4-16-2016

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

Tracy Edwards Douglas

Joan E. Strassmann
Washington University in St Louis, strassmann@WUSTL.EDU

David C. Queller
Washington University in St Louis, queller@WUSTL.EDU

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

Tracy E. Douglas*, Joan E. Strassmann*, and David C. Queller*

**Department of Biology, Washington University in St. Louis, St. Louis, MO 63130*

Correspondence: Tracy Edwards Douglas

Email: tracy.douglas@wustl.edu

Mailing Address: Department of Biology, Washington University in St. Louis

Campus Box 1137, 1 Brookings Drive, St. Louis, MO 63130

Phone: (314) 935-5302

Fax Number: (314) 935-4432

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

Abstract

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

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

Introduction

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

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

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

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

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

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

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

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

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

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

Materials and Methods

Study Populations

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

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

Gamete Size Measurement

To measure gamete size, we sampled multiple clones from each of the three self-incompatible mating types from two populations. Because two haploid cells fuse to form the reproductive zygote during the sexual cycle of *D. discoideum*, we measured the size of cells prepared in the absence of a compatible mating partner but in conditions conducive for sexual fusion, to get at their size right before fusion. These fusion-competent cells are considered at this point to be gametes (Saga et al., 1983; O'Day et al., 1987; Urushihara & Muramoto, 2006). Specifically, we plated $2x10^5$ spores on LP agar plates (0.1% lactose, 0.1% peptone, 1.5% agar) in an excess of Bonner's salt solution (SS: 0.06% NaCl, 0.03% $CaCl_2$, 0.075% KCl) with *K. pneumoniae* and incubated the plates in the dark for 3 days at 22° C. We then collected the resulting dark-grown cells and measured the cell diameters using a Nexcelom Cellometer Auto 1000 (Lawrence, MA). We used the default settings with the exception of a cell size minimum set to 5 um and a maximum set to 15 um. In each population, we measured 160 cell diameters from each of four to six clones per mating type.

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

sexual fusion. We plated $2x10^5$ spores on SM/5 agar plates with *K. pneumoniae* and allowed the plates to grow on a bench for ~36 hours. We collected pre-aggregate vegetative cells in buffer and used the same methods as previous for measuring cell diameters.

Mating Type Identification and Microsatellite Analysis

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

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

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

Statistical Analyses

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

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

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

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

Results

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

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

Frequencies of mating types are unequal and vary between locations

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

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

Balancing selection maintains mating type distributions across populations

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

Discussion

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

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

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

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

Conclusions and Implications for Future Research

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

Acknowledgments

This material is based upon work supported by the National Science Foundation under grant number DEB 1146375 and the John Templeton Foundation grant 43667. We thank members of the Strassmann-Queller lab for support and advice, especially undergraduates Julian Duodo, Amanda Boozalis, Olivia Williams, and Rohit Unni for their assistance in the laboratory. The authors declare that they have no conflict of interest.

References

Anderson, M. & ter Braak, C. 2003. Permutation tests for multi-factorial analysis of variance. *J. Stat. Comput. Simul.* **73**: 85-113.

Bachtrog, D., Mank, J.E., Peichel, C.L., Kirkpatrick, M., Otto, S.P., Ashman, T., et al. 2014. Sex determination: why so many ways of doing it? *PLoS Biol.* **12**: e1001899.

Balloux, F., Brünner, H., Lugon-Moulin, N., Hausser, J., & Goudet, J. 2000. Microsatellites can be misleading: an empirical and simulation study. *Evolution* **54:** 1414-1422.

Basu, S., Fey, P., Pandit, Y., Dodson, R., Kibbe, W.A. & Chisholm, R.L. 2013. dictyBase 2013: integrating multiple Dictyostelid species. *Nucleic Acids Res.* **41**: D676-D683.

Bell, G. 1978. The evolution of anisogamy. *J. Theor. Biol*. **73**: 247-270.

Benjamini, Y. & Hochberg, Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Statist. Soc. B* **57**: 289-300.

Billiard, S., López-Villavicencio, M., Devier, B., Hood, M.E., Fairhead, C. & Giraud, T. 2011. Having sex, yes, but with whom? Inferences from fungi on the evolution of anisogamy and mating types. *Biol. Rev.* **86**: 421-442.

Billiard, S., López-Villavicencio, M., Hood, M.E. & Giraud, T. 2012. Sex, outcrossing and mating types: unsolved questions in fungi and beyond. *J. Evol. Biol.* **25**: 1020-1038.

Blaskovics, J.C. & Raper, K.B. 1957. Encystment stages of Dictyostelium. *Biol. Bull.* **113**: 58-88.

Bloomfield, G., Skelton, J., Ivens, A., Tanaka, Y., & Kay, R.R. 2010. Sex determination in the social amoeba Dictyostelium discoideum. *Science* **330**: 1533-1536.

Bloomfield, G. 2013. Sex in Dictyostelia. In: *Dictyostelids* (M. Romeralo, S. Baldauf, R. Escalante, eds.), pp. 129-148. Springer Berlin Heidelberg.

Bulmer, M.G. & Parker, G.A. 2002. The evolution of anisogamy: a game-theoretic approach. *Proc. R. Soc. Lond. B.* **269**: 2381-2388.

Busch, J.W., Witthuhn, T. & Joseph, M. 2014. Fewer S-alleles are maintained in plant populations with saprophytic as opposed to gametophytic self-incompatibility. *Plant Species Biol.* **29**: 34-46.

Campbell, J. M., & Lawrence, M. J. 1981. The population genetics of the self-incompatibility polymorphism in *Papaver rhoeas*. II. The number and frequency of S-alleles in a natural population (R106). *Heredity* **46**: 81-90.

Castric, V., & Vekemans, X. 2004. INVITED REVIEW: Plant self-incompatibility in natural populations: a critical assessment of recent theoretical and empirical advances. *Mol. Ecol.* **13**: 2873-2889.

Cavender, J.C., Worley, A.C., & Raper, K.B. 1981. The yellow-pigmented Dictyostelia. *Am. J. Bot.* **68**: 373-382.

Cavender, J.C., Vadell, E., Landolt, J.C., & Stephenson, S.L. 2005. New species of small dictyostelids from the Great Smoky Mountains National Park. *Mycologia* **97**: 493-512.

Chang, M.T. & Raper, K.B. 1981. Mating types and macrocyst formation in *Dictyostelium rosarium*. *J. Bacteriol.* **147**: 1049-1053.

401 Charnov, E.L. 1982. The theory of sex allocation. Princeton University Press, Princeton, NJ.
402 Clark, M.A., Francis, D. & Eisenberg, R. 1973. Mating types in cellular slime molds. *Biochem.*
403 *Biophys. Res. Commun.* **52**: 672-678.
404 Collins, K. & Gorovsky, M.A. 2005. Tetrahymena thermophile *Curr. Biol.* **15**: R317-8.
405 Collins, O.R. & Tang, H. 1977. New mating types in *Physarum polycephalum*. *Mycologia* **69**:
406 421-423.
407 Delph, L. F., & Kelly, J. K. 2014. On the importance of balancing selection in plants. *New*
408 *Phytol.* **201**: 45-46.
409 Doerder, F. P., Gates, M. A., Eberhardt, F. P., & Arslanyolu, M. 1995. High frequency of sex
410 and equal frequencies of mating types in natural populations of the ciliate *Tetrahymena*
411 *thermophila*. *PNAS* **92**: 8715-8718.
412 Douglas, T.E., Kronforst, M.R., Queller, D.C., & Strassmann, J.E. 2011. Genetic diversity in the
413 social amoeba *Dictyostelium discoideum*: population differentiation and cryptic species.
414 *Mol. Phylogenet. Evol.* **60**: 455-462.
415 Erdos, G.W., Raper, K.B. & Vogen, L.K. 1973a. Mating types and macrocyst formation in
416 *Dictyostelium discoideum*. *PNAS* **70**: 1828-1830.
417 Erdos, G.W., Nickerson, A.W., & Raper, K.B. 1973b. The fine structure of macrocyst
418 germination of *Dictyostelium mucoroides*. *Dev. Biol.* **32**: 321-330.
419 Erdos, G.W., Raper, K.B., & Vogen, L.K. 1975. Sexuality in the cellular slime mold
420 *Dictyostelium giganteum*. *PNAS* **72**: 970-973.
421 Fijarczyk, A., & Babik, W. 2015. Detecting balancing selection in genomes: limits and
422 prospects. *Mol. Ecol.* **24**: 3529-3545.

423 Filosa, M.F. & Dengler, R.E. 1972. Ultrastructure of macrocyst formation in the cellular slime
424 mold, *Dictyostelium mucoroides*: extensive phagocytosis of amoebae by a specialized cell.
425 *Dev. Biol.* **29**: 1-16.
426 Fisher, R. A. 1930. The genetical theory of natural selection: a complete variorum edition.
427 Oxford University Press, Oxford.
428 Flowers, J. M., Li, S. I., Stathos, A., Saxer, G., Ostrowski, E. A., Queller, D. C., et al. 2010.
429 Variation, sex, and social cooperation: molecular population genetics of the social amoeba
430 Dictyostelium discoideum. *PLoS Genet*, ***6***: e1001013-e1001013.
431 Fortunato, A., Strassmann, J.E., Santorelli, L. & Queller, D.C. 2003. Co-occurrence in nature of
432 different clones of the social amoeba, *Dictyostelium discoideum*. *Mol. Ecol.* **12**: 1031-
433 1038.
434 Francis, D. 1975. Macrocyst genetics in *Polysphondylium pallidum*, a cellular slime mould. *J.*
435 *Gen. Microbiol.* **89**: 310-318.
436 Goudet, J. 2001. FSTAT, a program to estimate and test gene diversities and fixation indices
437 (version 2.9. 3).
438 Hamilton, W. D. 1967. Extraordinary sex ratios. *Science* **156**: 477-488.
439 Hedrick, P.W., 2005. A standardized genetic differentiation measure. *Evolution* **59**: 1633–1638.
440 Iwasa, Y. & Sasaki, A. 1987. Evolution of the number of sexes. *Evolution* **41**: 49-65.
441 Kawakami, S. & Hagiwara, H. 1999. Macrocyst formation in three dictyostelid species,
442 *Dictyostelium monochasioides, Polysphondylium candidum,* and *P. pseudo-candidum*.
443 *Mycoscience* **40**: 359-361.

444 Kato, S., & Mukai, Y. 2004. Allelic diversity of S-RNase at the self-incompatibility locus in
445 natural flowering cherry populations (Prunus lannesiana var. speciosa). *Heredity* **92**: 249-
446 256.
447 Keenan, K., McGinnity, P., Cross, T. F., Crozier, W. W., & Prodöhl, P. A. 2013. diveRsity: an R
448 package for the estimation and exploration of population genetics parameters and their
449 associated errors. *Methods Ecol. Evol.* **4**: 782-788.
450 Knowlton, N. 1974. A note on the evolution of gamete dimorphism. *J. Theor. Biol.* **46**: 283-285.
451 Kothe, E. 1996. Tetrapolar fungal mating types: sexes by the thousands. *FEMS Microbiol. Rev.*
452 **18**: 65-87.
453 Lawrence, M.J. 2000. Population genetics of the homomorphic self-incompatibility
454 polymorphisms in flowering plants. *Ann. Botany* **85**: 221-226.
455 May G, Shaw F, Badrane H, & Vekemans X 1999. The signature of balancing selection: Fungal
456 mating compatibility gene evolution. *PNAS* **96**: 9172–9177.
457 Meirmans, P.G. & Hedrick, P.W. 2011. INVITED TECHNICAL REVIEW: Assessing
458 population structure: F_{ST} and related measures. *Mol. Ecol. Resour.* **11**: 5-18.
459 Noh, S. & Henry, C.S. Within-species mate preferences do not contribute to the maintenance of
460 sexually monomorphic mating signals in green lacewings. *Ethol.* **121**: 714-724.
461 O'Day, D.H. 1979. Aggregation during sexual development in *Dictyostelium discoideum. Can. J.*
462 *Microbiol.* **25**: 1416-1426.
463 O'Day, D.H. & Durston, A.J. 1979. Evidence for chemotaxis during sexual development in
464 *Dictyostelium discoideum*. *Can. J. Microbiol.* **25**: 542-544.

O'Day, D.H., McConachie, D.R., & Rivera, J. 1987. Appearance and developmental kinetics of a unique cell type in *Dictyostelium discoideum*: is it the gamete of sexual development? *J. Exp. Zool.* **242**: 153-159.

O'Day, D.H. & Keszei, A. 2012. Signalling and sex in the social amoebozoans. *Biol. Rev.* **87**: 313-329.

Orias, E., & Rohlf, F. J. 1964. Population genetics of the mating type locus in Tetrahymena pyriformis, variety 8. *Evolution* **18**: 620-629.

Parker, G.A., Baker, R.R., & Smith, V.G. 1972. The origin and evolution of gamete dimorphism and the male-female phenomenon. *J. Theor. Biol.* **36**: 529-553.

Phadke, S.J. & Zufall, R.A. 2009. Rapid diversification of mating systems in ciliates. *Biol. J. Linnean Soc.* **98**: 187-197.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D. & R Core Team. 2014. nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-117, URL: http://CRAN.R-project.org/package=nlme/.

R Core Team. 2015. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL: http://www.R-project.org/.

Raper, J.R. 1966. Genetics of sexuality in higher fungi. Ronald Press, New York.

Randerson, J.P. & Hurst, L.D. 2001. The uncertain evolution of the sexes. *Trends Ecol. Evol.* **16**: 571-579.

Saga, Y., Okada, H., & Yanagisawa, K. 1983. Macrocyst development in *Dictyostelium discoideum*. II. Mating-type specific cell fusion and acquisition of fusion-competence. *J. Cell Sci.* **60**: 157-168.

487 Saga, Y. & Yanagisawa, K. 1983. Macrocyst development in *Dictyostelium discoideum* III. Cell-
488 fusion inducing factor secreted by giant cells. *J. Cell Sci.* **62**: 237-248.
489 Smith, M.H. 2004. Genotypic diversity and population structure in *Dictyostelium discoideum*.
490 M.A. Thesis. Rice University, Houston, TX.
491 Urushihara, H. & Muramoto, T. 2006. Genes involved in *Dictyostelium discoideum* sexual
492 reproduction. *Eur. J. Cell Biol.* **85**: 961-968.
493 Vekemans, X., & Slatkin, M. 1994. Gene and allelic genealogies at a gametophytic self-
494 incompatibility locus. *Genetics* **137**: 1157–1165.
495 West, S.A. 2009. Sex allocation. Princeton University Press, Princeton, NJ.
496 Wright, S. 1939. The distribution of self-sterility alleles in populations. *Genetics* **24**: 538.

Table 1. F_{ST} and G'_{ST} values show differentiation in mating type frequencies and microsatellite allele frequencies between populations of *Dictyostelium discoideum*. We included the 95% 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**

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

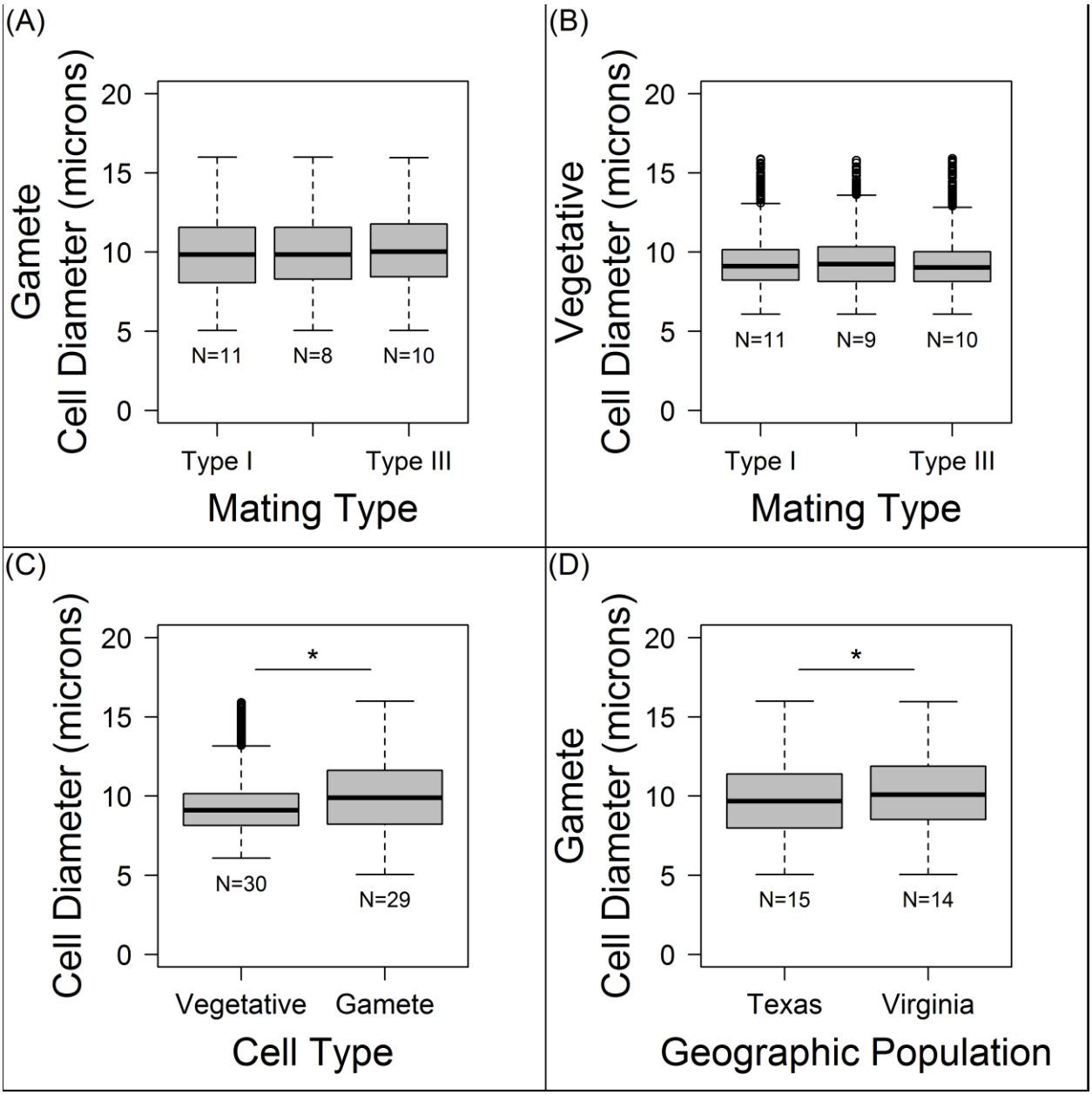

Figure 2. Mating type proportions vary by population. The pie charts show the distributions of mating types within each of the four geographic populations, with the large pie for North Carolina representing the combined totals from the two populations represented individually by the smaller pies. Stars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

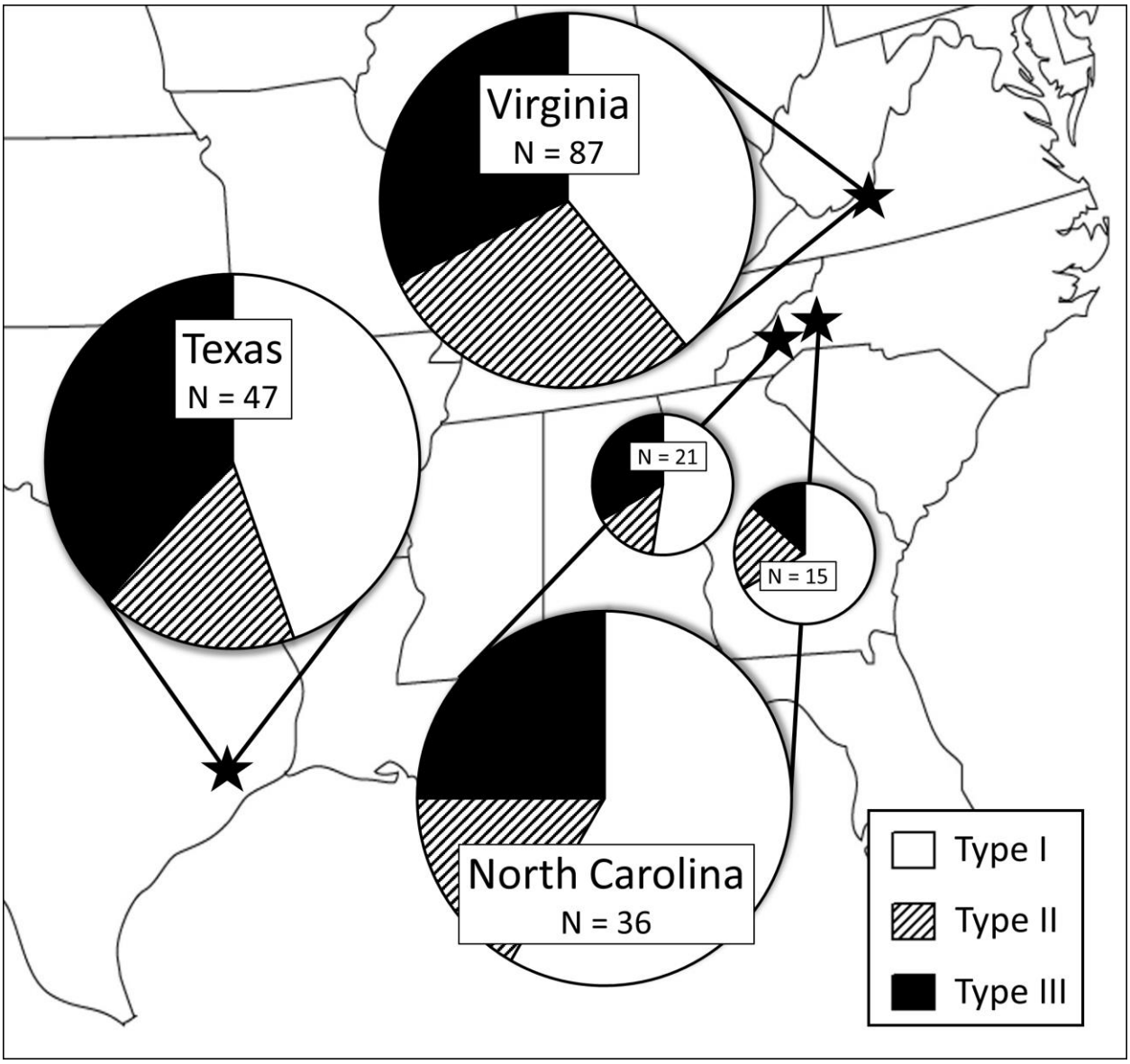

Table S1. *Dictyostelium discoideum* clones from the four populations used in this study (LF = Linville Falls [35°57.197' N, 81°56.516' W], LBG = Little Butts Gap [35°46' N, 82°20' W], H = Houston [29°46' N, 95°27' W], MLBS = Mountain Lake Biological Station [37°21' N, 80°31' W]) and their associated 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).

			Confirmed Mating Type Genes						Microsatellite Allele Size (bp)				
Clone Name	**Population**	**Type**	**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	GGATAGCCAAAAAACTAGTTT
	matT (partial)	Forward	CGAAAACAGTCAAAAGTCAA
		Reverse	CATTATATTGCATTTCAGTGG

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*