

A seven-sex species recognizes self and non-self mating-type via a novel protein complex

Reviewed Preprint

Published from the original preprint after peer review and assessment by eLife.

About eLife's process

Reviewed preprint version 1

January 8, 2024 (this version)

Posted to preprint server

November 11, 2023

Sent for peer review

November 8, 2023

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Abstract

Although most species have two sexes, multisexual species (i.e., those with multiple mating types) are also widespread. However, it is unclear how mating-type recognition is achieved at the molecular level in multisexual species. The unicellular ciliate *Tetrahymena thermophila* has seven mating types, which are determined by the MTA and MTB proteins. In this study, we found that both proteins are essential for cells to send or receive complete mating-type information, and transmission of the mating-type signal requires both proteins to be expressed in the same cell. We found that MTA and MTB form a mating-type recognition complex that localizes to the plasma membrane, but not to the cilia. Stimulation experiments showed that the mating-type-specific regions of MTA and MTB mediate both self- and non-self-recognition, indicating that *T. thermophila* uses a dual approach to achieve mating-type recognition. Our results suggest that MTA and MTB form an elaborate multifunctional protein complex that can identify cells of both self and non-self mating types in order to inhibit or activate mating, respectively.

Impact statement

A giant multifunctional protein complex mediates mating-type recognition through a non-ligand-receptor mechanism in a multisexual species.

eLife assessment

This **fundamental** study provides insight into the fascinating process of self- and non-self-recognition in the protist *Tetrahymena thermophila*, a species with seven distinct mating types. Using an elegant combination of phenotypic assays, protein studies, and imaging, the authors present **convincing** evidence that a large multifunctional protein complex at the cell surface mediates both self- and non-self mating-type recognition. This study extends our understanding of how more than two mating types/sexes may be specified in a species, and it will be relevant for anyone interested in sexual systems and cell-cell communication.

Introduction

Sexual reproduction is almost universal among eukaryotic organisms. Mating type (or sex) is a key regulatory feature of gamete fusion. Most species have only two mating types (e.g., male and female, + and -, or α and α) and species usually use either self- or non-self-recognition mechanism to achieve mating-type recognition (Goodenough & Heitman, 2014 [↗](#)). However, species in some lineages, such as some ciliates and basidiomycetes (Heitman, 2015 [↗](#); Phadke & Zufall, 2010 [↗](#)), possess multiple mating types, and multiple-alleles self-incompatibility system were observed in some plants, such as Brassicaceae (Iwano & Takayama, 2012 [↗](#); Takayama & Isogai, 2005 [↗](#); Vekemans & Castric, 2021 [↗](#)). This raises the interesting question of how mating types are recognized at the molecular level in multiple mating-type systems.

The model unicellular ciliate, *Tetrahymena thermophila*, has seven mating types (I–VII). Under starvation conditions, any cell of one mating type can mate with a cell of any of the other six mating types, but not with one of the same mating type (**Figure 1A** [↗](#), **Figure 1-figure supplement 1** [↗](#), Video 1–3) (Cervantes et al., 2013 [↗](#); Nanney, 1953 [↗](#); Orias et al., 2017 [↗](#); Yan et al., 2021 [↗](#)). Mating-type recognition in *Tetrahymena* depends on direct cell–cell contact, which suggests that mating-type proteins localize to the cell surface. However, there is no direct evidence to indicate whether they are ciliary proteins or not. When one cell comes into contact with a cell of a different mating type, a mating-type-dependent recognition event enables both cells to enter a pre-conjugation stage (called costimulation) (Bruns & Palestine, 1975 [↗](#); Finley & Bruns, 1980 [↗](#)). Even when cells of different mating types are mixed in unequal ratios (for example, 9 : 1), all cells become fully stimulated (Bruns & Palestine, 1975 [↗](#)). Processes that take place during costimulation include Tip transformation (Wolfe & Grimes, 1979 [↗](#)) and concanavalin A (Con-A) receptor appearance (**Figure 1B** [↗](#)) (Wolfe & Feng, 1988 [↗](#); Wolfe et al., 1986 [↗](#)). In preparation for pairing, costimulated cells of the same and different mating type(s) adhere to form very loose pairs. Heterotypic cell pairs form a stable conjugation junction, whereas homotypic pairs separate very quickly (Video 2 and 3) (Kitamura et al., 1986 [↗](#)).

The mating-type system of *T. thermophila* was described by Nanney and collaborators in the early 1950s (reviewed in (Orias, 1981 [↗](#); Orias et al., 2017 [↗](#))). We previously showed that mating type is determined by a pair of mating-type genes that are organized in head-to-head orientation: *MTA* and *MTB* (**Figure 1C** [↗](#)) (Cervantes et al., 2013 [↗](#)). Each gene has a terminal exon that encodes five transmembrane (TM) helices and a cysteine-rich growth factor receptor (GFR) domain. The region between the two terminal exons of the gene pair encodes the N-terminal mating-type-specific extracellular regions. Based on the mating-type-specific regions, the mating-type genes are called *MTA1-MTB1* for Mating type I, *MTA2-MTB2* for Mating type II and so on. We previously showed that Δ *MTB* cells do not form pairs or produce progeny and that Δ *MTA* cells retain mating-type specificity but pair extremely poorly and rarely produced progeny (Cervantes et al., 2013 [↗](#)); we

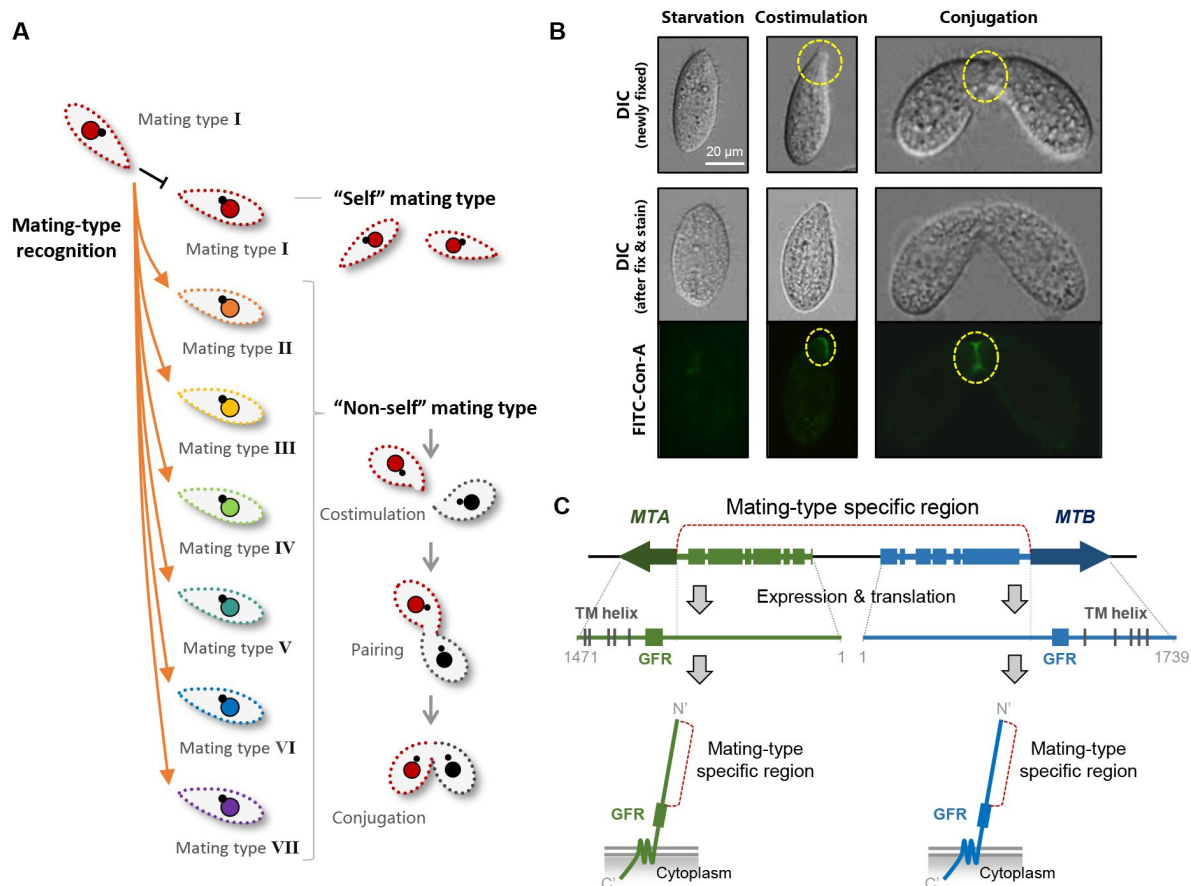


Figure 1.

Mating-type recognition in *T. thermophila*.

(A) Example of self and non-self mating-type recognition. When one cell of mating type I encounters another, costimulation and mating do not occur. When a cell of mating type I encounters a cell of another mating type (II-VII), the cells enter the costimulation stage and go on to form a pair. (B) Two typical phenotypes of the costimulation stage are Tip transformation and Con-A receptor appearance. Yellow dashed circle, transformed cell tip (center, single cell) or pairing junction (right, cell pair). Note that Tip transformation may become less obvious after Con-A staining. (C) *MTA* and *MTB* gene structure and *MTA* and *MTB* protein domain information (Cervantes et al., 2013). *MTA* and *MTB* form a head-to-head gene pair. For each gene, the terminal exon is shared by all mating types and the remainder is mating-type-specific (the sequence differs for each mating type). The mating-type-specific region of each protein is predicted to be extracellular.

concluded that the two genes are non-redundant and both are essential for mating. However, challenges such as multiple mating types; the high molecular weight, membrane localization and extremely low expression levels of mating-type proteins; and difficulty in genetically manipulating the mating-type gene locus have so far prevented elucidation of the mode of action of the MTA and MTB proteins and of whether they mediate self-or non-self mating-type recognition.

In this study, we provide direct evidence that the MTA and MTB form an elaborate multifunctional protein complex that can identify cells of both self and non-self mating types to inhibit or activate mating, respectively.

Results

Mating-type recognition cannot be explained by a simple receptor–ligand model

Receptor–ligand interactions are a critical mechanism for intercellular communication that may regulate mating-type recognition in *T. thermophila*, irrespective of whether self-or non-self-recognition mechanisms are employed. Therefore, we first assessed whether mating-type recognition conforms to a straightforward receptor–ligand model (in which one mating-type protein acts as the receptor and the other as the ligand) and whether self or non-self is recognized (**Figure 2–figure supplement 1A**, [1](#) and [2](#)). For this, we determined whether deletion of each mating-type gene affected the transmission and detection of mating signals to and from wild-type (WT) cells (**Figure 2A** shows the experimental procedure) by assessing the ability of cells to undergo costimulation (the prerequisite for mating). Our experiment allows us to test if cells missing one of the two mating-type proteins can still costimulate WT cells (**Figure 2–figure supplement 1A**, [3](#) and [6](#)).

In *T. thermophila*, cells normally enter into the fully costimulation stage within ~30 min after mixing starved WT cells of two different mating types, and start pair formation during the next ~30 min (**Figure 2B**, black line). Cells that have already been costimulated immediately start forming pairs with other costimulated cells of a different mating type (**Figure 2B**, red line).

To our surprise, the rate of pair formation in WT cells pre-incubated with either Δ MTA cells (**Figure 2B**, green line) or Δ MTB cells (**Figure 2B**, blue line) did not increase (i.e., costimulation did not occur). These results were not changed by extending the pre-incubation time (**Figure 2–figure supplement 1B**). Therefore, both MTA and MTB proteins are essential for the mating-type signal; there is no simple receptor–ligand relationship.

In addition, WT cells were not costimulated even when they were simultaneously incubated with both Δ MTA and Δ MTB cells (**Figure 2B**, teal line), although, according to the receptor–ligand model, they should have received “MTA stimulation” from Δ MTB cells and “MTB stimulation” from Δ MTA cells. This result indicates that the absence of a mating-type protein in one cell cannot be complemented by its presence in another cell in the same culture; the MTA and MTB proteins must be in the same cell to transmit the mating-type signal. This finding suggests that mating-type recognition cannot be explained by a simple receptor–ligand model. It is possible that the MTA and MTB proteins form a complex which either serves as a recognizer (functioning as both ligand and receptor) or a co-receptor (which influences ligand–receptor activity). But, since MTA and MTB are the only genes with mating-type-specificity, it is unlikely that the complex is acting as a co-receptor.

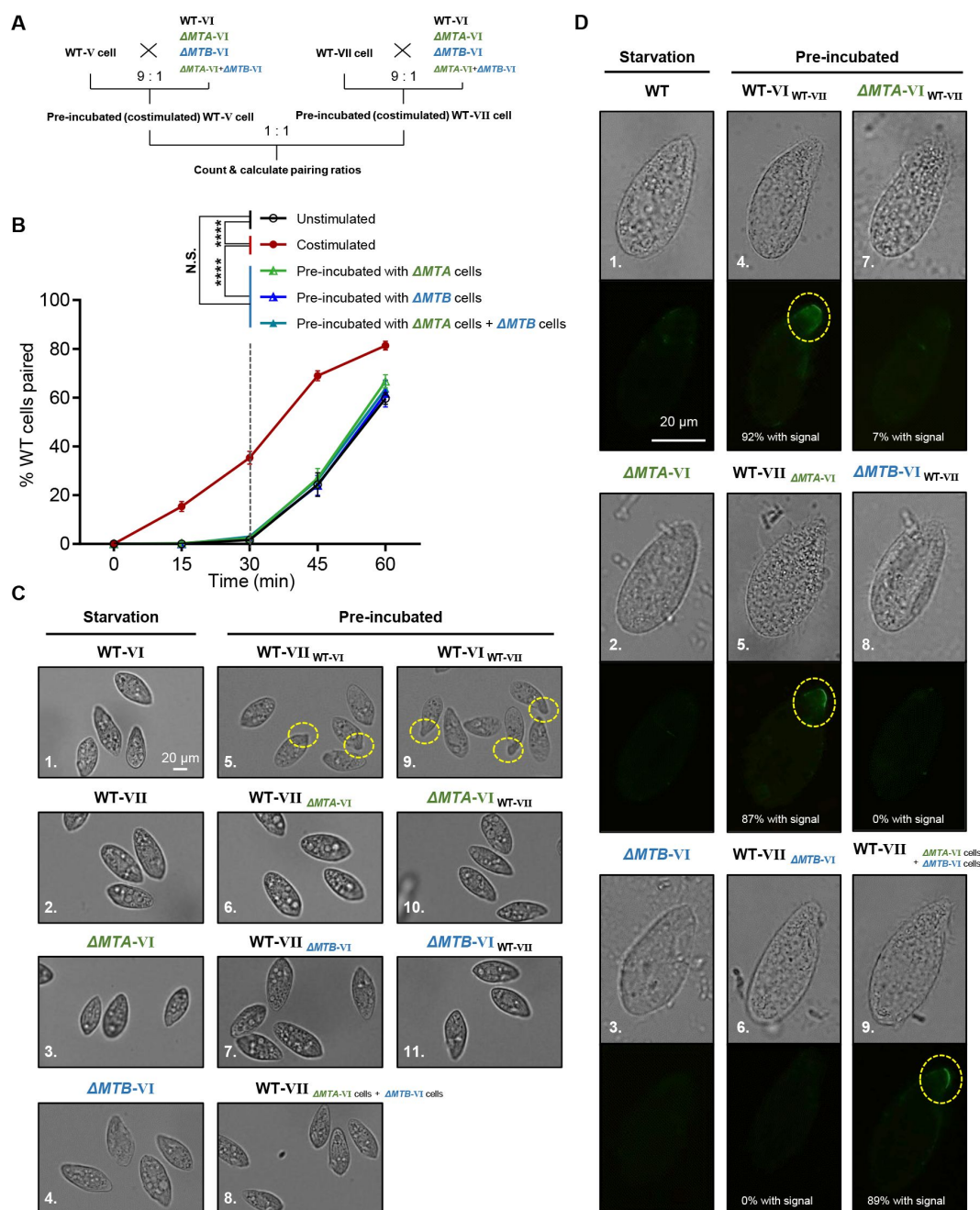


Figure 2.

Mating-type proteins are essential for mating-type recognition.

(A) Experimental procedure for the costimulation experiments. Starved WT cells of mating types V (WT-V) and VII (WT-VII) were separately pre-incubated with the indicated mating type VI mutant (9 : 1 ratio) for 30 min and then the pre-incubated cells were mixed at a 1 : 1 ratio. Note that before mixing the costimulated cells, any potentially pairing cells were separated by shaking. (B) Effect of pre-incubation with ΔMTA and ΔMTB on the rate of pair formation. Each experiment was repeated three times, with > 100 pairs counted at each time point. Matched two-way ANOVA was used for the statistical analysis. N.S., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Unpaired mutants were excluded when calculating the pairing rate (see Materials and Methods). (C) Tip transformation, a hallmark of costimulation. Costimulated cells from two different WT strains are shown because their tips have slightly different shapes. Each strain was pre-incubated with the strain shown in subscript. Yellow dashed circle, transformed cell tip. (D) Appearance of Con-A receptors, another hallmark of costimulation. In all, ~90% cells show Con-A receptor fluorescence (panels 4, 5 and 9). The low percentage of cells (7%) with fluorescence in panel 7 were probably WT cells, which comprised 10% of the pre-incubation culture. Each strain was pre-incubated with the strain shown in subscript. Yellow dashed circle, Con-A receptor fluorescence.

Mating-type proteins differentially regulate two steps of costimulation

During costimulation, cells undergo a sequence of developmental events that remodel the anterior cell membrane and its associated cytoskeleton (Cole, 2013). Two hallmarks of this process are Tip transformation (in which the anterior tip of the cell becomes curved) and Con-A receptor appearance (receptors bound by the plant lectin concanavalin A, which binds to mannose containing glycoproteins). When WT cells of one mating type were mixed with WT cells of another mating type, the cell tips became transformed (Figure 2C, 5 and 9) and Con-A receptors appeared in almost all cells (Figure 2D, 4). When WT cells were pre-incubated with Δ MTA, Δ MTB, or both cell types, Tip transformation was not observed in any cell (Figure 2C, 6–8). Similarly, when cells of each mutant were pre-incubated with WT cells, Tip transformation was not detected (Figure 2C, 10 and 11). The outcome was slightly different for Con-A receptor appearance. Con-A receptors were not observed in WT cells pre-incubated with Δ MTB cells (Figure 2D, 6) or in cells of either mutant pre-incubated with WT cells (Figure 2D, 7 and 8). In contrast, when WT cells were exposed to Δ MTA or “ Δ MTA cells plus Δ MTB cells”, the Con-A receptor was detected (Figure 2D, 5 and 9); this is consistent with Δ MTA cells retaining a very weak ability to pair (Cervantes et al., 2013). These results indicate that neither Δ MTA and Δ MTB cells can fully stimulate WT cells or be stimulated by WT cells. They also demonstrate that costimulation can be separated into two stages: (i) the appearance of Con-A receptors, which requires the expression of MTB only in partner cells, and (ii) morphological transformation of the cell tip, which requires both MTA and MTB.

Mating-type proteins form a complex with several coexpressed proteins

The inability of “ Δ MTA cells plus Δ MTB cells” to costimulate WT cells of a different mating type indicates that MTA and MTB proteins can only function when expressed on the same cell (Figure 2B, teal; Figure 2C, 8). We previously found that CDK19 and CYC9 proteins are also essential for mating. Δ CDK19 and Δ CYC9 cells cannot mate with other cells, but they still express MTA and MTB proteins (Ma et al., 2020). In WT cells, the pairing rate was increased following pre-incubation with Δ CDK19 or Δ CYC9 cells of a different mating type (Ma et al., 2020). These results further support the idea that MTA and MTB must be expressed on the same cell to be fully functional, leading us to hypothesize that MTA and MTB proteins form a mating-type recognition complex (MTRC).

To test whether MTA and MTB proteins physically interact, an HA-tag coding sequence was ligated to the 3' end of the MTA gene (Figure 3A); cellular proteins were co-precipitated with HA-tagged MTA and analyzed by immunoprecipitation-coupled mass spectrometry (IP-MS). As expected, the MTB protein co-purified with MTA (Figure 3B, C), as did another set of proteins, which we named MRC1–MRC6 (Figure 3B, C and Figure 3–Source data 1 and 2). Next, we produced strains expressing either HA-tagged MTB or MRC1 (Figure 3A), and found that each protein pulled down a subset of the proteins that co-purified with MTA (Figure 3B, C). Unfortunately, these pull-down experiments were not as successful as the MTA IPs, perhaps because of the higher molecular weight of MTB and MRC1. Taken together, our results suggest that MTA, MTB, and MRC1–MRC6 form the MTRC. Alternatively, MTA and MTB may interact with subsets of MRC proteins to form smaller complexes or alternative MTRCs. Different protein interactors were identified in extracts from cells at different mating stages. This may reflect conformational changes in the MTRC but the huge molecular weight of the complex and extremely low expression levels of its proteins make this possibility difficult to investigate.

Mating-type-related factors in other species are often relatively small (Iwano & Takayama, 2012; Kahmann & Bölker, 1996), but those of *T. thermophila* are large proteins (predicted size, 92–212 kDa). Figure 3D shows the predicted domains of the MRC1–MRC6 proteins. Like MTA and MTB,

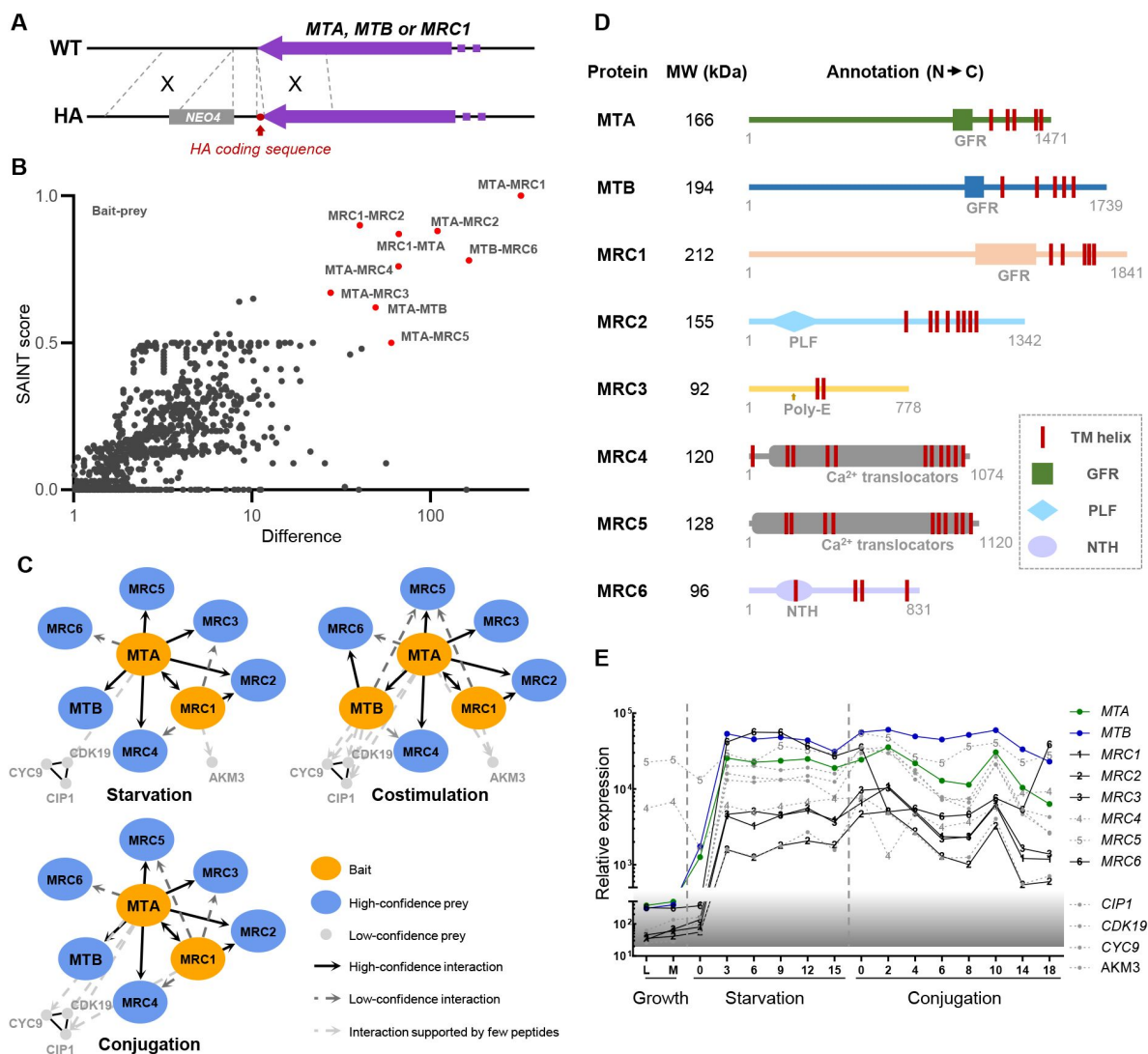


Figure 3.

Proteins that interact with MTA and MTB.

(A) Construction of HA-tagged strains. All of the tagged strains mated like WT cells. (B) Statistical analysis of IP-MS data. A total of 13 experiments were carried out. WT samples (untagged) were run in parallel for each sample. All 13 WT controls were combined as the background control. Red dot, high-confidence interaction; dark gray dot, low-confidence interaction. Gene identifiers are summarized in [Figure 3](#)–Source data 2. Note that the wash buffer contained 1% Triton X-100 and 600 mM NaCl. (C) Interaction network based on IP-MS data. Orange oval, bait; blue oval, high-confidence prey; light gray dot, low-confidence prey; black line, high-confidence interaction; dark gray dashed line, low-confidence interaction; light gray dotted line, interaction supported by a few peptides (these proteins were shown because their coding genes are coexpressed with *MTA* and *MTB* and deleting them affects mating behavior). (D) Diagram of functional domain annotation of MTRC components. GFR, growth factor receptor domain; PLF, pectin lyase fold; Poly-E, poly-glutamic acid region; NTH, P-loop-containing nucleotide triphosphate hydrolase. (E) Expression profiles of genes whose protein products were identified by IP-MS as potentially components of the MTRC. Expression data is derived from *TetraFGD* ([Xiong et al., 2011](#)).

MRC1 has five predicted TM helices and a GFR domain. MRC2 has eight TM helices and a pectin lyase-fold domain, suggesting a possible role in carbohydrate chain modification. MRC3 has two TM helices in the central region and an adjacent ~35 amino acid (aa) poly-E region. MRC4 and MRC5 (previously named TPA9 (Wang et al., 1997 [↗](#); Wang & Takeyasu, 1997 [↗](#))) are both P-type ATPases that are likely to function as calcium-translocators. MRC6 has four TM helices and a P-loop containing nucleoside triphosphate hydrolases. Most of the MRC genes are highly coexpressed with *MTA* and *MTB* (Figure 3E [↗](#)). Examination of the whole genome sequences of strains with mating types II–VII (Wang et al., 2020 [↗](#)), confirmed that only the sequences of *MTA* and *MTB* genes are mating-type-specific.

In addition to the MRC proteins, CDK19, CYC9, CIP1, and AKM3 were identified in IP-MS experiments, but with relatively few supporting peptides (Figure 3C [↗](#)). Our previous work showed that CDK19, CYC9, and CIP1 are components of a cyclin-dependent kinase complex. These genes are coexpressed with *MTA* and *MTB* (Figure 3E [↗](#)) and are important for mating, and the encoded proteins localize to the cell tip and pairing junction (Ma et al., 2020 [↗](#)). AKM3 is predicted to be a K⁺ channel of unknown biological function. It is coexpressed with *MTA* and *MTB* (Figure 3E [↗](#)), and we found that the *AKM3* deletion strain cannot pair. Therefore, these four proteins are also likely to interact with the mating-type proteins (perhaps indirectly and/or via weak interactions) and might be involved in downstream signaling following mating-type recognition.

Mating-type proteins localize to the cell surface but not to the cilia

We used the *MTA7*-HA strain to determine the localization of mating-type proteins. Cell fractionation (Figure 4-figure supplement 1 [↗](#)) revealed that *MTA7*-HA is a membrane protein (Figure 4A [↗](#)). Biotinylation and isolation of cell surface proteins confirmed that *MTA7*-HA is exposed on the cell surface (Figure 4B [↗](#)). To investigate whether *MTA7*-HA localizes to the cilia membrane, we isolated and collected cilia (Figure 4C [↗](#)) and then analyzed cilia protein extracts by IP-coupled western blotting (WB) and MS. MS analysis identified typical ciliary proteins, such as inner and outer arm dynein proteins (Figure 4 [↗](#)–Source data 1). However, both IP-WB (Figure 4D [↗](#)) and MS (Figure 4 [↗](#)–Source data 1) consistently failed to identify *MTA7*-HA protein in the cilia fraction. These results conclusively indicate that *MTA7*-HA localizes to the cell surface, but not to cilia. Unfortunately, we failed to detect *MTA7*-HA by immunofluorescence staining of cells at any mating stage (starvation, costimulation, or conjugation), probably due to the epitope masking and extremely low expression level.

To further examine localization of the mating-type proteins, eGFP-tagged *MTB2* was overexpressed from an exogenous locus (Figure 4E [↗](#)). This strain (which has a mating type VI background) mated normally with WT cells of all mating types except for VI and II. This result indicates the overexpressed *MTB2*-eGFP protein is fully functional for mating. Interestingly, cells of this strain can also mate with one another (self-mating); a similar phenotype was previously reported for strains expressing multiple mating-type proteins (Lin & Yao, 2020 [↗](#)). The overexpressed *MTB2*-eGFP protein was detected on the cell surface in a linear pattern radiating from the cell tip to the cell body along the ciliary rows (Figure 4F [↗](#), costimulated cell; Figure 4G [↗](#), mating pair), although signals are also apparent between ciliary rows. Co-staining with a tubulin dye showed that the *MTB2*-eGFP protein is adjacent to, rather than co-localizing with, the base of cilia (Figure 4F [↗](#), G, enlarged). Confocal images from the interior of the cell and through the cilia showed that *MTB2*-eGFP localizes to the cell surface (and also to intracellular structures, probably the endoplasmic reticulum (ER) and Golgi), but not to the cilia (Figure 4-figure supplement 2 [↗](#)), confirming our results with the *MTA7*-HA protein (Figure 4A–D [↗](#)). MS analysis showed that overexpressed *MTB2*-eGFP protein was not present in isolated cilia of the *MTB2*-eGFP strain (Figure 4–Source data 2 [↗](#)). Therefore, mating-type proteins localize to the cell surface, as might be expected since mating-type recognition depends on cell–cell contact.

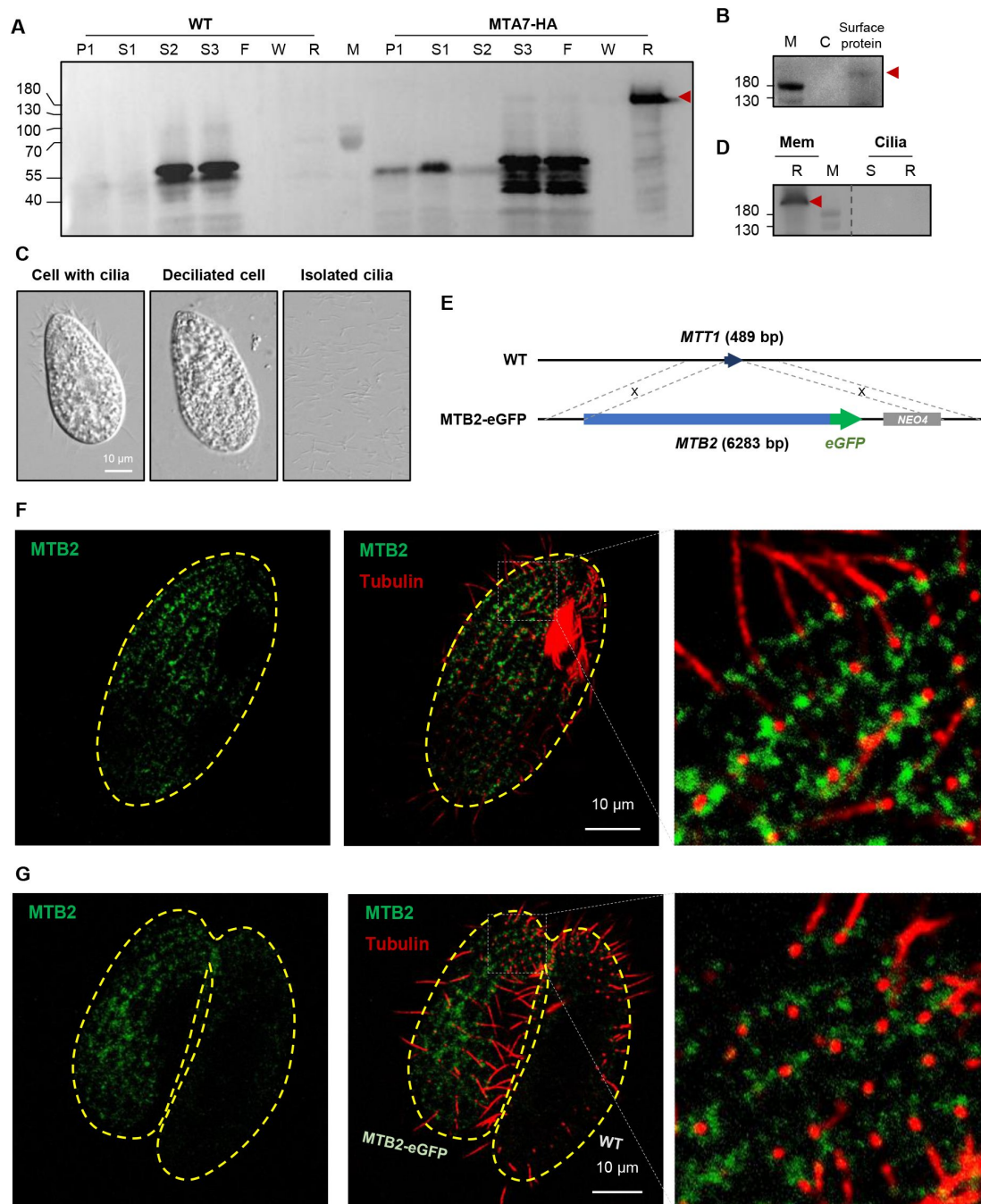


Figure 4.

Mating-type proteins are cell surface proteins but do not localize to cilia.

(A) Fractionation of *MTA7-HA* cells (please see [Figure 4-figure supplement 1A](#) for the experimental process). Red arrowhead, *MTA7-HA*; F, flow through; P, pellet; R, resin; S, supernatant; W, wash. The MTA signal is undetectable until S3 (enriched membrane proteins), and only appears after affinity chromatography (R). (B) WB analysis of cell surface proteins. Red arrowhead, *MTA7-HA*; M, marker; C, negative control (unbiotinylated). (C) Cilia isolation and purification. (D) WB analysis of IP products of membrane and ciliary proteins. Mem, membrane; R, resin; S, supernatant. The same amount of *MTA7-HA* cells was used for the membrane and ciliary protein IPs. The full blot is shown in [Figure 4-figure supplement 1B](#). (E) Construction scheme for eGFP-tagged MTB2 strains. (F) Costimulated MTB2-eGFP cell. (G) Paired MTB2-eGFP cell. To induce MTB2-eGFP overexpression, cells were treated with 10 ng/ml Cd^{2+} for 5 h. Green, eGFP signal; red, tubulin signal; yellow dashed line, cell outline. The focal plane of these images is the cell surface.

Mating-type proteins influence non-self-recognition

The mating-type-specific region of the *MTA* and *MTB* gene pair is the only known genetic locus with mating-type specificity; therefore, we next tested whether this region influences self-and/or non-self mating-type recognition. For this, we expressed the extracellular region of *MTA* (MTAxc) or *MTB* (MTBxc) in an insect cell secreted expression system, purified the recombinant proteins (**Figure 5–figure supplement 1** [↗](#)), and then tested their effect on mating behavior.

First, we tested whether MTAxc and MTBxc can influence mating in cells with a different mating-type specificity (i.e., non-self-recognition). When WT cells were incubated with MTAxc (and/or MTBxc) of a different mating type, markers of costimulation were not observed (**Figure 5–figure supplement 2** [↗](#)). Surprisingly, treated cells had a significantly increased pairing rate. Compared with controls (**Figure 5A–C** [↗](#), black), WT cells (VI and VII) pre-treated with MTAxc or MTBxc of different mating types (VII and VI, respectively) had an increased pairing rate (**Figure 5A–C** [↗](#), green or blue), with a stronger effect after pre-treatment with both MTAxc and MTBxc (**Figure 5A–C** [↗](#), teal). Dose–effect assays showed that pairing rates increased with increasing MTA6xc and MTB6xc concentrations between 3 pg/ml and 30 pg/ml, with the effect becoming saturated or weaker at higher concentrations (**Figure 6A–F** [↗](#)). MTAxc and MTBxc also stimulated mating for all other WT mating types (**Figure 6G** [↗](#), H), indicating that this is a general effect. Based on these results, we conclude that the mating-type-specific regions of *MTA* and *MTB* proteins are involved in non-self mating-type recognition.

Mating-type proteins also influence self-recognition

We used similar methods to examine whether pre-treatment with a cognate mating-type-specific region (i.e., self-recognition) affects mating. Treatment of WT cells (VI and VII) with MTAxc and/or MTBxc of the same mating type decreased the pairing rate (**Figure 5D** [↗](#), E, green or blue). No obvious difference was found between treatments with either MTAxc or MTBxc. Meanwhile, a significant synergistic effect was observed (**Figure 5D** [↗](#), E, teal). For all treatments (single or combined), the pairing rate was similar by 4 h (reaching >80%; **Figure 5D** [↗](#), F), indicating that the initial inhibitory effect on pairing was eventually overcome. Negative regulation by MTAxc and MTBxc was also observed for other mating-type combinations (**Figure 6I** [↗](#), J). These results support the idea that the mating-type-specific regions of *MTA* and *MTB* proteins mediate both non-self (between cells of different mating types) and self (between cells of same mating type) recognition.

Discussion

Although the basic biological features of the *T. thermophila* mating-type system were discovered over half a century ago, the mechanism for mating-type recognition remains unclear. Here, we identified a novel MTRC that contains *MTA*, *MTB*, and several other proteins and provide evidence that *MTA* and *MTB* mediate both self and non-self mating-type recognition.

In most species, mating signals are associated with relatively small proteins or molecules (Iwano & Takayama, 2012 [↗](#); Kahmann & Bölker, 1996 [↗](#)). However, in *T. thermophila*, the MTRC is likely to be over a million Daltons in size. This large size is consistent with its multiple functions: An elaborate MTRC is needed to inhibit mating between cells of the same mating type while also activating mating between cells of different mating types. This is likely to be a highly complex process, given that there are seven mating types in all. Further structural studies of this protein complex may reveal the specific self- and non-self-recognition mechanisms.

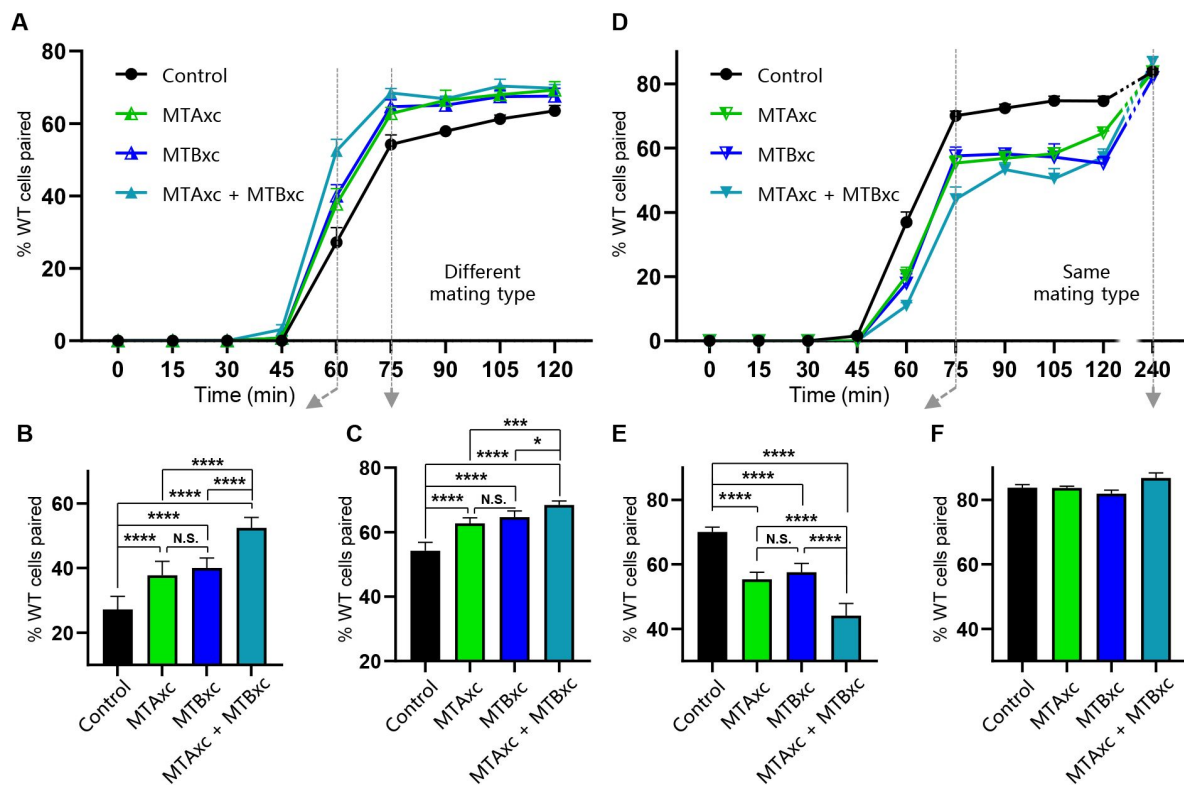


Figure 5.

Stimulation experiments using MTAXc and/or MTBxc.

(A) WT cells were treated with MTAXc and/or MTBxc proteins (30 pg/ml, 1 h) of different mating-type specificities. WT-VI cells were treated with MTA/B7xc protein, and WT-VII cells were treated with MTA/B6xc protein. Treated cells were washed twice before mixing to remove residual proteins from the starvation medium. Note that the starvation medium used for washing had been previously used for cell starvation because *T. thermophila* cells secrete mating-essential factors during starvation (Adair et al., 1978). Each experiment was repeated five times. (B, C) The percentages of cells paired at 60 min (B) and 75 min (C) were used for the statistical analysis (method described in Figure 2B). (D) WT cells (mating types VI and VII) were treated with MTAXc and/or MTBxc proteins of the same mating-type specificity (as described in (A)). (E, F) The percentages of cells paired at 75 min (E) and 240 min (F) were used for the statistical analysis (method described in Figure 2B).

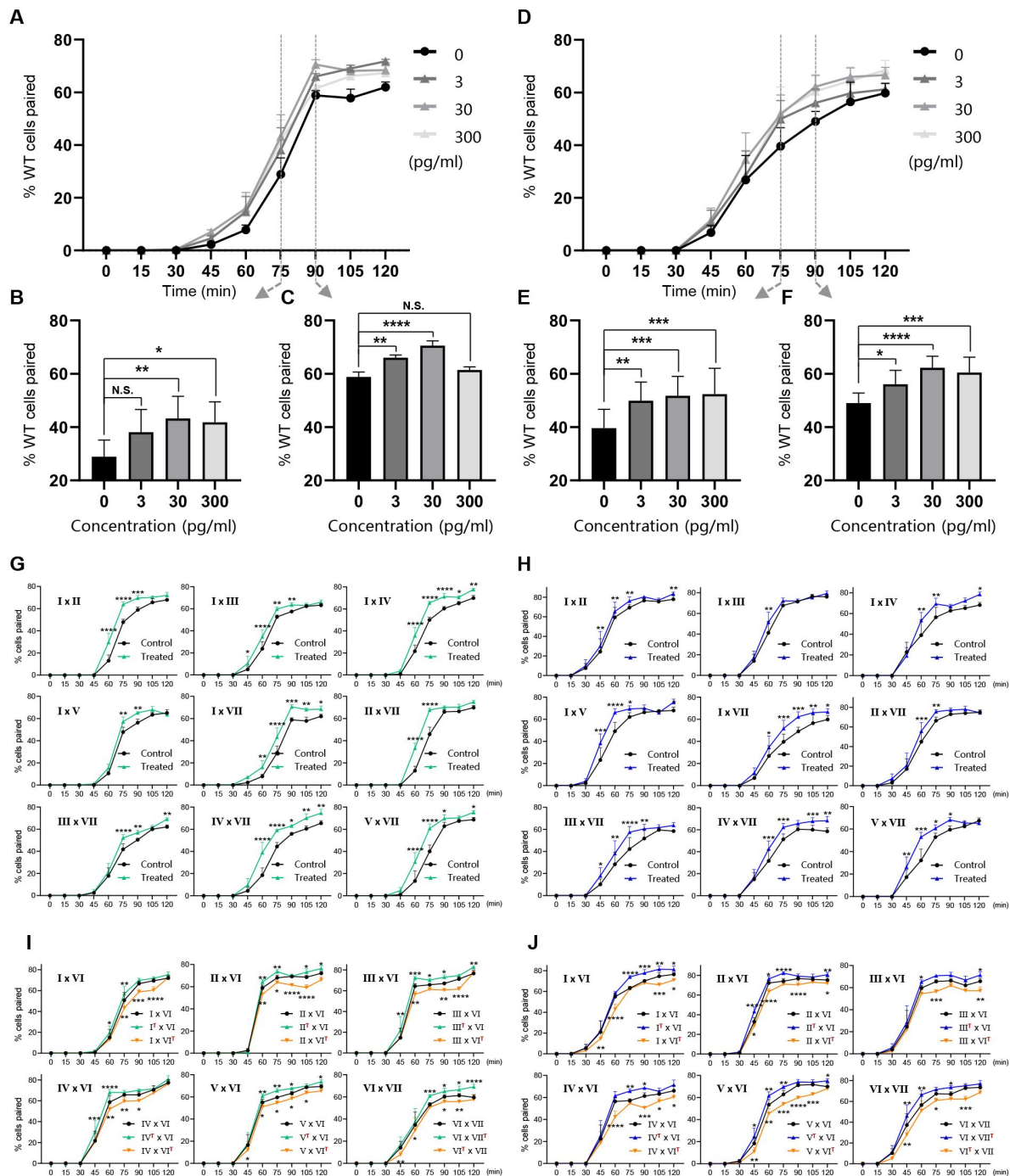


Figure 6.

Results of treatment with either MTA6xc or MTB6xc proteins.

(A-F) Dose-response effect of treatment with MTA6xc or MTB6xc protein. (A-C) MTA6xc results. (D-F) MTB6xc results. Cells of mating types I and VII were used for these experiments. Experimental and statistical methods were as described for [Figure 5](#), except for protein concentrations. (G-J) MTA6xc or MTB6xc proteins affect the mating of various combinations of other WT mating types. (G) MTA6xc results. Both mating partners were treated. (H) MTB6xc results. Both mating partners were treated. (I) MTA6xc results. Cells of only one mating type were treated. Note that mating type VI cells were used in these experiments. (J) MTB6xc results. Note that mating type VI cells were used in these experiments. Experimental and statistical methods were as described for [Figure 5](#). Red superscript "T" in (I) and (J) indicate the strain treated with MTA6xc or MTB6xc. The mating types used in each experiment is shown in the top-left corner.

An open question is why *T. thermophila* should use such a dual approach to achieving mating-type recognition. Recent research on basidiomycetes and flowering plants (other species with multiple mating types) has shown that their mating-type recognition (or self-incompatibility) mechanisms involve either self- or non-self-recognition (Fraser & Heitman, 2003 [↗](#); Iwano & Takayama, 2012 [↗](#); Vekemans & Castric, 2021 [↗](#)). Our previous evolutionary study showed that the length of the mating-type-specific region differs significantly among different *Tetrahymena* species (Yan et al., 2021 [↗](#)). Based on the massive difference in length, it is reasonable to speculate that different species might use different mechanisms for mating-type recognition. Therefore, dramatic evolution of the mating-type recognition mechanism seems to have occurred relatively soon after the emergence of the *Tetrahymena* genus. Further detailed functional and evolutionary studies may reveal how and why this recognition mechanism evolved and how its evolution contributed to speciation.

We still do not know what intracellular signals are transduced when the MTRCs on two cells interact. Mating-type self-recognition might generate an inhibitory signal or might simply inactivate the MTRC. In many species, such as *Papaver rhoeas* and *Ciona intestinalis*, interaction between mating-type proteins of the same mating type induces changes in cytoplasmic Ca^{2+} concentration that cause self-incompatibility (Giamarchi et al., 2006 [↗](#); Harada et al., 2008 [↗](#); Wu et al., 2011 [↗](#)). A similar system may exist in *T. thermophila*, since the MRC4 and MRC5 proteins are predicted to be Ca^{2+} -translocators. Interaction between MTRCs on cells of different mating types (non-self-recognition) should result in their activation to allow the cells to initiate pairing. We propose that the activation signal involves the CDK19 complex (a cyclin-dependent kinase complex) (Ma et al., 2020 [↗](#)) and AKM3 (a K^+ channel; **Figure 3C** [↗](#)) because they probably interact with the MTRC. We expect future studies to lead to the discovery of more detailed mechanisms for mating-type recognition and initiation of conjugation involving these proteins.

Paramecium tetraurelia, a closely related Oligohymenophorean ciliate, has only two mating types, which are determined by the expression or non-expression of a *Tetrahymena*-MTA/B-like protein called mtA (Singh et al., 2014 [↗](#); Yan et al., 2021 [↗](#)). An intriguing question is whether mtA also engages in the formation of a MTRC with other proteins by serving as a recognizer rather than mediating a straightforward receptor–ligand interaction. Future investigations of *Paramecium tetraurelia* may shed light on the origins and evolutionary aspects of this distinctive mating system. Moreover, due to the extremely long evolutionary distance, recognition mechanisms discovered in model species fall short of explaining many of the intricate biological events in protists. Insight into the detailed function of MTRC could contribute to our understanding of cell–cell recognition processes in other species, such as *Toxoplasma* and *Plasmodium*.

Materials and Methods

Biological methods

Strains used in this study are summarized in Appendix 1–table 1. All cell growth, starvation, and pairing experiments were conducted at 30°C. Cells were grown in Super Proteose Peptone (SPP) medium (1% Proteose Peptone, 0.1% yeast extract, 0.2% glucose, 0.003% Sequestrene) or Neff medium (0.25% Proteose Peptone, 0.25% yeast extract, 0.5% glucose, 0.003% Sequestrene). Cells were starved in 10 mM Tris-Cl (pH 7.4) for ~16 h before all pairing experiments. For normal pairing assays, equal numbers of starved cells of different mating types (at $\sim 2 \times 10^6$ cells/ml) were mixed. To obtain costimulated (pre-incubated) cells, two starved strains were mixed at a 9 : 1 ratio for ~30 min (unless otherwise stated). Before mixing costimulated cells, any potentially pairing cells were separated by shaking. **Figure 2A** [↗](#) shows the setup of costimulation experiments. For

all mating experiments (whether or not they involved mutant cells), the starting WT cell density was $\sim 2 \times 10^6$ cells/ml. The following formula was used to calculate pairing ratios and correct for the presence of mutant cells:

$$\% \text{cells paired} = \frac{2 \times \# \text{ pairs}}{(2 \times \# \text{ pairs} + \# \text{ unpaired cells}) \times \% \text{ WT cells}} \times 100$$

Somatic gene deletion, truncation, and protein tagging

To construct deletion strains, a ~ 1 kb fragment upstream of the gene's open reading frame (ORF) (#1), a ~ 0.5 kb fragment downstream of the gene's ORF (#2), and a ~ 1 kb fragment downstream of #2 (#3) were amplified. Fragments #2 and #3 were joined to the *NEO4* cassette (Cd²⁺-inducible *MTT1* promoter linked to the neomycin resistance gene) by fusion PCR and then cloned into the pBlueScript SK (+) vector together with fragment #1. In this way, #1-#2-*NEO4*-#3 constructs were obtained for the next transformation. HA-tagged strains were constructed in a similar way, except that fragment #1 was upstream of the stop codon or upstream of the terminal intron. To obtain the MTB2-eGFP construct, MTB2-coding sequences replaced the MTT1-coding sequence (Figure 4E) and the construct was made using the large DNA fragment assembly method (Jiang et al., 2022). Constructs were introduced into starved WT cells by biolistic transformation to obtain deletion strains (Mochizuki, 2008). Positive clones were selected in SPP medium containing decreasing Cd²⁺ concentrations (from 1 $\mu\text{g/ml}$ to 0.05 $\mu\text{g/ml}$) and increasing paromomycin concentrations (from 120 $\mu\text{g/ml}$ to 40 mg/ml) until all WT somatic chromosomes had been replaced by mutant ones, as determined by PCR using checking primers. All primers used are listed in Appendix 1–table 2.

Immunoprecipitation and mass spectrometry

The IP method was adapted from a published method (Tian et al., 2017). To pull down HA-tagged proteins from *T. thermophila*, cells were harvested from 500 ml cultures (density $\sim 3 \times 10^6$ cells/ml). Cells were then treated for 20 min with paraformaldehyde (PFA) (at a final concentration of 0.3%) to stabilize protein–protein interactions, washed with PB buffer (2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM K₂HPO₄), and blocked with 125 mM glycine. Cells were then resuspended in lysis buffer (1% Triton X-100, 30 mM Tris-HCl, 20 mM KCl, 2 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 150 mM NaCl, cOmplete proteinase inhibitor [Roche Diagnostics, Indianapolis, IN, USA]), lysed by ultrasonic treatment and incubated with EZview anti-HA agarose beads (Sigma-Aldrich, St Louis, MO, USA) for 2.5 h at 4°C. The beads were washed with wash buffer (1% Triton X-100, 600 mM NaCl, 30 mM Tris-HCl, 20 mM KCl, 2 mM MgCl₂, cOmplete proteinase inhibitor) to remove nonspecific-binding proteins and then HA-tagged proteins were eluted with HA peptides (Sigma-Aldrich). WT samples (not HA tagged) were run in parallel for each sample. In total, data for 13 WT controls were combined to identify non-specific binding proteins.

For MS, the EASY-nLC chromatography system (Thermo Scientific, Rockford, IL, USA) was coupled on-line to an Orbitrap Elite instrument (Thermo Scientific) via a Nanospray Flex Ion Source (Thermo Scientific). Xtract software (Thermo Scientific) and Proteome Discoverer 2.1 software were used for MS data analysis based on a database that combines the 2014 version of whole genome protein annotation (<http://ciliate.org/index.php/home/downloads>), which contains the whole length sequence of MTA6 and MTB6) and mating-type-specific regions of all other mating-type proteins. IP data were analyzed using CRAPome (Mellacheruvu et al., 2013).

Membrane protein extraction

Figure 4–figure supplement 1A shows the workflow used for membrane protein extraction. Cells were collected, resuspended in 20 ml lysis buffer (150 mM NaCl, 25 mM HEPES, 10% glycerol, 2 mM PMSF, 2.6 $\mu\text{g/ml}$ aprotinin, 1.4 $\mu\text{g/ml}$ pepstatin, 10 $\mu\text{g/ml}$ leupeptin, pH 7.4), and lysed by high-pressure homogenization. The lysate was clarified first at low speed (14,000 rpm, 4°C, 15 min), and then at high speed (150,000 g, 4°C, 1 h). The pellet was resuspended in 5 ml lysis buffer containing

1% DDM (Anatrace, Maumee, OH, USA) and rotated for 2 h at 4°C to extract the membrane proteins. Undissolved material was removed by centrifugation (14,000 rpm, 4°C, 30 min). The membrane protein extract was incubated with EZview anti-HA agarose beads for 2.5 h at 4°C and then washed with 5 ml lysis buffer.

Biotinylation and isolation of cell surface proteins

Pierce Cell Surface Protein Biotinylation and Isolation Kit (Thermo Scientific) was used to biotinylate and isolate cell surface proteins. For this, 75 ml cells (density $\sim 3 \times 10^6$ cells/ml) were harvested and washed once with BupH phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , 1.4 mM KH_2PO_4). Cells were then resuspended in 75 ml PBS containing 0.72 mg/ml Sulfo-NHS-SS-biotin and incubated at room temperature for 10 min. After two washes with 50 ml ice-cold BupH Tris buffer, cells were resuspended in 3 ml lysis buffer (PBS containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and cOmplete proteinase inhibitor [Roche Diagnostics]), lysed by ultrasonic treatment and incubated with 1.2 ml NeutrAvidin Agarose for 0.5 h at room temperature. The resin was washed four times with 0.5 ml wash buffer and then cell surface proteins were eluted with 1.2 ml elution buffer (with 10 mM DTT). Before WB, cell surface protein samples were concentrated into 0.1 ml volumes using a 30 kDa centrifugal concentrator (Merck Millipore).

Ciliary protein collection

To remove cilia, 500 ml cells (density $\sim 3 \times 10^6$ cells/ml) were harvested at room temperature and resuspended in 25 ml 10 mM Tris-Cl (pH 7.4), to which 50 ml medium A (10 mM EDTA_2Na , 50 mM sodium acetate, pH 6.0) was added. After 30 s, 25 ml cold distilled water was added; 1 min later, 0.25 ml 0.4 M CaCl_2 was added and incubated for 15 s. The cilia were detached from the calcium-shocked cells by vortexing three times for 5 s at 15 s intervals. To collect the cilia, cell bodies were removed by two rounds of centrifugation at 1500 rpm for 5 min at 4°C, and then cilia were collected by centrifugation at 15000 rpm for 15 min at 4°C.

Cytological methods

For tubulin staining, cells were collected and fixed in PHEM buffer (30 mM PIPES, 14 mM HEPES, 5 mM EGTA and 2 mM MgSO_4) containing 1% PFA and incubated for 30 min at 4°C. After three washes with PBS (10 min each), Tubulin-Atto 594 was added and incubated for 1 h at 25°C. Finally, cells were washed three times with PBS (10 min each). Fluorescein-labeled Con-A labeling was performed as previously reported (Ma et al., 2020 [DOI](#)). In brief, cells were fixed and stained with fluorescein-labeled Con-A (Vector Laboratories, Burlingame, CA, USA) at 37.5 $\mu\text{g/ml}$ for 5 min and then washed three times with PB. For analysis of Tip transformation, cells were observed and photographed as soon as possible after fixation with 1% PFA. To distinguish between cell strains in a pairing mixture, starved cells of one strain were labeled with 500 nM MitoTracker (Invitrogen, Eugene, OR, USA), followed by two washes with 10 mM Tris-Cl (pH 7.4) before mixing.

Expression and purification of the extracellular region of mating-type proteins

Coding sequences of the extracellular region of mating-type proteins (MTA6xc, MTB6xc, MTA7xc, and MTB7xc) were codon-optimized and synthesized for expression in an insect-cell system (*Trichoplusia ni* Hi5 cells). Codon-optimized sequences were cloned into pFastBac vectors containing an N-terminal hemolin signal peptide sequence and a C-terminal 10 $\times$ His tag sequence. The obtained constructs were transformed into competent DH10Bac cells and individual bacmids were transfected into *Spodoptera frugiperda* Sf9 cells. Recombinant Baculoviruses were collected after 4 days and used to infect *Trichoplusia ni* Hi5 cells for protein expression. Proteins were

harvested 60 h after infection and purified with Ni-NTA Superflow resin (Qiagen), anion-exchange chromatography (Source 15Q, GE Healthcare), and size-exclusion chromatography (Superdex-200 Increase 10/300, GE Healthcare).

Bioinformatics analysis

All microarray data were derived from *TetraFGD* (Xiong et al., 2011 <http://tfgd.ihb.ac.cn/>). DNA sequencing data for mating type II–VII cells are derived from a previous report (Wang et al., 2020 https://web.expasy.org/compute_pi/). Compute pI/Mw (https://web.expasy.org/compute_pi/) was used to predict protein molecular weight. InterProScan (<http://www.ebi.ac.uk/interpro/>) was used for function and domain annotation (Jones et al., 2014 <https://www.ebi.ac.uk/interpro/>).

Statistical analysis

For mating experiments, more than 100 unpaired cells or cell pairs were counted, with three to five independent replicates. GraphPad software (version 8.0.2) was used for statistical analysis based on ANOVA (matched, Fisher's LSD test).

Acknowledgements

We thank Eduardo Orias (University of California Santa Barbara), Eileen P. Hamilton (University of California Santa Barbara), and Kazufumi Mochizuki (University of Montpellier) for their help and suggestions about experiment design and manuscript writing, and Yunfei Wei (Huazhong University of Science and Technology) for tubulin staining using Tubulin-Atto 594. We also thank members of the Protist 10,000 Genomes Project (P10K) consortium for helpful suggestions. We would like to thank Min Wang at the Analysis and Testing Center of Institute of Hydrobiology, Chinese Academy of Sciences for her help with mass spectrometry. The bioinformatics analysis was supported by the Wuhan Branch, Supercomputing Center, Chinese Academy of Sciences, China. Culture and maintenance of *Tetrahymena* cells were supported by the National Aquatic Biological Resource Center (NABRC).

Funding information

This work was funded by Bureau of Frontier Sciences and Education, Chinese Academy of Sciences (No. ZDBS-LY-SM026), National Natural Science Foundation of China (No. 32130011, 32200344) and China Postdoctoral Science Foundation (No. 2021M703433).

Author Contributions

G.Y. performed the experiments and analyzed the data. Y.M and H.C helped to construct mutants and perform cellular experiments. Y.W. and F.T. performed protein expression and purification experiments. J.Z helped to maintenance of *Tetrahymena* strains. G.Y. wrote the manuscript. All of the authors helped to conceptualize the work, and edit the text and the figures. W.M. and P.Y. supervised the work.

Conflict of Interest

Authors declare that they have no competing interests.

Supplementary Information

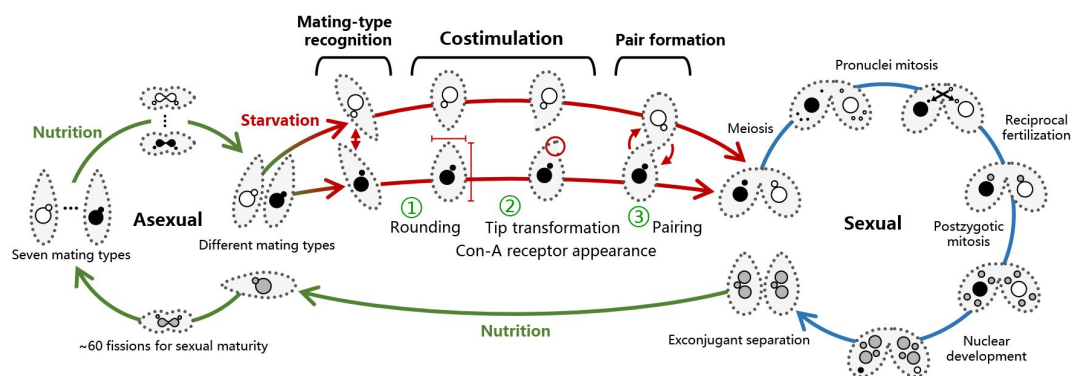


Figure 1 – figure supplement 1.

T. thermophila lifecycle.

T. thermophila has seven mating types. Cells divide asexually when nutrition is adequate. After starvation, cells of any two different mating types recognize each other and enter a pre-conjugation stage called costimulation. In this stage, cells first become round (Fujishima et al., 1993 [\[1\]](#)); subsequently, their cell tips are transformed into a curved shape (Wolfe & Grimes, 1979 [\[2\]](#)) and the Con-A receptor becomes detectable (Wolfe & Feng, 1988 [\[3\]](#); Wolfe et al., 1986 [\[4\]](#)). Costimulated cells then form pairs via a rotating behavior (Video S1, S2). During the sexual life cycle (conjugation), cells undergo a series of sexual events such as meiosis and reciprocal fertilization (for details refer to (Orias et al., 2011 [\[5\]](#))). Exconjugants (separated pairs) finally form parent-like progeny when nutrition becomes available, but remain sexually immature. After ~60 fissions, cells mature and their mating type is determined and becomes fixed (for details of mating-type determination, refer to (Cervantes et al., 2013 [\[6\]](#); Lin & Yao, 2020 [\[7\]](#); Orias et al., 2017 [\[8\]](#))).

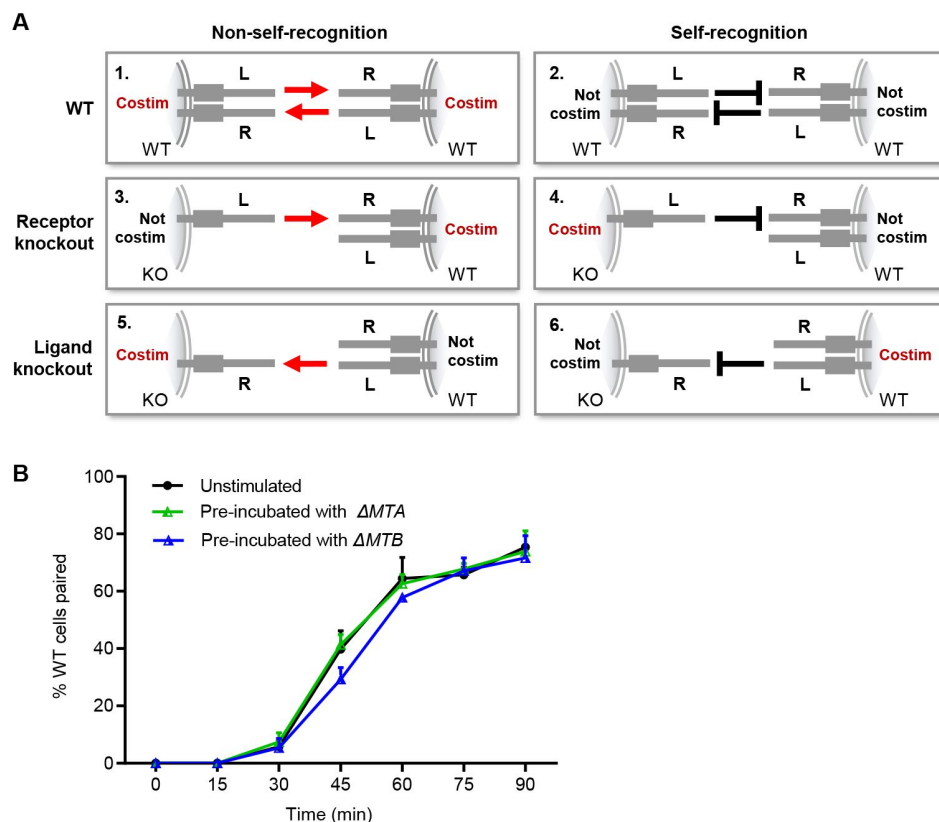


Figure 2 – figure supplement 1.

Mating-type gene deletion strains do not costimulate WT cells. **(A)** Pre-incubation results predicted by a simple receptor–ligand model of mating-type recognition, in which one mating-type protein is designed ligand (L) and the other receptor (R). The type of cell used to costimulate is shown on the left. The expected result of costimulation if *T. thermophila* were to use non-self mating-type recognition (1, 3, 5) or self mating-type recognition (2, 4, 6). If this simple model were true, deleting the receptor would have a different effect on costimulation to deleting the ligand. However, the experimental results showed that neither ΔMTA nor ΔMTB can fully transmit/receive mating signal to/from WT cells. Therefore, mating-type recognition in *T. thermophila* does not occur via the simple receptor–ligand binding mechanism. Costim, costimulation. **(B)** Pre-incubation with ΔMTA or ΔMTB does not influence the pairing rate. The experimental method was as described in [Figure 2A](#) except that the pre-incubation time was 16 h.

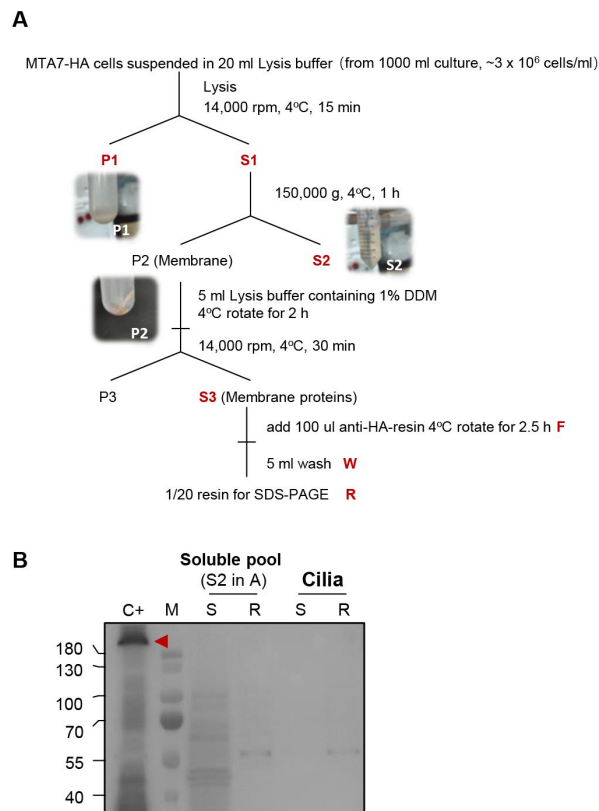


Figure 4 – figure supplement 1.

Cell fractionation MTA7-HA cells.

(A) Experimental process for cell fractionation. (B) Affinity purification results of the soluble pool (S2, shown in panel A) and ciliary protein. C+, positive control (i.e., IP product of MTA7-HA cell membrane); R, resin; S, supernatant. No MTA7-HA signal was detected in the soluble pool or in ciliary protein samples, even after affinity purification.

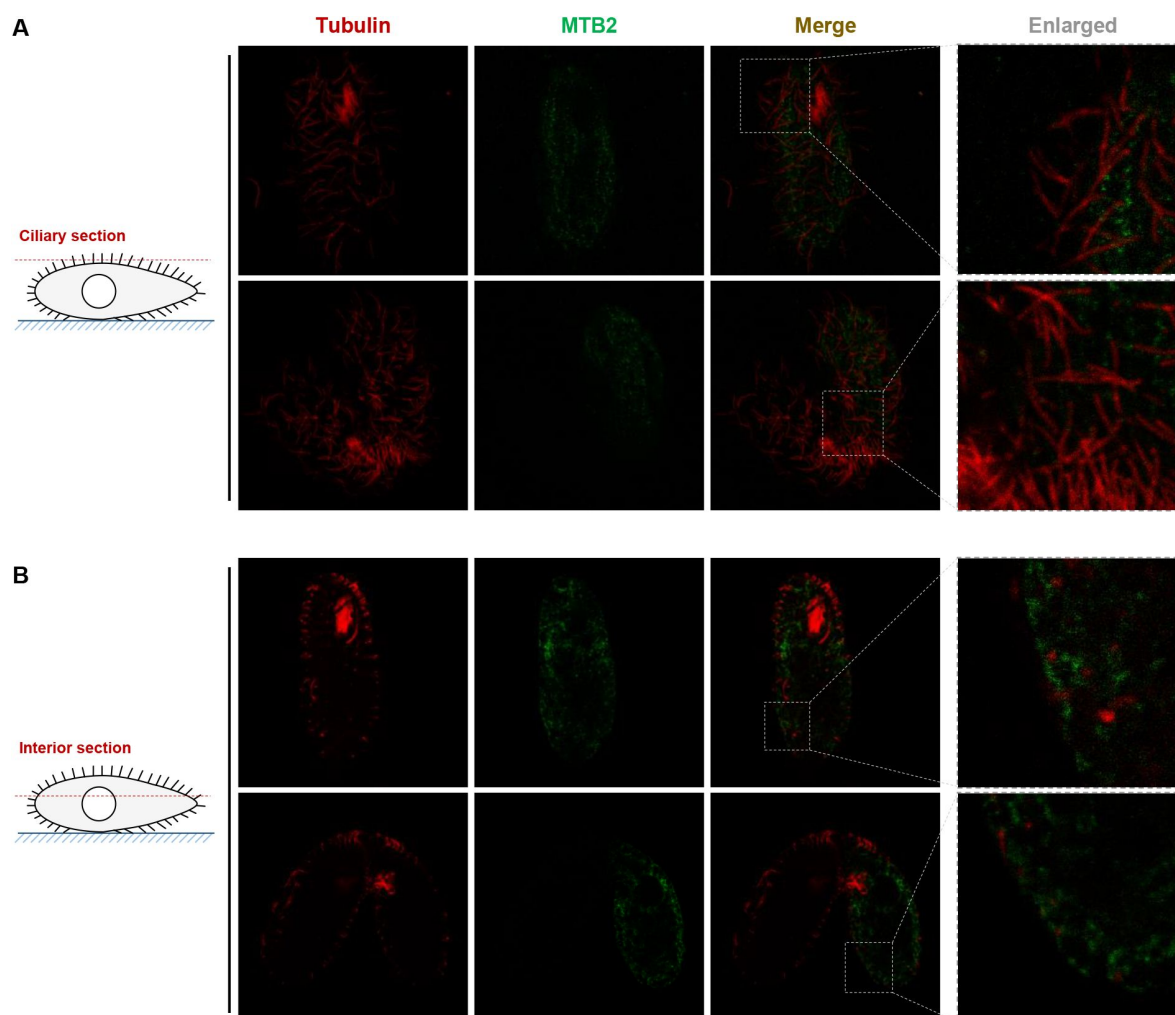


Figure 4 – figure supplement 2.

Confocal images of ciliary sections and cell interior sections of MTB2-eGFP cells.

(A) No MTB2-eGFP signal was associated with cilia. (B) In the cell interior section, MTB2-eGFP protein was detected on the plasma membrane and in intracellular structures, probably the ER and Golgi.

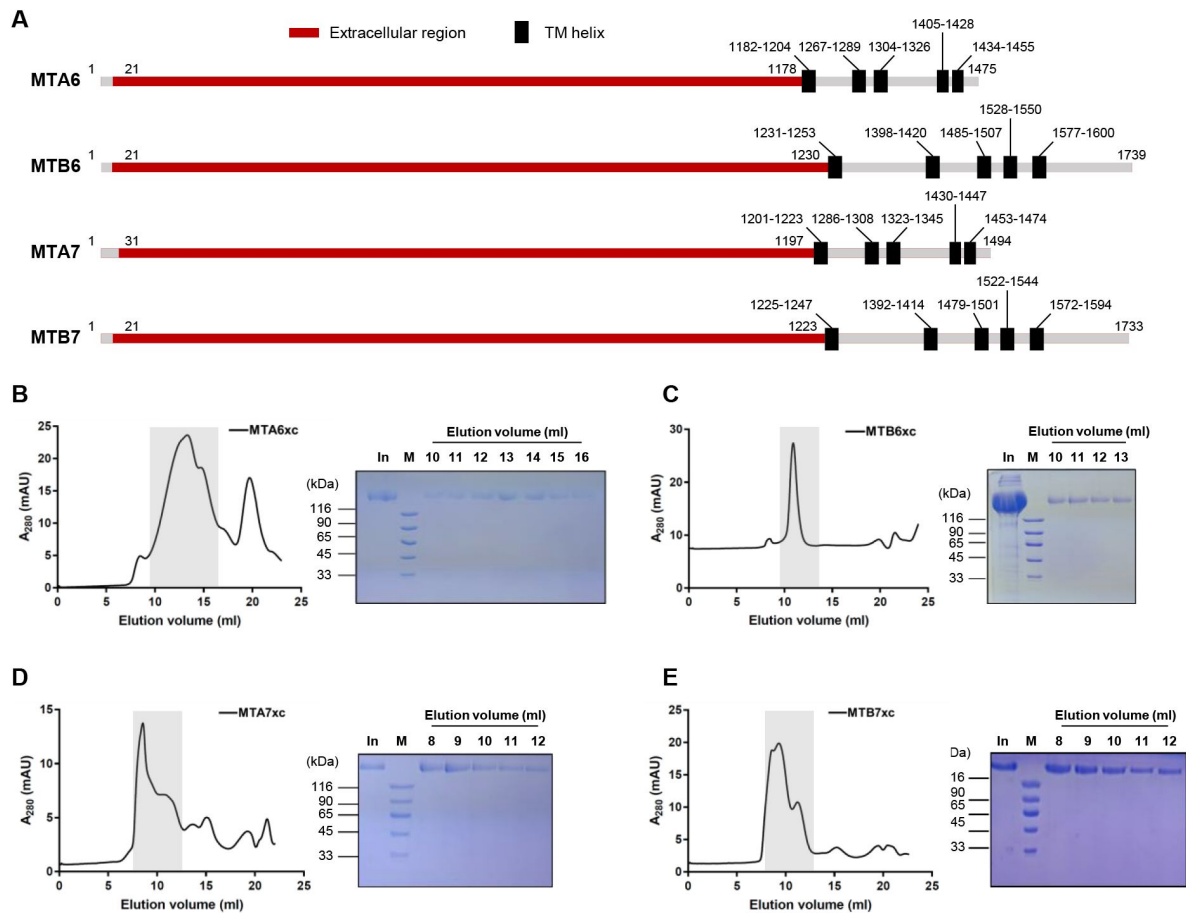


Figure 5 – figure supplement 1.

Expression and purification of extracellular regions of mating-type proteins.

(A) Schematic diagram showing truncated and full-sized proteins. (B–E) Size-exclusion chromatography for MTA6xc (B), MTB6xc (C), MTA7xc (D), and MTB7xc (E). Left, elution profile; right, Coomassie blue staining. In, input; M, marker.

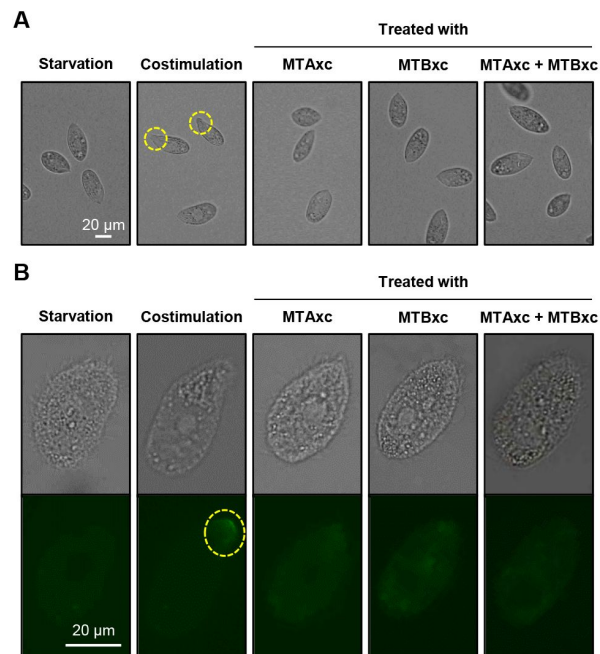


Figure 5 – figure supplement 2.

Treatment with MTA6xc and/or MTB6xc proteins fails to induce costimulation.

(**A**) Tip transformation. Yellow dashed circle, transformed cell tip. (**B**) Con-A receptor appearance. Yellow dashed circle, Con-A signal. Cells of mating type VII were used for these experiments.

Video 1. Mating behavior of *T. thermophila*.

Video 2. Mating behavior of *T. thermophila*. To distinguish cells of different mating types, smaller mating type VI cells and larger mating type VII cells were used in this experiment.

Video 3. Cells of different mating types form a pair, whereas cells of the same mating type become separated after a short contact. To distinguish cells of different mating types, smaller mating type VI cells and larger mating type VII cells were used in this experiment.

Figure 3–Source data 1. IP-MS results. Figure 3–Source data 2. Gene identifiers.

Figure 4–Source data 1. MS analysis of MTA7-HA cilia protein.

Figure 4–Source data 2. MS analysis of MTB2-eGFP cilia protein.

Appendix 1—table 1. Strains used in this study.

Appendix 1—table 2. Primers used in this study.

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Reviewer #1 (Public Review):**Summary:**

In this study, Yan et al. investigate the molecular bases underlying mating type recognition in *Tetrahymena thermophila*. This model protist possesses a total of 7 mating types/sexes and mating occurs only between individuals expressing different mating types. The authors aimed to characterize the function of mating type proteins (MTA and MTB) in the process of self- and non-self recognition, using a combination of elegant phenotypic assays, protein studies, and imaging. They showed that the presence of MTA and MTB in the same cell is required for the expression of concavalin-A receptors and for tip transformation - two processes that are characteristic of the costimulation phase that precedes cell fusion. Using protein studies, the authors identify a set of additional proteins of varied functions that interact with MTA and MTB and are likely responsible for the downstream signaling processes required for mating. This is a description of a fascinating self- and non-self-recognition system and, as the authors point out, it is a rare example of a system with numerous mating types/sexes. This work opens the door for the further understanding of the molecular bases and evolution of these complex recognition systems within and outside protists.

The results shown in this study point to the unequivocal requirement of MTA and MTB proteins for mating. Nevertheless, some of the conclusions regarding the mode of functioning of these proteins are not fully supported and require additional investigation.

Strengths:

1. The authors have established a set of very useful knock-out and reporter lines for MT proteins and extensively used them in sophisticated and well-designed phenotypic assays that allowed them to test the role of these proteins in vivo.
2. Despite their apparent low abundance, the authors took advantage of a varied set of protein isolation and characterization techniques to pinpoint the localization of MT proteins to the cell membrane, and their interaction with multiple other proteins that could be downstream effectors. This opens the door for the future characterization of these proteins and further elucidation of the mating type recognition cascade.

Weaknesses:

The manuscript is structured and written in a very clear and easy-to-follow manner. However, several conclusions and discussion points fall short of highlighting possible models and mechanisms through which MT proteins control mating type recognition:

1. The authors dismiss the possibility of a "simple receptor-ligand system", even though the data does not exclude this possibility. The model presented in Figure 2 S1, and on which the authors based their hypothesis, assumes the independence of MTA and MTB proteins in the generation of the intracellular cascade. However, the results presented in Figure 2 show that both proteins are required to be active in the same cell. Coupled with the fact that MTA and MTB proteins interact, this is compatible with a model where MTA would be a ligand and MTB a receptor (or vice-versa), and could thus form a receptor-ligand complex that could potentially be activated by a non-cognate MTA-MTB receptor-ligand complex, leading to an intracellular cascade mediated by the identified MRC proteins. As it stands, it is not clear what is the proposed working model, and it would be very beneficial for the reader for this to be clarified by having the point of view of the authors on this or other types of models.
2. The presence of MTA/MTB proteins is required for costimulation (Figure 2), and supplementation with non-cognate extracellular fragments of these proteins (MTA_{xc} or MTB_{xc}) is a positive stimulator of pairing. However, alone, these fragments do not have the ability to induce costimulation (Figure 5). Based on the results in Figures 5 and 6 the authors suggest that MT proteins mediate both self and non-self recognition. Why do MTA_{xc} and

MTBxc not induce costimulation alone? Are any other components required? How to reconcile this with the results of Figure 2? A more in-depth interpretation of these results would be very helpful, since these questions remain unanswered, making it difficult for the reader to extract a clear hypothesis on how MT proteins mediate self- and non-self-recognition.

- <https://doi.org/10.7554/eLife.93770.1.sa2>

Reviewer #2 (Public Review):

This manuscript reports the discovery and analysis of a large protein complex that controls mating type and sexual reproduction of the model ciliate *Tetrahymena thermophila*. In contrast to many organisms that have two mating types or two sexes, *Tetrahymena* is multi-sexual with 7 distinct mating types. Previous studies identified the mating type locus, which encodes two transmembrane proteins called MTA and MTB that determine the specificity of mating type interactions. In this study, mutants are generated in the MTA and MTB genes and mutant isolates are studied for mating properties. Cells missing either MTA or MTB failed to co-stimulate wild-type cells of different mating types. Moreover, a mixture of mutants lacking MTA or MTB also failed to stimulate. These observations support the conclusion that MTA and MTB may form a complex that directs mating-type identity. To address this, the proteins were epitope-tagged and subjected to IP-MS analysis. This revealed that MTA and MTB are in a physical complex, and also revealed a series of 6 other proteins (MRC1-6) that together with MTA/B form the mating type recognition complex (MTRC). All 8 proteins feature predicted transmembrane domains, three feature GFR domains, and two are predicted to function as calcium transporters. The authors went on to demonstrate that components of the MTRC are localized on the cell surface but not in the cilia. They also presented findings that support the conclusion that the mating type-specific region of the MTA and MTB genes can influence both self- and non-self-recognition in mating.

Taken together, the findings presented are interesting and extend our understanding of how organisms with more than two mating types/sexes may be specified. The identification of the six-protein MRC complex is quite intriguing. It would seem important that the function of at least one of these subunits be analyzed by gene deletion and phenotyping, similar to the findings presented here for the MTA and MTB mutants. A straightforward prediction might be that a deletion of any subunit of the MRC complex would result in a sterile phenotype. The manuscript was very well written and a pleasure to read.

- <https://doi.org/10.7554/eLife.93770.1.sa1>

Reviewer #3 (Public Review):

The authors describe the role, location, and function of the MTA and MTB mating type genes in the multi-mating-type species *T. thermophila*. The ciliate is an important group of organisms to study the evolution of mating types, as it is one of the few groups in which more than two mating types evolved independently. In the study, the authors use deletion strains of the species to show that both mating types genes located in each allele are required in both mating individuals for successful matings to occur. They show that the proteins are localized in the cell membrane, not the cilia, and that they interact in a complex (MTRC) with a set of 6 associated (non-mating type-allelic) genes. This complex is furthermore likely to interact with a cyclin-dependent kinase complex. It is intriguing that *T. thermophila* has two genes that are allelic and that are both required for successful mating. This coevolved double recognition has to my knowledge not been described for any other mating-type recognition system. I am not familiar with experimental research on ciliates, but as far as I can judge, the experiments

appear well performed and mostly support the interpretation of the authors with appropriate controls and statistical analyses.

The results show clearly that the mating type genes regulate non-self-recognition, however, I am not convinced that self-recognition occurs leading to the suppression of mating. An alternative explanation could be that the MTA and MTB proteins form a complex and that the two extracellular regions together interact with the MTA+MTB proteins from different mating types. This alternative hypothesis fits with the coevolution of MTA and MTB genes observed in the phylogenetic subgroups as described by Yan et al. (2021 iScience). Adding MTAXc and/or MTBxc to the cells can lead to the occupation of the external parts of the full proteins thereby inhibiting the formation of the complex, which in turn reduces non-self interactions. Self-recognition as explained in Figure 2S1 suggests an active response, which should be measurable in expression data for example. This is in my opinion not essential, but a claim of self-recognition through the MTA and MTB should not be made.

The authors discuss that *T. thermophila* has special mating-type proteins that are large, while those of other groups are generally small (lines 157-160 and discussion). The complex formed is very large and in the discussion, they argue that this might be due to the "highly complex process, given that there are seven mating types in all". There is no argument given why large is more complex, if this is complex, and whether more mating types require more complexity. In basidiomycete fungi, many more mating types than 7 exist, and the homeodomain genes involved in mating types are relatively small but highly diverse (Luo et al. 1994 PMID: 7914671). The mating types associated with GPCR receptors in fungi are arguably larger, but again their function is not that complex, and mating-type specific variations appear to evolve easily (Fowler et al 2004 PMID: 14643262; Seike et al. 2015 PMID: 25831518). The large protein complex formed is reminiscent of the fusion patches that develop in budding or fission yeasts. In these species, the mating type receptors are activated by ligand pheromones from the opposite mating type that induce polarity patch formation (see Sieber et al. 2023 PMID: 35148940 for a recent review). At these patches, growth (shmooing) and fusion occur, which is reminiscent (in a different order) of the tip transformation in *T. thermophila*. The fusion of two cells is in all taxa a dangerous and complex event that requires the evolution of very strict regulation and the existence of a system like the MTRC and cyclin-dependent complex to regulate this process is therefore not unexpected. The existence of multiple mating types should not greatly complicate the process, as most of the machinery (except for the MTA and MTB) is identical among all mating types.

The Tetrahymena/ciliate genetics and lifecycle could be better explained. For a general audience, the system is not easy to follow. For example, the ploidy of the somatic nucleus with regards to the mating type is not clear to me. The MAC is generally considered "polyploid", but how does this work for the mating type? I assume only a single copy of the mating type locus is available in the MAC to avoid self-recognition in the cells. Is it known how the diploid origin reduces to a single mating type? This does not become apparent from Cervantes et al. 2013. Also, the explanation of co-stimulation is not completely clear (lines 49-60). Initially, direct cell-cell contact is mentioned, but later it is mentioned that "all cells become fully stimulated", even when unequal ratios are used. Is physical contact necessary? Or is this due to the "secrete mating-essential factors" (line 601)? These details are essential, for interpretation of the results and need to be explained better.

Abstract and introduction: Sexes are not mating types. In general, mating types refer to systems in which there is no obvious asymmetry between the gametes, beyond the compatibility system. When there is a physiological difference such as size or motility, sexes are used. This distinction is of importance because in many species mating types and sexes can occur together, with each sex being able to have either (when two) or multiple mating types. An example are SI in angiosperms as used as an example by the authors or mating

types in filamentous fungi. See Billiard et al. 2011 [PMID: 21489122] for a good explanation and argumentation for the importance of making this distinction.

- <https://doi.org/10.7554/eLife.93770.1.sa0>