The pattern of inheritance and mechanism of sex determination can have important evolutionary consequences. We studied probabilistic sex determination in the ciliate *Tetrahymena thermophila*, which was previously shown to cause evolution of skewed sex ratios. We find that the genetic background alters the sex determination patterns of *mat* alleles in heterozygotes and that allelic interaction can differentially influence the expression probability of the 7 sexes. We quantify the dominance relationships between several *mat* alleles and find that A-type alleles, which specify sex I, are indeed recessive to B-type alleles, which are unable to specify that sex. Our results provide additional support for the presence of modifier loci and raise implications for the dynamics of sex ratios in populations of *T. thermophila*.

**Key words:** ciliate, inheritance, mating type, sex determination, sex ratio

Eukaryotes exhibit great diversity in sex, or mating type, determination mechanisms. Chromosomal sex determination (SD) is widespread among plants and animals with the exceptions of reptiles in which environment often interacts with the genetic factors to determine sexes, and some insects in which cytoplasmic elements contribute largely to SD (Bull 1983). Among microbial eukaryotes, free-living ciliates as well as fungal and apicomplexan parasites together reveal a variety of SD mechanisms, including strictly genetic determination involving one to many loci and environmentally controlled temperature-dependent and circadian SD (Casselton 2002; Smith et al. 2002; Phadke and Zufall 2009).

The genetic and environmental details of SD mechanisms may have important evolutionary consequences, for example, in determining population sex ratio. The ciliate *Tetrahymena thermophila* has 7 self-incompatible mating types (sexes) and skewed sex ratios in natural populations (Doerder et al. 1995; Arslanyolu and Doerder 2000). The sexes are determined by alleles at the *mat* locus. In contrast to sex-specific alleles, for example, *mat-a* and *mat-alpha* in some yeast (Jackson and Hartwell 1990), each *T. thermophila* *mat* allele can specify multiple sexes with distinct probabilities (Doerder et al. 1995). We previously demonstrated that this probabilistic SD can lead to the evolution of skewed population sex ratios (unequal frequencies of the 7 sexes) by imposing constraints on the maintenance of genetic variation (Paixão et al. 2011), a result consistent with the observation of skewed sex ratios in natural population of *T. thermophila*.

The *mat* locus in *T. thermophila* resides at chromosome 1L (Orias 1981; Orias E, personal communication). Prior to the recent discovery of the structure of the locus (Cervantes et al. 2013), *mat* alleles in *T. thermophila* were distinguished based on their sex determination pattern (SDP) (Orias 1981; Lynch et al. 1995). The SDP of a *mat* allele specifies the probability that an individual homozygous for that allele will express 1 of the 7 sexes (Arslanyolu and Doerder 2000). Thus, two individuals that are genetically identical, and homozygous for the same allele at the *mat* locus, may develop different sexes (Figure 1). A unique SDP, that is, the distribution of probabilities with which the allele specifies different sexes, characterizes every *mat* allele.

Two types of *mat* alleles were found in various natural populations of *T. thermophila* (Nanney 1959; Doerder et al. 1995; Arslanyolu and Doerder 2000): alleles that code for all sexes except sex I (e.g., *mat-2*, B-type alleles) and alleles that code for all sexes except the sexes IV and VII (e.g., *mat-3*, A-type alleles). Some evidence exists for the sensitivity of an allele’s SDP to the growth temperature: at different temperatures, individuals homozygous for the same *mat* allele express the sexes with different probabilities (Arslanyolu and Doerder 2000).

We empirically analyzed SDPs of the 2 alleles, *mat-2* and *mat-3*, in homozygous and heterozygous states. Our results, together with the previously published observations (Nanney 1959), suggest that unlike the growth temperature, the genetic background does not affect the SDP of *mat* alleles in the homozygous state; however, it alters...
the dominance relationships and hence the heterozygote SDP in *T. thermophila*. We also provide the first estimate of degree of dominance in *mat* alleles of *T. thermophila*.

**Materials and Methods**

**Strains and Media**

*Tetrahymena thermophila* strains SB2364H and SB2374H (Table 1) were obtained from the Tetrahymena Stock Center (Cornell University). These strains are whole-genome homozygotes derived from heterozygous F1 progeny of a cross between inbred strains B and C3 (Lynch et al. 1995) followed by genomic exclusion (Allen 1967). SB2364H is homozygous for the allele *mat*-2 and SB2374H is homozygous for *mat*-3 at the SD locus in the germ line (Hamilton E, Orias E, personal communication). *mat*-2 specifies 6 sexes (II–VII) and *mat*-3 specifies 5 sexes (I–III, V, and VI). Both strains are homozygous in the germ-line genome for an allele that confers resistance to cyclohexamide (Cy, 15 ng/μL; Ares...

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**Figure 1.** SDPs of *mat* genotypes. SDPs of the homozygous genotypes (*mat*-2/*mat*-2 or *mat*-3/*mat*-3) and the heterozygote (*mat*-2/*mat*-3) were measured using 3 separate crosses (Table 2). Cartoon of the genotypes (top panel) in the germ-line nucleus (Cervantes et al. 2013, Cervantes M., Orias E., Hamilton E., personal communication) and the various progeny produced (middle panel) are shown for each cross. The bottom panels represent SDPs measured in the present study (black bars) or published previously (gray bars) for (A) homozygous genotype (*mat*-2/*mat*-2), (B) homozygous genotype (*mat*-3/*mat*-3), and (C) heterozygous genotype (*mat*-2/*mat*-3). SDP expected under codominance is marked by filled circles in panel (C). SDPs measured in this study are shown as the average of 2 replicates and do not differ significantly from the expectation under codominance (Table 2). Error bars indicate standard error. SDP of the heterozygous genotype measured in the previous study is significantly different from the expectation under codominance (Table 2).
Table 1  Strains used in analyses of SDPs

<table>
<thead>
<tr>
<th>Parental strain</th>
<th>Genotype at the mat locus in germ-line genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB2364H</td>
<td>mat-2/mat-2</td>
</tr>
<tr>
<td>SB2374H</td>
<td>mat-3/mat-3</td>
</tr>
<tr>
<td>B*VI</td>
<td>—</td>
</tr>
</tbody>
</table>

* The strain B*VI has a deteriorated germ-line nucleus (Allen 1967) and does not contribute any genes to the progeny, which inherit the mat alleles only from the germ-line nucleus of the strain SB2364H or SB2374H.

and Bruns et al. 1985) and homozygous for the susceptible allele in the somatic genome (Lynch et al. 1995). Strain B*VI lacks a functional germ-line genome and hence does not contribute its genome to progeny. Mating with B*VI is used to produce whole-genome homozygotes (Allen 1967).

Stocks of all strains were maintained frozen under liquid nitrogen for the entire duration of the study. Frozen stocks were thawed and cells were grown to log phase for 48 h prior to use in the experiments. We used 2% w/v Proteose Peptone (PP) to grow cells asexually. To induce mating, all strains were starved in 2% bacterized peptone (BP). To make 2% BP, an overnight culture of Klebsiella pneumoniae grown in 2% PP was diluted 1:50 with sterile water. In this medium, cells grow asexually by feeding on the bacteria and starve on exhausting the bacteria in about 48 h (Hamilton and Orias 1999). We used 2% BP, instead of conventional starvation media (e.g., 10 mM Tris), to closely mimic starvation in natural environment. All matings were performed in autoclaved distilled water. All culturing and mating experiments were performed at 26 °C.

Determination of SDPs

We crossed each strain with the strain B*VI, which does not contribute its genome to the progeny (genomic exclusion, Allen 1967). After successful completion of 2 rounds of genomic exclusion, the progeny express a Cy-resistant phenotype and are sexually immature for ~100 asexual divisions (Hamilton and Orias 1999). Sexual immaturity was confirmed by attempted mating of progeny cells with the strains SB2364H and SB2374H, which are sexes I and VII, respectively; immature progeny did not mate with either strain. We grew drug-resistant, immature progeny until they reached sexual maturity. Each progeny inherits the mat genotype (mat-2/mat-2 or mat-3/mat-3) of its parental strain and expresses 1 of the 7 sexes (Figure 1A,B). We identified the sexes of several progeny of each parental strain using tester strains (Orias et al. 1999). Progeny were mixed separately with cultures of the 7 different sexes. Because T. thermophila sexes are self-incompatible, the sex of the progeny was identified as the only sex that it did not mate with.

To determine the SDP of the heterozygote, we performed a cross between clonal cultures of the two homozygotes (SB2364H and SB2374H; Table 1), and isolated single cells after mating pairs separated. We identified and retained several immature progeny among the isolated cells using immaturity tests as described above. Immaturity indicates successful mating between the parents and confirms heterozygosity (mat-2/mat-3) at the mat locus of the progeny cell. We grew the immature progeny asexually until they reached sexual maturity and identified the sex of each progeny culture.

Dominance Relationships between mat Alleles

We calculated the degree of dominance of mat alleles assuming the absence of large effects of any modifier loci on SD. Under this assumption, the SDP of a heterozygote can be expressed as a linear combination of SDPs of the homozygotes by the equation $E = d \times M_i + (1 - d) \times M_j$, where $M_i$ and $M_j$ are the SDPs of the homozygotes, respectively, and $d$ is the dominance coefficient. $d$ equals 0.5 under codominance.

To calculate the dominance coefficients, we resampled the SDPs by generating a multinomial sample of the same size as the experimental sample, for each of the genotypes $M_i$, $M_j$, and $M_k$. We then minimized the chi-square distance $\chi^2 = \sum(O_i - E_i)^2/E_i$ with respect to the dominance coefficient $d$ for each set of genotypes using the NMinimize function in Mathematica 8 (http://www.wolfram.com/mathematica/). This results in an estimate of dominance coefficient that is averaged across all sexes. We repeated this procedure $10^3$ times for each of the heterozygote genotypes and calculated the mean and 2.5% and 97.5% quantiles as confidence intervals (CIs). Because this procedure includes the sampling effects of all SDPs, comparisons between values of $d$ will be conservative. We have deposited the primary data used for these analyses with Dryad.

Results and Discussion

Our aim was to determine how alleles at the mat locus interact in T. thermophila. Our study confirms the previous claim that SDPs of the alleles in homozygous state are reproducible across genetic backgrounds (Orias 1981; Doerder et al. 1996). However, our analysis shows that the SDP of a heterozygote may vary depending on the genetic background, providing further support for the presence of epistatic effects with modifier loci (Arslanyolu and Doerder 2000). The SDP of a mat allele is a distribution of probabilities with which the allele (or its homozygote) specifies different sexes. We analyzed SDPs of the alleles mat-2 and mat-3 by identifying the sex of several progeny homozygous for either allele. Our experiments were conducted at the same temperature as the previous studies (Nanney 1959). Table 2 shows the relative frequencies with which the 7 sexes were represented in the progeny. The SDPs found in our study are not significantly different (tested using the goodness-of-fit $G$ test) from the previously published SDPs of these alleles in a different genetic background (Nanney 1959; Table 2). The reproducibility of SDPs across genetic backgrounds supports a strong genetic basis of SD at a single locus or tightly linked loci consistent with the array of sex-specific segments discovered at the mat locus (Orias 1981; Cervantes et al. 2013).

The previously published SDPs of mat-2 and mat-3 were established in the genetic backgrounds of the wild isolates collected from Massachusetts and Vermont, respectively (Nanney 1959). The genetic backgrounds of strains used in our study are a hybrid between those previously used for
Table 2. SDPs of homozygotes and heterozygotes

<table>
<thead>
<tr>
<th>Cross</th>
<th>mat-2 × mat-2</th>
<th>mat-2/ mat-3</th>
<th>mat-3 × mat-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB2364H × BVI</td>
<td>0.598</td>
<td>0.056</td>
<td>0.305</td>
</tr>
<tr>
<td>SB2374H × SB2374H</td>
<td>0.537</td>
<td>0.180</td>
<td>0.345</td>
</tr>
<tr>
<td>Source of SDP</td>
<td>This study</td>
<td>This study</td>
<td>This study</td>
</tr>
<tr>
<td>G value (P value)</td>
<td>0.006</td>
<td>0.005</td>
<td>0.003</td>
</tr>
<tr>
<td>Expected SDP</td>
<td>0.180</td>
<td>0.113</td>
<td>0.215</td>
</tr>
<tr>
<td>SDP of heterozygote</td>
<td>0.604</td>
<td>0.118</td>
<td>0.305</td>
</tr>
</tbody>
</table>

The goodness-of-fit test was used to compare the SDPs observed in this study with the corresponding SDPs published previously (Nanney 1959) for the heterozygotes or with the SDPs expected under codominance (Nanney 1959).

The SDPs of the heterozygote (mat-2/ mat-3) by identifying the sex of many heterozygous progeny (Table 2 and Figure 1C). If the alleles were codominant, the SDP of a heterozygote would be the arithmetic mean of SDPs of the two homozygotes. In homozygous state, mat-2 specifies 6 sexes and mat-3 specifies 5 sexes (Nanney 1959; Bleyman et al. 1992). The presence of 7 sexes in the heterozygous progeny indicates that both alleles contributed to SD (Table 2 and Figure 1C). Also, the frequencies of the 7 sexes observed in our analyses are not significantly different from those expected under codominance (Table 2); however, a significant deviation from codominance was observed previously (Nanney 1959). Because both of these studies (i.e., our present study and that described in Nanney 1959) analyze the SDP of the same alleles (mat-2 and mat-3) in different backgrounds, the conflicting results of dominance analysis indicate the presence of modifier loci that affect allelic interaction in heterozygotes, but not SDP of the homozygotes.

We calculated the degree of dominance for the heterozygote SDP established in the earlier data set (Nanney 1959) and found mat-2 partially dominant over mat-3 at 26 °C (d = 0.55; Table 3). Although CIs of d do not exclude the possibility of codominance, our CIs represent conservative estimates (see Materials and Methods) and we find a significant deviation from codominance using a goodness-of-fit G test (Table 2). Also, the heterozygote SDP found by Nanney (1959) is significantly different from that established in the present study (G = 30.9; P value = 2.542e-05). mat-2 is an example of B-type alleles, which have been found to be partially dominant over another A-type allele in various genetic backgrounds (Table 3; Arslanyolu and Doerder 2000). Also, the expression probability of only a few sexes appears to vary substantially from the codominance expectation, in both the earlier (Nanney 1959) and our data sets. For example, in the mat-2/ mat-3 heterozygotes analyzed by Nanney (1959), sex I is underrepresented and sex VI is overrepresented compared with that expected under codominance (Table 2 and Figure 1C). This indicates that the interaction between the mat alleles varies for different sexes and depends on the genetic background (Arslanyolu and Doerder 2000). A mechanism contributing to dominance may not affect all sexes monotonically making the expression probability of a sex adjustable independently of the other sexes. The structure of the mat locus, that is, sex-specific segments arranged in a tandem (Cervantes et al. 2013), may allow for independent control of sex expression because the different sexes specified by an individual mat allele may be regulated independently.

either allele. Given that recombination is not suppressed between the wild strains (Lynch et al. 1995), our results verify that SDPs are indeed reproducible in different genetic backgrounds. Previous studies have indicated that modifier loci may affect SDPs (Arslanyolu and Doerder 2000). Such loci do not appear to affect the SDPs of homozygotes (mat-2/ mat-2; mat-3/ mat-3) measured here. Given the close relationship between the strains used here and the previously studied wild isolates (Nanney 1959), it is however possible that any modifier loci contain the same alleles across strains or that modifier loci are linked to the mat locus and did not undergo recombination.

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Because SD mechanisms control inheritance of sex and in turn the sex ratio, selection on sex ratio is an important factor in evolution of SD mechanisms (Bull 1983). The empirically estimated SDPs and the degree of dominance are important parameters for predicting equilibrium allele frequencies and sex ratios in populations. Using our population genetic model (Paixão et al. 2011) and assuming codominance, we predicted the equilibrium in populations containing the alleles mat-2 and mat-3 at the relative frequency of mat-2 = 0.67 and a skewed sex ratio. Variable dominance interactions dependent on genetic background will influence mating dynamics among the self-incompatible sexes by generating variation in SDPs. The varying strength of dominance relationships among alleles may change the equilibrium sex ratio and allele frequencies, as well as the evolutionary trajectory for the approach to equilibrium. These predictions are empirically testable using populations initiated away from the equilibrium at the respective sex ratios.

The ability of a single allele (or its homozygote) to specify many sexes with reproducible probabilities signifies stable DNA rearrangements during SD by the homoygous genotype. In contrast, differences in the SDP of a heterozygote likely reflect epistatic effects of the genetic background including the genetic factors responsible for an assembly of a sex-specific gene segment in the somatic nucleus. Sequence variation in different mat alleles may provide the basis for dominance relationships such that sex-specific segments of the alleles in a heterozygote may compete for binding sites of the regulatory elements responsible for the programmed site-specific deletion and joining events. Environmental factors may further influence this competition generating variation in SDPs of a heterozygote. To understand how such a locus may have evolved, it will be necessary to investigate the reproducibility of SDPs in more species with stochastic SD. Homologues of a few sex-specific gene segments were found in 4 other species of Tetrahymena, two of which have stochastic SD (Phadke and Zufall 2009; Cervantes et al. 2013). The relative importance of the environment and genetics in SD may vary among these species.

SD in most microbial eukaryotes with multiple, self-incompatible sexes occurs via sex-specific alleles. For example, Dictyostelium discoideum has 3 sexes, each specified by a different sex-specific allele (Bloomfield 2011). Such sex-specific alleles are also found in most algae (Ferris and Goodenough 1994; Peng et al. 2011) and fungi, such as the yeasts of the Saccharomyces sensu stricto group (Casselton 2002). In contrast to these, probabilistic mat alleles in T. thermophila provide a mixed SD strategy, which may give an evolutionary advantage over sex-specific alleles observed in other microbial eukaryotes (Paixão et al. 2011). Our finding of variable dominance effects dependent on genetic background further extends the amount of phenotypic variation that a mat allele can produce, providing an additional potential evolutionary advantage of this SD system.

### Table 3
Dominance between A-type and B-type mat alleles

<table>
<thead>
<tr>
<th>Heterozygous genotype (B-type/A-type)</th>
<th>Degree of dominance (CI)a</th>
<th>Source of SDPs</th>
</tr>
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<tr>
<td>mat-2/mat-3</td>
<td>0.55 (0.46, 0.64)</td>
<td>Nanney (1959)</td>
</tr>
<tr>
<td>mat-2/mat-3</td>
<td>0.33 (0.29, 0.37)</td>
<td>Replicate 1, this study</td>
</tr>
<tr>
<td>AR4-7/AR6-5</td>
<td>0.66 (0.58, 0.74)</td>
<td>Arslanyolu and Doerder (2000)</td>
</tr>
<tr>
<td>AR5-4/AR6-5</td>
<td>0.83 (0.77, 0.89)</td>
<td>Arslanyolu and Doerder (2000)</td>
</tr>
<tr>
<td>AR7-19/AR6-5</td>
<td>0.81 (0.75, 0.87)</td>
<td>Arslanyolu and Doerder (2000)</td>
</tr>
</tbody>
</table>

a Degree of dominance is calculated as described in Materials and Methods and shown for the B-type alleles, mat-2, AR4-7, AR5-4, and AR7-19. 95% CIs are shown in parentheses.

### Funding

### Acknowledgments
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