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ORIGINAL ARTICLE

Mutational Robustness of Morphological Traits in the Ciliate *Tetrahymena thermophila*

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ABSTRACT

Ciliate nuclear architecture, in particular the sequestration of a transcriptionally silent germline genome, allows for the accumulation of mutations that are "hidden" from selection during many rounds of asexual reproduction. After sexual conjugation, these mutations are expressed, potentially resulting in highly variable phenotypes. Morphological traits are widely used in ciliate taxonomy, however, the extent to which the values of these traits are robust to change in the face of mutation remains largely unknown. In this study, we examine the effects of mutations accumulated in the germline genome to test the mutational robustness of four traits commonly used in ciliate morphological taxonomy (number of somatic kineties, number of postoral kineties, macronuclear size, and cell size). We found that the number of postoral kineties is robust to mutation, confirming that it should be preferentially used in taxonomy. By contrast, we found that, as in other unicellular and multicellular species, cell and macronucleus sizes change in response to mutation. Thus, we argue that cell and macronucleus sizes, which are widely used in taxonomy, should be treated cautiously for species identification. Finally, we found evidence of correlations between cell and macronucleus sizes and fitness, suggesting possible mutational pleiotropy. This study demonstrates the importance of, and methods for, determining mutational robustness to guide morphological taxonomy in ciliates.

MUTATIONS can result in changes in a variety of life history or morphological traits. For example, spontaneous mutations can decrease longevity, survival rate, and body size in *C. elegans* (Azevedo et al. 2002; Keightley and Ohnishi 1998; Vassilieva and Lynch 1999). The extent to which a trait remains unchanged in the face of mutational input is its mutational robustness (reviewed in Wilke and Adami 2003). In addition, change in a morphological trait may also be coupled with changes in fitness, e.g. in some microbes, larger individuals have shorter generation times (Molenaar et al. 2009; Mongold and Lenski 1996). Neither mutational robustness, nor associations between morphological and fitness traits have previously been explored in ciliates.

A distinguishing feature of ciliates is the presence of two distinct types of nuclei in each cell: the micronucleus, which has germline functions and is transcriptionally silent during vegetative growth, and the transcriptionally active somatic macronucleus (Prescott 1994). Since the micronuclear genome is not transcribed during asexual growth, any mutations that occur in this genome are functionally neutral. Only after conjugation, when those mutations get expressed from the newly formed macronucleus, will they affect the phenotype and thus be exposed to selection. Therefore, if there are long periods of asexual growth prior to conjugation, we expect that after conjugation, many previously "hidden" mutations will be expressed, resulting in diverse phenotypes, including morphological and fitness associated traits (Long et al. 2013).

Mutations affecting morphological traits are especially important for taxonomy in ciliates, which is historically mostly based on morphology (Lynn 2008). Many new morphological species are being reported every year, due to the wide application of silver staining methods and improved microscopy, e.g. Heber et al. (2014), Jiang et al. (2013). Morphological traits, like the number of postoral kineties (No. PK, ciliary rows posterior to the oral region, also called postoral meridians, POM) (Fig. 1), which is used as a species distinguishing trait in the genus *Tetrahymena* (Corliss 1973), have never been tested with evolutionary genetic techniques to determine whether they are robust to environmental or genetic influences to provide precise morphological species identification. Resolving such uncertainty in morphological traits will contribute to our understanding of the true protist species diversity, which is under debate among ecologists and taxonomists

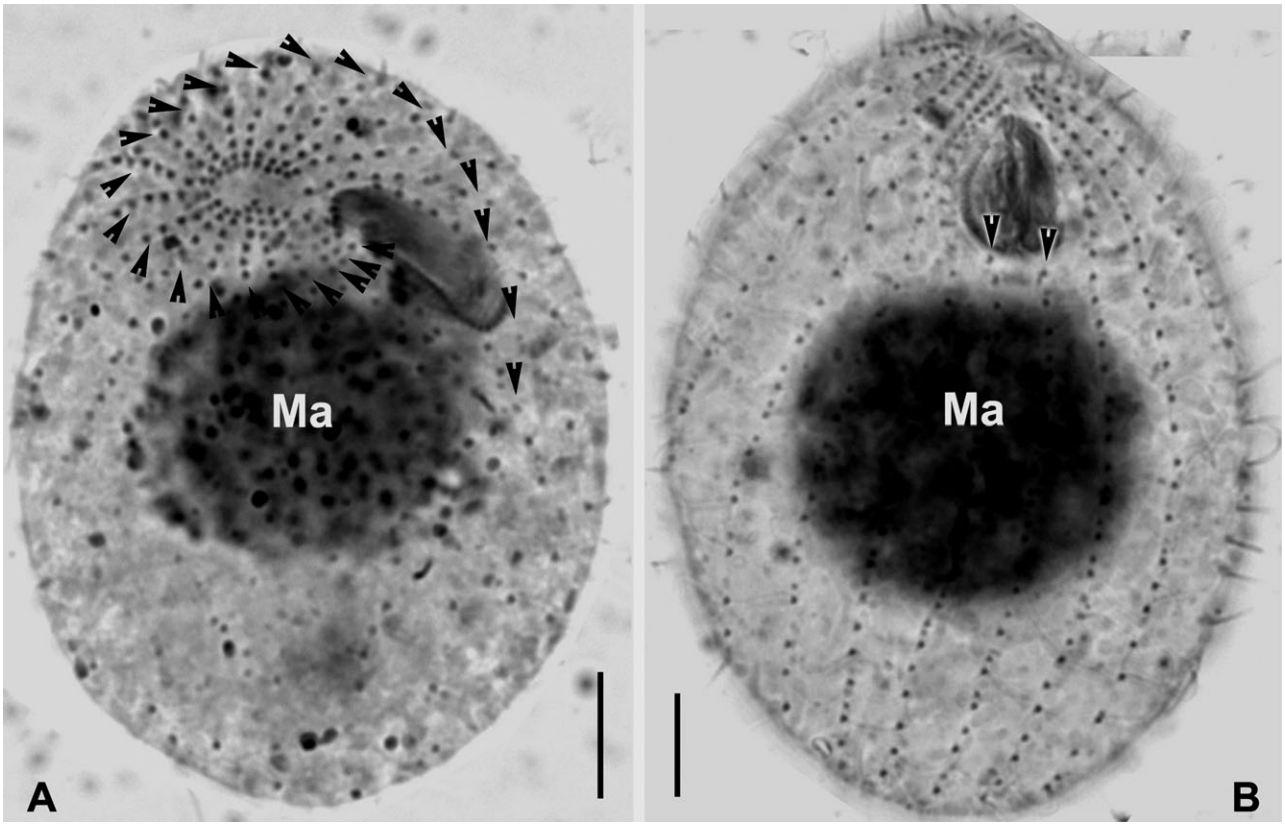

Figure 1 Protargol-stained *T. thermophila* cells (A from the GE line 4-0-A, B from GE line 51-1000-A). Arrowheads in A show all the somatic kineties (SK, including the two postoral kineties) of the cell, arrowheads in B show the postoral kineties (PK). The dark regions in the center in both pictures are the macronucleus (Ma). Both scale bars are 4.5 μm.

(Caron 2009; Fenchel and Finlay 2004; Foissner et al. 2008; Weisse and Rammer 2006).

In this study, we examined four morphological traits that are widely used in ciliate taxonomy and morphometrics studies (Fig. 1) (Agatha and Tsai 2008; Fan et al. 2011; Foissner and Stoeck 2011; Jung et al. 2012; McManus et al. 2010; Tokiwa et al. 2010; Tsai et al. 2010) and estimated fitness based on maximum population growth rate. Cell lines were grown for 1,000 generations of asexual reproduction, during which spontaneous mutations accumulated in the micronucleus but were not expressed (Long et al. 2013). We then assessed the morphological and fitness effects of these mutations after conjugation, which results in expression of the micronuclear mutations in progeny cells (Prescott 1994). This allowed us to test whether these four morphological traits were likely to change in response to spontaneous mutations over a relatively short evolutionary time and thus are suitable for precise morphological taxonomy.

MATERIALS AND METHODS

Cell lines and media

T. thermophila strains SB210 and B*VII were acquired from the *Tetrahymena* Stock Center (Cornell University). SB210 is a commonly used lab strain. It carries mating type VI, and its macronuclear genome is published (Eisen et al. 2006). B*VII has a dysfunctional germline nucleus and carries mating type VII.

SSP medium was used during mutation accumulation (MA): 2% proteose peptone (EMD Chemicals, New York, NY), 0.2% glucose, 0.1% yeast extract (BD, Sparks, MA), and 0.003% Fe-EDTA (Acros Organics, Morris Plains, NJ) (Gorovsky et al. 1975). Tris buffer (10 mM Tris-HCl pH 7.5) was used to starve cells in preparation for conjugation (Bruns and Brussard 1974). 2% proteose peptone was used for mating pair refeeding during conjugation (Bruns and Cassidy-Hanley 1999).

Mutation accumulation and genomic exclusion lines

The mutation accumulation protocol is described in Long et al. (2013). Briefly, SB210 (the ancestor) was subcultured in multiple replicate lines and maintained under asexual reproduction with frequent bottlenecking for 1,000 generations. Nine MA lines were studied: three replicates of the ancestral line and six lines that had evolved independently for 1,000 generations. Genomic exclusion (GE) cross procedures were performed according to Bruns and Cassidy-Hanley (1999) to express mutations that had accumulated in the micronuclear genome. GE involves conjuga-

tion with the mating partner B*VII with a dysfunctional micronucleus and, after two rounds of crosses, results in genetically identical progeny that are homozygous in both micro- and macronuclear genomes for ~1/2 of all mutations that have accumulated in the micronuclear genome during MA. 12 GE lines derived from the MA lines were studied: six GE lines from the ancestor and one GE line for each of the six evolved MA lines. All MA and GE lines were from the experiment described in Long et al. (2013) and were stored in liquid nitrogen. Cell lines included in this study were chosen to evenly sample all of the MA lines, with respect to fitness of the corresponding GE lines. The fitness metric used in this study is the maximum exponential growth rate, estimated as in Long et al. (2013).

Morphological traits

All morphological traits and fitness are shown in detail in Table S1. Cell lines were thawed from liquid nitrogen following Bruns et al. (1999): cryovials from a liquid nitrogen tank were quickly transferred to a 42 °C water bath; thawed cells were then transferred to 4 ml SSP medium supplemented with 1X Penicillin, Streptomycin, and Amphotericin B (PSA; final concentration; Amresco, Solon, OH) in wells of a 6-well plate, prewarmed in a 30 °C incubator. While this medium provides a standardized basis for experimentation, it should be noted that results may differ under different growth conditions, e.g. bacterized peptone. For each line, the plate with thawed cells was incubated in the 30 °C incubator for 48 h; then, cells were centrifuged in a 50 ml conical tube. The supernatant in the conical tube was removed and cells were resuspended with 12.5 ml Tris buffer and transferred to a clean 125 ml flask and starved for 24 h to synchronize growth. Starved cells were then transferred to a 6-well plate and photomicrographs were taken on an Olympus inverted microscope. Cell (60–95 cells/line) and macronucleus (49–133 cells/line) length and width were acquired from the photomicrographs analyzed in Image J (1.46r, Schneider et al. 2012). Cell and macronucleus sizes were then calculated based on the length and width measurements, by assuming a prolate spheroid-shape of the cell, using the formula from Hellung-Larsen and Anderson (1989):

$$V = (\pi/6) \times L \times W^2$$

Where V is the macronuclear or cellular volume (size), L is the macronuclear or cellular length, and W is the macronuclear or cellular width.

Another 100 μl of starved cells from each flask were stained with protargol (Wilbert 1975): cells were fixed with Bouins' fluid and saturated $HgCl_2$ mixture (volume ratio 1:1) for 1 min; then fixatives were removed and fixed cells were rinsed with DI water at least six times. After this, cells were bleached with sodium hypochlorite solution (NaClO) 1:600 diluted from the stock solution (CAS Number 7681-52-9, Sigma-Aldrich Co. LLC, St. Louis, MO); this procedure was finished within 2 min to avoid over-bleaching. Bleached cells were then immerged with 1% protargol solution (protargol S™ from Polysciences Inc., Warrington, PA, Cat No.: 01070) in an embryo dish and heated at 60 °C for 1 h before silver developing and fixing. Numbers of somatic kineties (10–40 cells investigated) and postoral kineties (10–41 cells investigated) of protargol stained specimens were determined using a compound Olympus microscope.

Each morphological trait value was standardized by dividing by the corresponding mean value of that trait in the ancestor.

Plotting and statistical analyses

Plots, linear regression, and statistical tests (*t*-tests and correlation tests) were all done in R (3.1.0) (R Development Core Team 2014).

RESULTS AND DISCUSSION

We tested whether four morphological traits commonly used in ciliate taxonomy are robust to mutations. Mutations that occurred in the macronucleus were continually exposed to selection, but mutations that occurred in the micronucleus were not expressed and therefore hidden from selection throughout the course of the experiment. This is likely similar to what happens in natural populations, where lineages reproduce asexually for many generations prior to undergoing sexual conjugation (Doerder et al. 1995).

We found no significant change in any of the four morphological traits, nor in fitness, due to mutations in the macronucleus (Ma; Fig. 2A; Table 1). This is likely due to the selective elimination of deleterious mutations during the course of the experiment. This result suggests that any of the morphological traits that are not robust to mutations (see below), may nonetheless experience directional or stabilizing selection in our growth environment. In nature, cell size may also be under selection due to the avoidance of size-selective predators (e.g. copepods, amoebae), which are extremely abundant in the freshwater habitats of *T. thermophila* and known to induce morphological change in other ciliates (Kusch 1993), however the natural predators of *T. thermophila* remain unknown.

By contrast, mutations that accumulated in the micronucleus throughout the course of the experiment and were expressed via genomic exclusion (GE) did result in changes in three traits (Fig. 2B; Table 1). Macronuclear size, cell size, and fitness of GE lines all decreased from those of the ancestral GE lines (decrease of 26%, 23%, and 26% respectively) (Fig. 2B). Cell size and macronuclear size in the evolved GE lines showed a significant drop from those of the ancestral GE lines (Table 1; Student *t*-test, $p < 0.05$). The drop in fitness between ancestral and evolved GE lines was marginally significant, however, this was likely due to the small sample size, since we previously demonstrated a significant decrease based on a larger sample size of $N = 19$ GE lines (including the samples in this study; Long et al. 2013). No signifi-

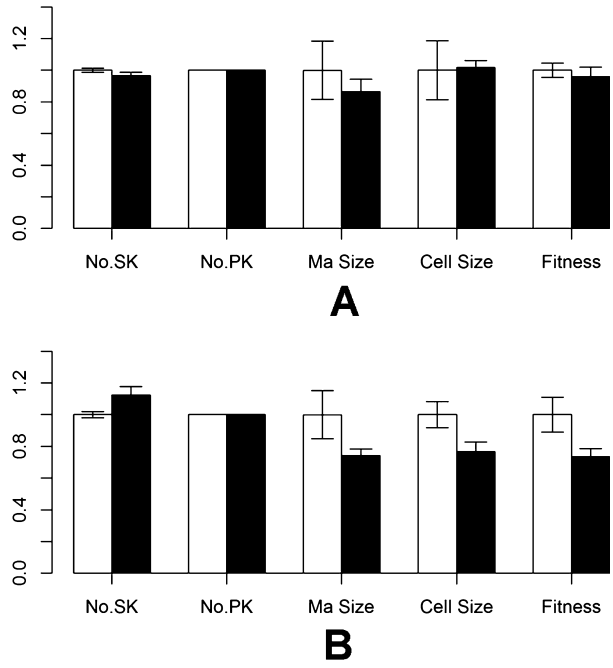

Figure 2 Morphological traits and fitness from ancestor (white bars) and evolved (black bars) MA lines (**A**) and GE lines (**B**). Note that each trait or fitness was standardized by dividing the observed values with the ancestral mean. Error bars are standard errors.

Table 1. *p*-values from two-sided Student *t*-tests comparing the ancestral to the evolved cell lines

Cell lines	No. SK	No. PK	Ma size	Cell size	Fitness
MA	0.21	N/A	0.55	0.94	0.60
GE	0.28	N/A	**0.01**	**0.008**	0.08

GE, genomic exclusion lines with germline mutations expressed; MA, mutation accumulation lines without germline mutations expressed; No. SK, number of somatic kineties, No. PK, number of postoral kineties, Ma, macronuclear. Significant *p*-values are in bold.

cant change was detected for the number of somatic kineties of the evolved GE lines (Fig. 2B; Table 1), nor for the number of postoral kineties, which remained consistently two in all lines. These results indicate that while some morphological traits are robust to mutations (number of postoral and somatic kineties), other traits (macronucleus and cell size) do vary in response to mutation.

We also find that several of these traits are correlated with one another. Cell size and macronuclear size are positively correlated (Pearson product moment correlation test, $r = 0.71$, $p < 0.01$), and both of these traits are positively correlated with fitness (Fig. 3, lower two plots). These results are suggestive of mutational pleiotropy, i.e. that the same mutations in one line affect multiple traits, although our experimental design does not allow us to explicitly test this hypothesis. Such positive correlation between growth rate and cell size appears to be widespread (Mongold and Lenski 1996; Stearns 1992). Mon-

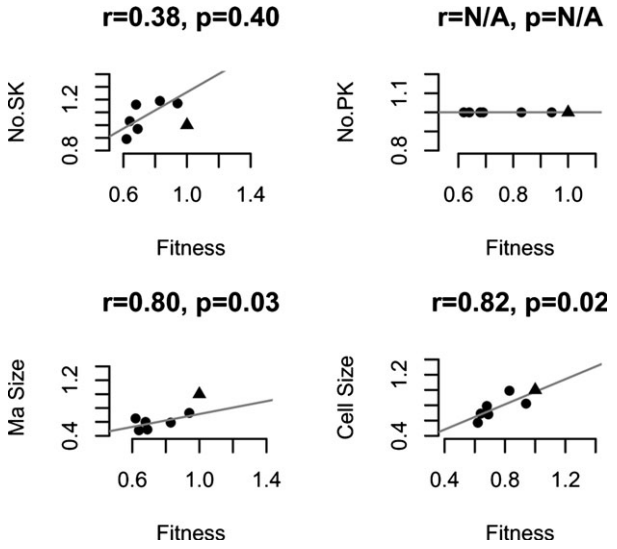

Figure 3 Linear regressions between morphological traits and fitness of GE lines. Triangles show the ancestor and black rounded dots show the evolved lines. No. SK: number of somatic kineties; No. PK: number of postoral kineties.

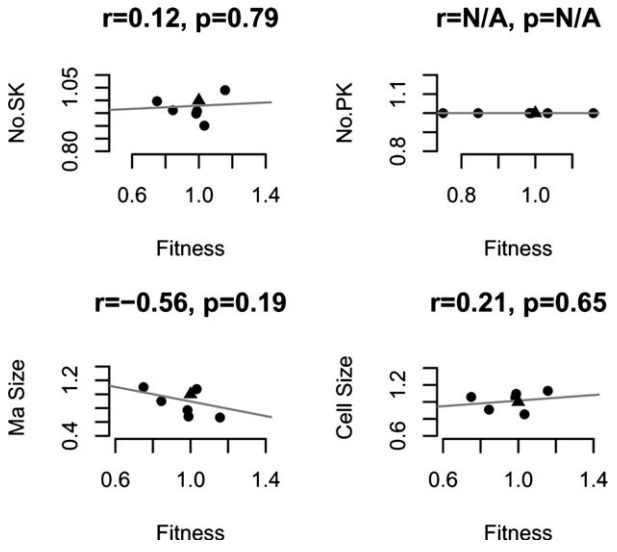

Figure 4 Linear regressions between morphological traits and fitness of MA lines. Triangle shows the ancestor and black rounded dots show the evolved lines. No. SK: number of somatic kineties; No. PK: number of postoral kineties.

gold and Lenski (1996) propose that larger cells have more reserves for growth and that this is the cause of the correlation between growth rate and cell size. By contrast, neither number of somatic kineties nor postoral kineties, either in the MA or GE lines, show a significant correlation with fitness (Fig. 3 and 4).

Number of postoral kineties and cell size are two of the most widely recorded traits in ciliate morphological taxon-

Table 2. Three morphological traits of 10 *Tetrahymena* species

Species	L × W	No. SK	No. PK	References
T. thermophila	50 × 20	18–21	2	Nanney and Simon (1999)
T. thermophila[a]	37.5 × 18.4	15–24	2	This study
T. thermophila[b]	34.8 × 16.6	16–23	2	This study
T. setifera	40 × 25	22–26	1–3	Corliss (1973)
T. chironomi	40 × 23	23–28	2	Corliss (1973)
T. rostrata[c]	20–80[e]	27–35	1–4	Corliss (1973)
T. limacis[d]	40–45[e]	24–32	1–4	Corliss (1973)
T. corlissi	47 × 31	25–31	2	Corliss (1973)
T. stegomyiae	60–100[e]	25–30	2	Corliss (1973)
T. patula[f]	45 × 28	32–41	3–5	Corliss (1973)
T. vorax[f]	31–115[e]	18–23	2	Corliss (1973)
T. paravorax[f]	70–90[e]	22–30	2	Corliss (1973)

[a]Ancestral strain after GE.
[b]Evolved strain after GE.
[c]Trophont life stage data.
[d]Free-living phase data.
[e]Cell length.
[f]Microstome life stage data.
Cell length and width (L × W) in μm. No. SK = number of somatic kineties, No. PK = number of postoral kineties.

omy. Number of postoral kineties has limited variability across *Tetrahymena* species, and as we show here, is highly robust to mutations (Table 2). By contrast, cell size varies among *Tetrahymena* species, consistent with our finding that it varies widely in response to mutations (Table 2). This is worthwhile to note, given that *Tetrahymena* may experience long periods of asexual reproduction, followed by sexual conjugation, which would expose the mutations accumulated in the micronuclear genome during asexual reproduction. Differences in morphology after conjugation thus have the potential to cause confusion in morphological species identification. Cell size is often considered in determining species identity in *Tetrahymena*, as in most other ciliate genera (Table 2), however, our results indicate that cell size should be treated cautiously. Morphological traits, like the number of postoral kineties, which are robust to mutations, should be weighted more heavily in species identification. Fortunately, morphological taxonomists have long noticed the evolutionary stability of such traits and have consequently given them more weight in species identification. For example, the 3–5 (mostly 4) postoral kineties is the species-unique trait of *Tetrahymena patula* (Table 2).

Previous studies have suggested that additional morphological traits may also be subject to change in response to mutation, e.g. number of adoral membranelles (Kaczanowski 1975) and cell shape (Doerder et al. 1975). Thus, we suggest that the mutational robustness of more quantitative morphological traits as well as morphological variance from more populations for a single species should be studied. In addition, this study has been conducted under constant conditions, however, many morphological traits are also likely to vary in response to differences in both the environment and cell cycle stage (e.g. Nanney (1967, 1970), Nanney and Doerder (1972), Doerder and DeBault (1978)). Thus, future work would also benefit from additional analysis of morphological trait plasticity. Such studies would provide guidance for current ciliate morphological taxonomy.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Morphological traits (before standardization) and fitness of the cell lines in this study**.** Cell line names containing two hyphens indicate GE lines, with one hyphen refer to MA lines, 0 and 1000 after the first hyphen indicate generation numbers. No. SK: number of somatic kineties; No. PK: number of postoral kineties; Ma: macronuclear size, both cell and macronuclear sizes are in μm^3; Cell: cell size; Sd: standard deviation; n: number of specimens observed, n*: the number of replicates in the fitness assay.