | | | | | | | 234 | 166 | 161 | 140 | 250 |
| V323C1a | Virginia (MLBS) | 3 | | | | | X | X | 255 | 214 | 161 | 158 | 217 |
| V323C1b | Virginia (MLBS) | - | | | | | | | 255 | 163 | 161 | 158 | 256 |
| V323D1 | Virginia (MLBS) | 1 | X | | | | | | 234 | 166 | 161 | 140 | 250 |
| V324B1 | Virginia (MLBS) | 1 | X [†] | | | | | | 234 | 163 | 161 | 140 | 217 |
| V324B3 | Virginia (MLBS) | 1 | X* | | | | | | 234 | 163 | 161 | 140 | 250 |
| V324D1 | Virginia (MLBS) | 1 | X | | | | | | 255 | 211 | 161 | 158 | 256 |
| V324D2 | Virginia (MLBS) | - | | | | | | | 255 | 211 | 158 | 158 | 256 |
| V325A1a | Virginia (MLBS) | 1 | X | | | | | | 255 | 211 | 161 | 158 | 256 |

| | | | | | | | | | | | | | |
|---------|-----------------|---|----------------|----------------|----------------|----------------|---|---|-----|-----|-----|-----|-----|
| V325A1b | Virginia (MLBS) | - | | | | | | | 255 | 211 | 161 | 158 | 172 |
| V325B4 | Virginia (MLBS) | 3 | | | | | | X | 255 | 214 | 161 | 158 | 256 |
| V325D1 | Virginia (MLBS) | 2 | | X | X | X | | | 234 | 208 | 182 | 167 | 172 |
| V326A1 | Virginia (MLBS) | 2 | | X | X | X | | | 255 | 214 | 161 | 158 | 256 |
| V326B1 | Virginia (MLBS) | - | | | | | | | 255 | 208 | 161 | 158 | 256 |
| V326D1 | Virginia (MLBS) | 3 | | | | | X | X | 282 | 178 | 161 | 230 | 250 |
| V327A1 | Virginia (MLBS) | 2 | | X | X | X | | | 234 | 205 | 182 | 167 | 172 |
| V327A2 | Virginia (MLBS) | 2 | | X | X | X | | | 234 | 208 | 182 | 167 | 172 |
| V327B1 | Virginia (MLBS) | 3 | | | | | X | X | 234 | 163 | 158 | 191 | 172 |
| V327C1 | Virginia (MLBS) | 2 | | X | X | | | | 255 | 211 | 161 | 158 | 256 |
| V327C2 | Virginia (MLBS) | 1 | X | | | | | | 234 | 208 | 182 | 167 | 172 |
| V327D1 | Virginia (MLBS) | - | | | | | | | 234 | 208 | 182 | 167 | 172 |
| V327D2 | Virginia (MLBS) | 1 | X | | | | | | 255 | 211 | 158 | 158 | 256 |
| V329C1 | Virginia (MLBS) | - | | | | | | | 264 | 163 | 158 | 158 | 232 |
| V330A | Virginia (MLBS) | 3 | | | | | X | | 228 | 205 | 176 | 227 | 184 |
| V330B1 | Virginia (MLBS) | 2 | | X | X | X | | | 234 | 208 | 182 | 167 | 172 |
| V330B2 | Virginia (MLBS) | 2 | | X | X | X | | | 228 | 205 | 176 | 227 | 184 |
| V330D2 | Virginia (MLBS) | 1 | X [†] | | | | | | 279 | 205 | 176 | 140 | 178 |
| V331B1 | Virginia (MLBS) | 2 | | X | X | X | | | 255 | 208 | 182 | 170 | 172 |
| V331C1 | Virginia (MLBS) | 1 | X | | | | | | 234 | 214 | 161 | 158 | 256 |
| V331C2 | Virginia (MLBS) | 1 | X | | | | | | 255 | 214 | 161 | 158 | 256 |
| V331D1 | Virginia (MLBS) | 2 | | X | X | | | | 234 | 208 | 182 | 167 | 172 |
| V331D2 | Virginia (MLBS) | 3 | | | | | X | X | 255 | 214 | 161 | 158 | 256 |
| V335B1 | Virginia (MLBS) | 3 | | | | | X | X | 255 | 214 | 179 | 158 | 256 |
| V335C1 | Virginia (MLBS) | 1 | X | | | | | | 255 | 208 | 161 | 158 | 172 |
| V335D1 | Virginia (MLBS) | - | | | | | | | 255 | 214 | 161 | 158 | 256 |
| V336B1 | Virginia (MLBS) | 2 | | X [†] | X [†] | X [†] | | | 228 | 205 | 176 | 227 | 184 |
| V336D1 | Virginia (MLBS) | 1 | X | | | | | | 228 | 205 | 176 | 227 | 184 |
| V337C1 | Virginia (MLBS) | 3 | | | | | X | X | 282 | 181 | 161 | 233 | 250 |
| V337D1 | Virginia (MLBS) | 1 | X | | | | | | 255 | 214 | 161 | 158 | 256 |
| V341A2 | Virginia (MLBS) | 1 | X | | | | | | 255 | 211 | 161 | 158 | 256 |
| V341C2 | Virginia (MLBS) | 3 | | | | | X | X | 288 | 205 | 161 | 158 | 250 |
| V341D1 | Virginia (MLBS) | - | | | | | | | 234 | 205 | 176 | 140 | 178 |
| V342A2 | Virginia (MLBS) | 2 | | X | X | X | | | 234 | 163 | 161 | 161 | 253 |
| V342B2 | Virginia (MLBS) | 1 | X [†] | | | | | | 255 | 208 | 161 | 158 | 256 |
| V345D1 | Virginia (MLBS) | 1 | X | | | | | | 279 | 205 | 176 | 140 | 178 |
| V53A | Virginia (MLBS) | 2 | | X | X | X | | | 279 | 205 | 176 | 140 | 178 |
| V53B | Virginia (MLBS) | 2 | | X | X | X | | | 234 | 163 | 161 | 161 | 253 |

| | | | | | | | | | | | | | |
|-------|-----------------|---|----|---|---|---|---|---|-----|-----|-----|-----|-----|
| V53D1 | Virginia (MLBS) | 1 | X | | | | | | 234 | 163 | 161 | 161 | 253 |
| V55A1 | Virginia (MLBS) | 2 | | X | X | X | | | 228 | 205 | 176 | 227 | 184 |
| V55A2 | Virginia (MLBS) | 1 | X | | | | | | 228 | 205 | 176 | 227 | 250 |
| V55A5 | Virginia (MLBS) | 2 | | | X | | | | 255 | 211 | 161 | 158 | 256 |
| V55C1 | Virginia (MLBS) | - | | | | | | | 234 | 208 | 161 | 140 | 253 |
| V55C2 | Virginia (MLBS) | 3 | | | | | X | X | 234 | 205 | 161 | 140 | 253 |
| V55D2 | Virginia (MLBS) | 3 | | | | | X | X | 255 | 214 | 161 | 158 | 256 |
| V56A1 | Virginia (MLBS) | 1 | X | | | | | | 255 | 211 | 161 | 158 | 256 |
| V56A2 | Virginia (MLBS) | 1 | X* | | | | | | 264 | 163 | 176 | 212 | 178 |
| V56B2 | Virginia (MLBS) | 2 | | X | X | X | | | 228 | 205 | 176 | 227 | 184 |
| V56C1 | Virginia (MLBS) | 3 | | | | | X | X | 234 | 205 | 161 | 146 | 250 |
| V64A | Virginia (MLBS) | 3 | | | | | X | X | 255 | 214 | 161 | 158 | 256 |
| V64D1 | Virginia (MLBS) | 3 | | | | | X | X | 279 | 229 | 176 | 140 | 178 |
| V64D2 | Virginia (MLBS) | 1 | X | | | | | | 255 | 214 | 161 | 158 | 256 |
| V72A1 | Virginia (MLBS) | 3 | | | | | X | X | 234 | 208 | 161 | 233 | 250 |
| V77A | Virginia (MLBS) | 1 | X | | | | | | 234 | 205 | 161 | 146 | 253 |
| V77B | Virginia (MLBS) | 1 | X | | | | | | 225 | 205 | 161 | 158 | 256 |
| V78B | Virginia (MLBS) | 2 | | X | X | X | | | 264 | 163 | 158 | 212 | 229 |
| V78C | Virginia (MLBS) | 1 | X | | | | | | 234 | 205 | 176 | 179 | 172 |

* = Mating type gene confirmed using unpublished primers.

† = Mating type gene confirmed from whole genome sequencing.

Table S2. PCR primer pairs for amplification of mating type genes. Primer design based on the published DNA sequence data from Bloomfield et al. (2010).

| Mating Type | Gene | Direction | Primer Sequence (5' to 3' direction) |
|--------------------|----------------|------------------|---|
| Type I | matA | Forward | CACACTAAACATGGACCCAC |
| | | Reverse | CCCCTAAATCTTTACCAAGTCA |
| Type II | matC | Forward | GGGTACAAATATTACAGTGAG |
| | | Reverse | CCCCTTTAAAAATGTATTCATAT |
| | matB | Forward | CCCCGAATAAACATTTTAATGA |
| | | Reverse | GCGAACTCAATTACTATGGG |
| | matD (partial) | Forward | CCCATAGTAATTGAGTTCGC |
| | | Reverse | GGGCACTGTTATCTTGTTAAT |
| Type III | matS | Forward | CGATCAGTTGGAAAACATTAC |
| | | Reverse | GGATAGCCAAAAAAGTATT |
| | matT (partial) | Forward | CGAAAACAGTCAAAGTCAA |
| | | Reverse | CATTATATTGCATTTTCAGTGG |

Table S3. Standardized chi-square residuals for each population. Standardized residuals greater than 2 indicate significantly more individuals than expected of that mating type in the population and standardized residuals less than -2 indicate fewer than expected. Asterisks denote significance.

| Population | Standardized Residuals | | |
|-------------------|-------------------------------|----------------|-----------------|
| | Type I | Type II | Type III |
| Texas | 1.84 | -2.45* | 0.61 |
| North Carolina | 3.18* | -2.12* | -1.06 |
| Virginia | 1.14 | -0.91 | -0.23 |
| Overall | 3.24* | -2.92* | -0.32 |