

1 **Genome analysis of the unicellular eukaryote *Euplotes vannus* provides insights**  
2 **into mating type determination and tolerance to environmental stresses**

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20

21 **Abstract**

22 **Background:**

23 The genus *Euplotes* is a clade of free-living and cosmopolitan ciliated protists. As a model  
24 organism in studies of cell and environmental biology, *Euplotes vannus* has more than ten  
25 mating types (sexes) and shows strong resistance to environmental stresses such as low  
26 temperature and high salinity. However, the molecular basis of its mating type  
27 determination mechanism and how the cell responds to stress are still largely unknown.  
28 Here we focus on these topics by genome analysis of different mating types of *Euplotes*  
29 *vannus*.

30  
31 **Results:**

32 This work combines analysis of *de novo* assembled high-quality macronucleus (MAC; i.e.  
33 somatic) genome and partial micronucleus (MIC; i.e. germline) genome of *Euplotes*  
34 *vannus*. MAC genomic and transcriptomic data from several mating types of *E. vannus*  
35 were investigated and gene expression levels were profiled under different environmental  
36 stresses, including stresses from nutrient scarcity, extreme temperature, salinity and the  
37 presence of free ammonia. The results indicate that: 1) *E. vannus*, which possesses  
38 "gene-sized" nanochromosomes in its MAC, shares a similar pattern on frameshifting and  
39 stop codon usage as *Euplotes octocarinatus* and may be undergoing incipient sympatric  
40 speciation with *Euplotes crassus*; 2) *E. vannus* possesses two Type-I and four Type-II  
41 pheromones, including two novel alleles Ev-4 and Ev-beta, based on the genome  
42 investigation of six mating types; 3) the coding regions of pheromone genes in the MAC  
43 genome of *E. vannus* consist of multiple macronuclear destined sequences (MDS) regions  
44 in the MIC; 4) different mating types of *E. vannus* have mating type-specific chromatin and  
45 expression profiling of Type-II pheromone loci; 5) the HSP70 gene of *E. vannus* does not  
46 carry either unique amino acid substitutions of potential significance for cold adaptation nor  
47 mRNA destabilization ARE elements in its 3' regulatory region. Additionally, the genome  
48 resources generated in this study are available online at *Euplotes vannus* DB  
49 (<http://evan.ciliate.org>).

50

51 **Conclusions:**

52 Based on the results of the current study, the following conclusions are put forward: 1) the  
53 high similarity of the pheromones of *E. vannus* and *E. crassus* reveals the molecular basis  
54 of hybridization between these two "morphospecies" under laboratory conditions; 2)  
55 somatic pheromone loci of *E. vannus* are generated from programmed DNA  
56 rearrangements of multiple germline MDS segments, which are similar to the complex  
57 rearrangements of mating type determination in *Tetrahymena*; 3) however, unlike  
58 *Tetrahymena*, *E. vannus* does not possess mating type-specific genes. Instead, the mating  
59 types are distinguished by the different combinations of pheromone loci. This finding  
60 supports the allelic codominance or non-hierarchical dominance relationship among  
61 pheromone loci during *Euplotes* pheromone-mediated cell-cell signaling and cross-mating;  
62 4) as a common species in global waters, the HSP70 gene of *E. vannus* has evolved to be  
63 insensitive to environmental temperature change.

64

65 **Keywords:**

66 Ciliated protist, DNA rearrangement, environmental stress, frameshifting, mating type  
67 determination.

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69

## 70 **Background**

71 Single-celled, ciliated protists are abundant in diverse habitats across the globe, where  
72 they are among the most important components of food webs in aquatic ecosystems [1].  
73 Ciliate diversity, physiology and abundance has been linked to studies of environmental  
74 change [2-4], pollution monitoring [5-8], biogeography [9-13], adaptive evolution [14-20]  
75 and epigenetics [21-23]. *Euplotes* is a genus of free-living marine ciliates that play  
76 important roles as both predators of microalgae and preys of multicellular eukaryotes like  
77 flatworms [24]. For decades, euplotids, including *Euplotes vannus*, have been widely used  
78 as model organisms in studies of predator/prey relationships [25-31], cell signaling [32, 33],  
79 toxicology of marine pollutants [34, 35] and experimental ecology [36-38]. For example, a  
80 previous molecular study revealed that *Euplotes* species have a large number of genes  
81 requiring +1 frameshifts (i.e. addition of base pairs post-transcriptional) for expression at  
82 the post-transcriptional level, which is much higher than viruses, prokaryotes and other  
83 eukaryotes [39].

84 Ciliates, including the models *Paramecium* and *Tetrahymena*, have been shown to  
85 present a wide variety of mating type numbers and modes of inheritance [40]. *Paramecium*  
86 has two mating types and its mating type determination (MTD) is controlled by scnRNA-  
87 dependent excision of the MTD gene promoter [41]. *Tetrahymena* has seven mating types,  
88 and different mating types specificities are encoded in the single pair of mating type genes  
89 in the MAC from all the six pairs in the MIC [42]. More than ten mating types have been  
90 identified in *Euplotes* [43-48], yet the molecular basis and MTD mechanism are unknown.  
91 Previous studies identified two subfamilies of mating-type specific pheromones in euplotids,  
92 a "shared" pheromone (designated Ec-alpha and named as Type-I pheromone in this work)  
93 and a "mating type-specific compositional" subfamily (designated Ec-1, Ec-2 and Ec-3, and  
94 named as Type-II pheromone) [49, 50]. They are considered as the key mediator during  
95 the cell-cell signaling that regulates cross-mating processes by controlling self/nonself  
96 recognition [51, 52]. Previous studies reported that the euplotid model implicates  
97 relationships of hierarchical (or serial) dominance among the pheromone alleles [53-55].  
98 Yet the results from a recent work indicated that these pheromone genes were expressed  
99 without relationships of hierarchical dominance (i.e. heterozygous genotypes behaving like  
100 homozygous cells) in the *Euplotes* MAC genome [50].

101 Euplotids also feature a strong tolerance to environmental stresses. *Euplotes* spp.  
102 were reported to have a conserved molecular defense mechanism to heavy metal  
103 contamination for homeostasis by modulating mRNA expression [56]. In contrast, *E.*  
104 *crassus* and *E. focardii* had a barely detectable inducible HSP70 response to salinity and  
105 temperature stresses [14, 57]. These findings add weight to the argument that the lack of  
106 the classical heat shock response might be an adaptation strategy of euplotids to extreme  
107 environmental stresses. Additional studies reported *E. vannus*, as a microzooplanktonic  
108 grazer, had considerably strong tolerance to ammonia, which may enable it to survive in  
109 intensive aquaculture ecosystems with high levels of ammonium, potentially causing great  
110 damage in microalgal industry [36, 37]. Thus, it is important to elucidate the molecular  
111 mechanism of *Euplotes* cell response to external stresses under the background of global  
112 warming.

113 In this work, we analyze genomic and transcriptomic data of different mating types  
114 of *E. vannus* to study its genomic features, which lead us to reveal the molecular basis of  
115 mating type determination in euplotids. Furthermore, the gene expression profiling of *E.*  
116 *vannus* cells under different environmental stresses allows us to evaluate how this species  
117 tolerates the varying harsh conditions that it encounters.

118

119

## 120 **Results**

121 General description of genome sequencing and assembly of *Euplotes vannus*

122 In the current work, we acquired the MAC genomic data of four different mating types (EVJ,  
123 EVK, EVL and EVM), which were experimentally confirmed (Table S1). The MAC genome  
124 assemblies of these mating type had an average size of 164.2 Mb with a mean coverage  
125 of 61X (Table 1 and supplementary information, Figure S1 and Table S2).

126 After the contigs identified as noise (coverage < 5X) or contamination of bacteria  
127 and mitochondria were removed, the final genome assembly of *E. vannus* was generated  
128 by merging the genome assemblies of these four mating types (assembled genome size is  
129 85.1 Mb and N50 = 2,685 bp, Table 1). We compared these data with those of two other  
130 euplotids, *E. crassus* and *E. octocarinatus*. Although *E. vannus* and *E. crassus* shared a

131 similar %GC (36.95% and 38.65%, respectively), the genome size, number of 2-telomere  
132 contigs and N50 value of *E. vannus* were more comparable with those of *E. octocarinatus*  
133 (Table 1).

134 The contig N50 values of these three *Euplotes* species were all smaller than 3 kb,  
135 because chromosomes of *Euplotes* species are "nanochromosomes", similar to that of  
136 *Oxytricha trifallax* [39]. The 2-telomere contig percentage of the merged genome of *E.*  
137 *vannus* is 66.7%, consistent with *E. octocarinatus* in which 70.3% contigs contained  
138 telomeres on both ends. Among the four mating types with genomic sequencing data, the  
139 genome assembly of EVJ was of highest quality with a 2-telomere contig percentage of  
140 81.6% and N50 of 2,954 bp (Supplementary information, Table S2).

141 To evaluate the completeness of the genome assembly of *E. vannus*, gene content  
142 from single-copy orthologs of protists was identified by BUSCO, and the result indicated  
143 that the current assembly had a comparable percentage of complete ortholog sequences  
144 with other species (Figure 1 and supplementary information, Figure S2). Furthermore, the  
145 majority of genomic DNA sequencing reads of four mating types (EVJ, EVK, EVL and EVM)  
146 and RNA-seq reads of six mating types (EVJ, EVK, EVL, EVM, EVP and EVX) in both  
147 starvation and vegetative stages can successfully be mapped back to the merged  
148 reference genome assembly with a mean mapping ratio of 80.1% (Supplementary  
149 information, Table S3). Furthermore, 109 tRNAs which consist of 48 codon types for 21  
150 amino acids, were detected in the final genome assembly (Supplementary information,  
151 Table S4). These results indicated that our genome assembly of *E. vannus* was largely  
152 complete. The information of repeat regions and functional annotation of genes are  
153 summarized in supplementary information, Figure S3, Table S5 and Table S6.

154 The size distribution of complete chromosomes of *E. vannus* (i.e. those bearing  
155 telomeric repeats "C4A4" and "T4G4" on both ends) is quite close to that of another two  
156 euplotids, *E. octocarinatus* and *E. crassus*, with the peak values around 1.5 kb (Figure 1B).  
157 A similar result was found in the size distribution of telomeres, in which most telomeres of  
158 all three euplotids had a length of 28 bp, with an increment of 8 bp (Figure 1C). Most  
159 identified *E. vannus* introns were around 25 bp in length, with a canonical sequence motif  
160 5'-GTR(N)nYAG-3' at either end (Figure 1DE and supplementary information, Figure S4).

161

162 Evolution and synteny/comparative genomic analyses among euplotids

163 "*Joint*" nanochromosomes

164 Most chromosomes (37501/38245, 98.1%) in *E. vannus* are nanochromosomes, or so-  
165 called gene-sized chromosomes, bearing a single gene on each. A similar result was  
166 observed in *E. octocarinatus* (40396/41980, 96.2%). There was a small proportion of  
167 chromosomes that contains more than one gene (Figure 2A). These "joint"  
168 nanochromosomes were then divided into two groups by the consistency and  
169 inconsistency of the transcription directions of the genes on them (cis and trans,  
170 respectively). The trans-joint nanochromosomes had close numbers, considering the total  
171 chromosome numbers were similar in these two euplotids (Table 1). However, *E.*  
172 *octocarinatus* possessed 2-fold more cis-joint nanochromosomes than *E. vannus*.

173

174 *Homologous genes*

175 *E. vannus* and *E. crassus* shared 25026 closely related contigs (E-value cutoff = 1e-5),  
176 which was equivalent to 65.4% and 44.2% of total contigs for each species, respectively  
177 (Figure 2B). However, only 469 contigs were shared between these two species and *E.*  
178 *octocarinatus*. Furthermore, *E. vannus* and *E. crassus* shared not only more homologous  
179 contigs, but also more sequence identity (Figure 2C). For example, the sequence identity  
180 between *E. vannus* and *E. crassus* of the corresponding region on the chromosome that  
181 contains the coding gene of dynein heavy chain protein was 99.6%, while the sequence  
182 identity between *E. vannus* and *E. octocarinatus* was 71.2%.

183

184 *Frameshifting*

185 Frameshifting events in *E. vannus* and *E. octocarinatus* were detected by identifying the  
186 adjacent region between two BLASTX hits that are in different frames, targeting to a same  
187 protein sequence (illustrated by supplementary information, Figure S5). The E-value cutoff  
188 (1e-5) ensured the accuracy of the prediction process and a small inner distance cutoff (10  
189 nt) was applied to get rid of the interference from introns, because all introns of euplotids  
190 were larger than 20 nt as described above (Figure 2D). The result indicated that the high  
191 frequency of +1 programmed ribosomal frameshifting (PRF) was a conserved feature in

192 euplotids. However, intriguingly, more +2 and -1 PRF events were found in *E. vannus*  
193 (16.6%) than in *E. octocarinatus* (4.4%). With more cases of +2 and -1 PRF events being  
194 spotted, a novel motif rather than the canonical motif 5'-AAA-TAR-3' (R = A or G) was  
195 revealed as 5'-WWW-TAR-3' (W = A or T) (Figure 2E).

196

### 197 *Stop codon usage*

198 Based on the genomic and transcriptomic sequencing data, the stop codon usage was  
199 analyzed in *E. vannus* and *E. octocarinatus* and compared between the regular transcripts  
200 and the slippery sites of PRF transcripts (Figure 2F). In these two euplotids, UAA was  
201 preferentially used in the regular termination signal (73.7% and 76.0%, respectively) and in  
202 the slippery signal (91.3% and 91.0%, respectively). Moreover, the frequency of UAA  
203 codon usage in slippery signal is significantly higher than that in the regular termination  
204 signal ( $p = 0.005024 < 0.01$ , Analysis of variance), which suggested that UAA may be  
205 favorable for frameshifting in both *E. vannus* and *E. octocarinatus*.

206

### 207 *Profiling and development of Euplotes pheromone genes*

208 Pheromone alleles were successfully identified in MAC genomes of four *E. vannus* mating  
209 types (Supplementary information, Table S7). Other than the pheromones Ev-1, Ev-2, Ev-3  
210 and Ev-alpha that were orthologous to Ec-1, Ec-2, Ec-3 and Ec-alpha in *E. crassus*,  
211 respectively, a novel Type-II pheromone Ev-4 and a novel Type-I pheromone Ev-beta were  
212 found in *E. vannus* (Figure 3A and supplementary information, Figure S6, Figure S7 and  
213 Figure S8). Although Ev-4 used "TAG" as stop codon rather than using "TAA" in other  
214 three Type-II pheromones (Supplementary information, Figure S6), together with Ev-beta,  
215 they showed a significant sequence similarity with the other three known pheromones,  
216 especially in the pre- and pro-regions of the cytoplasmic precursor (marked by red arrows  
217 in Figure 3A) and retained highly conserved cysteine residues in the secreted region  
218 (marked by red dots), which was an important feature of pheromone allele sequences.

219 The phylogenetic analysis of *Euplotes* pheromones, including the homologs in each  
220 mating type of *E. vannus* and the corresponding consensus sequences, was performed  
221 (Figure 3A). Although these MAC loci were generated by alternative processing of MIC



222 regions in each species, the result indicated that the pheromones of *E. vannus* clustered  
223 together with those of *E. crassus*, distinct from those of other Euplotids, *E. octocarinatus*, *E.*  
224 *nobili* and *E. raikovi*.

225 Chromatin profiling of pheromone genes indicated that the combination of Type-II  
226 pheromone genes, Ev-1, Ev-2, Ev-3 and Ev-4, exhibited a mating type-specific feature on  
227 genic level (Figure 3B and supplementary information, Figure S9). Different mating types  
228 retained 1-3 Type-II pheromone genes in the MAC genome and no duplicate events  
229 existed. Gene expression profiling of pheromone genes consistently showed that both two  
230 Type-I pheromones Ev-alpha and Ev-beta were highly expressed in all six mating types in  
231 *E. vannus* and confirmed the mating type-specific chromatin profiling of Type-II pheromone  
232 genes in different mating types at the transcriptional level. As another independent  
233 verification, PCR amplification of pheromone loci was carried out to verify the presence of  
234 the pheromone-related contigs in the MAC genome of each mating type (Figure 3C). The  
235 results of PCR amplification of pheromone loci indicated that Type-II pheromone gene Ev-  
236 1 was absent from the mating types EVL, EVM and EVP, Ev-2 was absent from EVK, EVM,  
237 EVP and EVX, Ev-3 was absent from EVJ and Ev-4 was absent from EVK and EVP, which  
238 were mostly consistent with the results from chromatin and gene expression profiling  
239 (Figure 3B). In brief, each of the six mating types we identified contained a unique  
240 combination of four Type-II pheromone genes.

241 To further study the development process of the pheromone genes during the  
242 programmed DNA rearrangement from germline MIC to somatic MAC in *E. vannus*, MIC  
243 genomic DNA was acquired by single-cell sequencing and its draft genome was  
244 assembled (Table 2). Then the germline genome (MIC) origins of Ev-1 (MAC Contig16568),  
245 Ev-2 (Contig28896), Ev-3 (Contig29423) and Ev-4 (Contig34058) were mapped to MIC  
246 contigs (Figure 3C and supplementary information, Table S8, E-value cutoff = 1e-5). The  
247 coding region of pheromone genes Ev-1, Ev-2 and Ev-3 consisted of three MDS regions  
248 from the MIC genome while Ev-4 consisted of at least two MDS regions. However, the  
249 germline source of a part of the pro-region and the secreted region of Ev-4 was not found.

250

251 Molecular basis of strong tolerance to extreme environmental stresses

252 *Stress from nutrient scarcity: starvation-induced and mating type-specific transcripts*

253 Starvation stress (i.e. nutrient scarcity) had limited impact on the expression pattern of  
254 different mating types by global transcription profile (Figure 4A and supplementary  
255 information, Figure S10). However, differential gene expression analysis revealed some  
256 mating type-specific transcripts under nutrient scarcity (Figure 4B). Transcripts induced by  
257 starvation tended to be associated with mating type-specific genes (42.8%; see  
258 supplementary information, Figure S11). Gene functional annotation reveals that these  
259 starvation-induced and mating type-specific transcripts are related to protein transport and  
260 phosphorylation process in cells (Figure 4C and supplementary information, Figure S12).  
261 The expression of these genes may facilitate the cell response to pheromone-mediated  
262 cell-cell signaling and cross-mating behavior under the stress from nutrient scarcity.

263

264 *Stresses from extreme temperature, salinity or the presence of free ammonia*

265 PCA analysis based on the differential gene expression of *E. vannus* EVJ cells under  
266 different extreme environmental stresses was performed (Supplementary information,  
267 Figure S13A). The result revealed changes in the gene expression profile of cells under  
268 high temperature (35 °C), low temperature (4 °C), high or low salinity (60 and 10 psu,  
269 respectively). Also, the presence of free ammonia had substantial impact on transcription  
270 patterns (Supplementary information, Figure S13). Surprisingly, cells under high salinity  
271 and low salinity shared a similar gene expression profile (Supplementary information,  
272 Figure S13A).

273 To further dissect the relationships between co-expression of genes associated with  
274 the regulation of cellular processes and pathways under extreme environmental changes,  
275 a weighted gene co-expression eigengene network was constructed (Figure 5A). The  
276 network clustered different eigengenes into six modules based on their co-expression  
277 profile (Supplementary information, Figure S13B). A strongly co-expressed eigengene  
278 module was up-regulated in cells under both high and low salinity stresses (colored in steel  
279 blue in Figure 5A and supplementary information, Figure S13B). This module was involved  
280 with an extensive activation of many pathways, mainly related to tRNA aminoacylation,  
281 tRNA and rRNA processing, nucleosome assembly and pseudouridine synthesis (p.adjust

282 < 0.05). In addition, two small eigengene modules were up-regulated in cells under high  
283 salinity stress (purple) and low salinity stress (dark green), respectively, and low salinity  
284 stress activated an extra pathway related to the glutamine metabolic process.

285 Intriguingly, low temperature stress induced a large module cluster of eigengenes  
286 related to small GTPase mediated signal transduction, while very few eigengenes co-  
287 expressed under the high temperature (blue and purple, respectively, in [Figure 5](#) and  
288 supplementary information, [Figure S13B](#)). The homolog of the highly conserved heat-  
289 shock protein 70 (Hsp70), which many organisms upregulate under environmental stress,  
290 was identified in *E. vannus* and compared with its counterparts in *E. nobilii* and *E. focardii*  
291 ([Figure S14](#)). The result revealed that only the *E. focardii* Hsp70 sequence had numerous  
292 amino acid substitutions within its two major functional domains, i.e. the ATP-binding and  
293 substrate-binding domains ([Figure 6A](#)). However, the transcription of the HSP70 gene in *E.*  
294 *vannus* did not respond to temperature stresses while being responsive to other stresses  
295 like salinity and chemical stresses ([Figure 6B](#)).

296 To gain a better understanding of the molecular basis of the lack of change in  
297 response of the HSP70 gene to temperature stress in *E. vannus*, we analyzed the  
298 structure of non-coding regions flanking the gene in *E. vannus* and compared the structure  
299 of this gene between *E. vannus* and *E. focardii* ([Figure 6CD](#)). The result indicated that no  
300 substantial difference was detected in the 5' promoter region between the HSP70 genes of  
301 these two species, both bearing canonical regulatory *cis*-acting elements that bind  
302 transcriptional trans-activating factors, including heat-shock elements (HSE) and stress-  
303 response elements (StRE) ([Figure 6C](#)). Furthermore, neither *E. vannus* nor *E. focardii*  
304 retained mRNA destabilization ARE elements in their 3' promoter region ([Figure 6D](#)).

305 The gene expression of cells under the presence of free ammonia were very similar  
306 to those under high temperature stress, and there was a small cluster of eigengenes that  
307 responded related to the lipid metabolic process ([Figure 5](#) and supplementary information,  
308 [Figure S13B](#)). However, chemical stress activated the expression of HSP70 gene in *E.*  
309 *vannus* significantly ([Figure 6B](#)).

310

311

## 312 **Discussion**

313 Genomes of ciliates are divergent to each other and largely unexplored

314 Besides euplotids, we also evaluated the assembly completeness of genomes or  
315 transcriptomes of other ciliates and found a large divergence among them ([Figure 1A](#) and  
316 supplementary information, [Figure S2](#)). It reflects two facts: 1) ciliates have great genetic  
317 distances, even those closely related on phylogeny; 2) ciliate genomic data sequenced so  
318 far has not been collected by assembly completeness evaluation tools like BUSCO (the  
319 only ciliate covered is *Tetrahymena thermophila*). Nevertheless, among these ciliates, the  
320 completeness of transcriptome assembly of three species, *Anophryoides haemophila*,  
321 *Uronema* sp. and *Condylostoma magnum*, were not evaluated. One of the most likely  
322 reasons is that they possess small genome volume and thus share few genes with other  
323 ciliates [\[58\]](#). For instance, *Anophryoides* and *Uronema* are well-known parasitic species  
324 that cause disease in fish and lobsters in aquaculture facilities and have very small  
325 genome sizes [\[59-63\]](#). Another reason is that stop codon rearrangement occurs in some  
326 ciliates, such as *Condylostoma magnum*, all standard stop codons are reassigned to  
327 amino acids in a context-dependent manner [\[64, 65\]](#). These unusual features could  
328 dramatically increase the difficulty to precisely evaluate the assembly completeness of  
329 ciliate genomes/transcriptomes. Overall, the genomic investigation in ciliates is still waiting  
330 to be explored and the evaluation of ciliates genome assemblies calls for further  
331 improvement.

332

333 Molecular basis of the mating type determination in *E. vannus*

334 *Genomic data and pheromone gene assay supports the hypothesis that E. vannus and E.*  
335 *crassus may be undergoing incipient sympatric speciation*

336 An early study reported that *E. vannus* could interbreed with *E. crassus* under laboratory  
337 conditions [\[66\]](#). Other studies reported that *E. vannus* and *E. crassus* might be undergoing  
338 sympatric speciation [\[67, 68\]](#). The result in the current work increased the weight of this  
339 argument in three aspects: 1) *E. vannus* and *E. crassus* are closely clustered with each  
340 other among euplotid species ([Figure 1A](#)); 2) these two species shared both a large  
341 number of homologous sequences and at high levels of sequence identity, in contrast to

342 comparisons with *E. octocarinatus* (Figure 2BC); 3) the homologs of pheromones of *E.*  
343 *vannus* and *E. crassus* are closely related and distinct from those from other *Euplotes*  
344 species (Figure 3AB). Orthologs of each pheromone allele from different mating types of *E.*  
345 *vannus* shares identical sequences on both gene and protein levels in most cases except  
346 pheromone gene Ev-2 (Figure 3A and supplementary information, Figure S6, Figure S7  
347 and Figure S8). Our findings might describe a pattern that mating type loci evolve rapidly  
348 after a recent speciation between *E. crassus* and *E. vannus*.

349

350 *The combination of Type-II pheromone loci is mating type-specific in E. vannus MAC*

351 Type-I pheromone Ec-alpha has large sequence differences with the Type-II pheromones  
352 and has been considered as an "adaptor" that interacts with the other pheromones, as it  
353 has a strong propensity to oligomerize and retains a hydrophilic domain for putative  
354 interaction [50, 52]. This argument is supported by the results of expression profiling for  
355 pheromone genes in the current work (Figure 3B).

356 Studies on the pheromones from other euplotids, including *E. raikovi* [69-75], *E.*  
357 *nobilii* [51, 76-78] and *E. octocarinatus* [79-82], revealed that highly enriched and  
358 conserved cysteine residues in secreted region is the most outstanding sequence motif of  
359 *Euplotes* pheromones [52]. The novel pheromone Ev-4 identified in this study from *E.*  
360 *vannus* retains 10 cysteines, as same as other Type-II pheromones in *E. vannus* and *E.*  
361 *crassus*, and thus matches this characteristic perfectly (Figure 3A).

362 As the Ec-1 and the other two Type-II pheromones, Ec-2 and Ec-3, were identified  
363 in different mating types of *E. crassus* by pheromone purification and molecular mass  
364 determination after chromatographic separation, confirmed by PCR amplification and  
365 sequencing, Type-II pheromones have been considered as mating type-specific [49, 50].  
366 However, our study demonstrated that six *E. vannus* mating types retain different  
367 combinations of Type-II pheromone loci in their MAC genomes (Figure 3C), and thus they  
368 exhibited highly different pheromone gene expression profiling instead of possessing  
369 exclusive, mating type-specific genes (Figure 3B). Furthermore, mating types EVL and  
370 EVP have the same set of pheromone genes but with different abundance (Figure 3C).  
371 Therefore, it suggests that the mating type-specific combination of the Type-II pheromone

372 loci might not be an all-or-none phenomenon, but a manner related to the composition or  
373 copy number of Type-II pheromone genes. Although further studies are expected, the  
374 observations of mating type-specific combination in the current study support the allelic  
375 codominance or non-hierarchical dominance relationship among signaling pheromone  
376 genes in euplotids [50, 52].

377

### 378 *A new model for mating type determination in ciliates*

379 Taken together, the current work revealed that euplotids have a novel MTD manner by  
380 which mating types are determined through mating type-specific combination of four Type-  
381 II pheromone genes (Figure 7C). Unlike *Paramecium tetraurelia*, there is no excision event  
382 on promoter regions of *E. vannus* pheromone genes (Figure 3C, Figure 7A and  
383 supplementary information, Figure S6). On the other hand, the MAC of *E. vannus* does not  
384 possess exclusive mating type-specific MTD loci as *Tetrahymena thermophila* (Figure 3  
385 and Figure 7B). This mating type-specific feature of Type-II pheromones comes from the  
386 programmed DNA rearrangement between germline and somatic genomes. Intriguingly,  
387 none of the *E. vannus* mating types we have identified possesses all four Type-II  
388 pheromone genes in MAC (Figure 3B and supplementary information, Figure S9). Thus,  
389 the results of current study described a third MTD type in ciliate (Figure 7C).

390

### 391 Molecular basis of the HSP70's lack of response to temperature stress

392 A previous study indicated that response of the HSP70 gene expression to temperature  
393 change, no matter gradually or abruptly, was divergent between *E. nobilii* and *E. focardii*  
394 [14]. When transferred from 4 to 20 °C, a strong transcriptional activity of HSP70 genes  
395 was induced in *E. nobilii* cells, while no measurable change was found in cells of *E.*  
396 *focardii*. In contrast, HSP70 expression increased with oxidative and chemical stresses  
397 such as tributyltin and sodium arsenite [15]. Furthermore, together with the results from the  
398 previous studies [67, 83], the current work strongly suggest HSP70 gene of *E. vannus*,  
399 which is largely divergent from that of with *E. focardii*, does not carry unique amino acid  
400 substitutions of potential significance for cold adaptation (Figure 6A).

401 The cosmopolitan species *E. vannus* has a similar pattern of HSP70 gene activation  
402 to the Antarctic psychrophilic euplotid *E. focardii*, in contrast to the euplotid *E. nobilii* in  
403 which the HSP70 gene expression changed with both thermal and chemical stresses  
404 (Figure 6B). A previous study reported no substantial difference in the organization of the  
405 HSP70 5' promoter region between *E. focardii* and *E. nobilii*, but an adenine-rich element  
406 which would exclude a rapid mRNA degradation was detected in the HSP70 3' regulatory  
407 region of *E. nobilii* [16]. In both two euplotids, the 5' promoter region harbors the *cis*-acting  
408 elements like heat-shock elements (HSE) and stress-response elements (StRE), which are  
409 known to be targets of trans-acting transcriptional activators characterized in a variety of  
410 organisms in association with their stress-inducible genes [84-86]. It's also argued that the  
411 HSE-modulated HSP70 gene transcription is more specific for a response to temperature  
412 stress while the StRE-modulated HSP70 gene transcription is more specific for a response  
413 to a broader range of non-temperature stresses [16]. Combining these data with  
414 observations in the current work, we argue that structural divergence of transcriptional  
415 *trans*-activating factors underlies that lack of change of HSP70 gene expression in  
416 response to temperature stress in *E. vannus* and *E. focardii*

417

418

## 419 **Conclusions**

420 In the current work, we present a high-quality macronuclear and a partial micronuclear  
421 genome assemblies of a unicellular eukaryote, *Euplotes vannus*, which possesses "gene-  
422 sized" MAC nanochromosomes. Comparative genome analysis reveals that *E. vannus*  
423 shares similar pattern on frameshifting and stop codon usage with *E. octocarinatus* and is  
424 undergoing incipient sympatric speciation with *E. crassus*. The further investigation on  
425 *Euplotes* pheromones indicates that *E. vannus* has a set of orthologous pheromones with  
426 the reported ones in *E. crassus* as well as a novel type of pheromone named as Ev-4  
427 which also shares close homology with *E. crassus*, and thus explains the hybridization  
428 between these two species on the molecular level. Besides, the homologous search  
429 between MAC and MIC genomes reveals that pheromone genes in *E. vannus* develop by  
430 programmed DNA rearrangement. Furthermore, chromatin and expression profiling of  
431 pheromone genes indicated that the combination of these genes is mating type-specific on

432 genic level and thus provides new evidence for common pheromone-mediated cell-cell  
433 signaling and cross-mating. According to the analyses of transcriptomes under different  
434 environmental stresses, although the HSP70 gene of *E. vannus* does not carry unique  
435 amino acid substitutions of potential significance for cold adaptation, it has evolved to be  
436 insensitive to temperature change by losing mRNA destabilization ARE elements in the 3'  
437 regulatory region of HSP70.

438

439

## 440 **Methods**

### 441 Cell culture

442 Six mating types of *Euplotes vannus* (EVJ, EVK, EVL, EVM, EVP and EVX) were collected  
443 from seawater along the coast of Yellow Sea at Qingdao (36°06' N, 120°32' E), China.  
444 Cells of each mating type were cultured separately in filtered marine water at 20°C for 10  
445 days, with a monoclonal population of *Escherichia coli* as the food source, until reaching  
446  $10^6$  cells.

447

### 448 Experimental treatment simulating environmental stresses

449 To simulate the stress from nutrient scarcity,  $10^6$  cells of each mating type (EVJ, EVK, EVL,  
450 EVM, EVP and EVX) of *E. vannus* were starved for 24 hours before harvest. For stresses  
451 from low and high temperature,  $10^6$  cells of *E. vannus* mating type EVJ were cultured  
452 under the temperature of 4 °C and 35 °C, respectively, for 6 hours before harvest. For  
453 stress from low and high salinity,  $10^6$  cells of mating type EVJ were cultured under the  
454 salinity of 10 psu and 60 psu, respectively, for 6 hours before harvest. For stress from the  
455 presence of free ammonia,  $10^6$  cells of mating type EVJ were cultured in filtered marine  
456 water with 100 mg/L NH<sub>4</sub>Cl (pH 8.3, 20 °C and 35 psu), as described in the previous study  
457 [36]. Cells in two negative control groups were culture under pH 7.8 and pH 8.2,  
458 respectively, in filtered marine water under 20 °C and 35 psu. Each group had two  
459 biological replicates.

460



461 High-throughput sequencing and data processing

462 For regular genomic and transcriptomic sequencing to acquire macronucleus (MAC)  
463 genome information, cells were harvested by centrifugation at 300 g for 3 min. The  
464 genomic DNA was extracted using the DNeasy kit (QIAGEN, #69504, Germany). The total  
465 RNA was extracted using the RNeasy kit (QIAGEN, #74104, Germany) and digested with  
466 DNase. The rRNA fraction was depleted using GeneRead rRNA Depletion Kit (QIAGEN,  
467 #180211, Germany).

468 For single-cell whole-genome amplification to acquire micronucleus (MIC) genome  
469 information, a single vegetative cell of the mating type EVJ of *E. vannus* was picked and  
470 washed in PBS buffer (without Mg<sup>2+</sup> or Ca<sup>2+</sup>) and its MIC genomic DNA was enriched and  
471 amplified by using REPLI-g Single Cell Kit (QIAGEN, #150343, Germany), which was  
472 based on the whole-genome amplification (WGA) technology and tended to amplify longer  
473 DNA fragments.

474 Illumina libraries were prepared from amplified single-cell MIC genomic DNA of *E.*  
475 *vannus* according to manufacturer's instructions and paired-end sequencing (150 bp read  
476 length) was performed using an Illumina HiSeq4000 sequencer. The sequencing adapter  
477 was trimmed and low-quality reads (reads containing more than 10% Ns or 50% bases  
478 with Q value <= 5) were filtered out.

479

480 Genome assembly and annotation

481 Genomes of four mating types (EVJ, EVK, EVL and EVM) were assembled using SPAdes  
482 v3.7.1 (-k 21,33,55,77), respectively [87, 88]. Mitochondrial genomic peptides of ciliates  
483 and genome sequences of bacteria were downloaded from GenBank as BLAST databases  
484 to remove contamination caused by mitochondria or bacteria (BLAST E-value cutoff = 1e-  
485 5). CD-HIT v4.6.1 (CD-HIT-EST, -c 0.98 -n 10 -r 1) was employed to eliminate the  
486 redundancy of contigs (with sequence identity threshold = 98%) [89]. Poorly supported  
487 contigs (coverage < 5 and length < 300 bp) were discarded by a custom Perl script.

488 A final genome assembly of *E. vannus* was merged from the genome assemblies of  
489 four mating types (EVJ, EVK, EVL and EVM) by CAP3 v12/21/07 [90]. Completeness of  
490 genome assembly was evaluated based on expectations of gene content by BUSCO v3

491 (dataset "Alveolata") and the percentage of both genomic and transcriptomic reads  
492 mapping to the final assembly by HISAT2 v2.0.4 [91, 92]. Reads mapping results were  
493 visualized on GBrowse v2.0 [93]. Genome assemblies of *E. crassus* (accession numbers:  
494 GCA\_001880385.1) and *E. octocarinatus* (accession numbers: PRJNA294366) and their  
495 annotation information were acquired from NCBI database and the previous studies [39,  
496 94, 95].

497 Telomeres were detected by using a custom Perl script which recognized the  
498 telomere repeat 8-mer 5'-(C4A4)n-3' at the ends of contigs, as described in a previous  
499 study [96]. The repeats in the merged genome assembly were annotated by combining *de*  
500 *novo* prediction and homology searches using RepeatMasker (-engine wublast -species  
501 '*Euplotes vannus*' -s -no\_is) [97]. *De novo* genome-wide gene predictions were performed  
502 using AUGUSTUS v3.2.2 (--species = euplotes, modified from the model "tetrahymena",  
503 rearranging TAA/TAG as stop codon, TGA as Cys) [98]. ncRNA genes were detected by  
504 tRNAscan-SE v1.3.1 and Rfam v11.0 [99, 100].

505

#### 506 Gene modeling and functional annotation

507 After mapping RNA-seq data of each mating type of *E. vannus* back to the merged  
508 reference genome assembly, the transcriptome of six mating types as well as a merged  
509 transcriptome were acquired by using StringTie v1.3.3b [101]. Annotation of predicted  
510 protein products were matched to domains in Pfam-A database by InterProScan v5.23 and  
511 ciliate gene database from NCBI GenBank by BLAST+ v2.3.0 (E-value cutoff = 1e-5) [102,  
512 103].

513

#### 514 Comparative genomic analysis

515 BLAST+ v2.3.0 was employed to search ciliate gene database from NCBI GenBank to  
516 identify corresponding homologous sequences in euplotids (E-value cutoff = 1e-1 and  
517 match length cutoff = 100 nt) [103]. "Joint" chromosomes were detected by using a custom  
518 Perl script which recognized the chromosomes containing multiple genes (cutoff of  
519 distance between two genes = 100 nt). Frameshifting events were detected by using a  
520 custom Perl script which recognized the frame change between two BLASTX hits (E-value

521 cutoff = 1e-5 and inner distance  $\leq$  10 nt), modified from the protocol in a previous study  
522 [39], with the addition of a strict criterion (the distance between two adjacent hits with  
523 different frames  $\leq$  10 bp) to make sure no intron was involved. 30 bp sequences from the  
524 upstream and downstream of each type of frameshifting site (+1, +2 or -1) were extracted  
525 to identify the motif. Local motifs of nearby frameshifting sites were illustrated by WebLogo  
526 3 [104]. The frequency of stop codon usage was estimated by a custom Perl script which  
527 recognized the stop codon TAA or TAG in transcripts of euplotids.

528

### 529 Differential gene expression analysis

530 Transcript abundances were estimated and differential gene expression was analyzed by  
531 using featureCounts [105] and R packages "Ballgown" and "DESeq2" (p.adjust < 0.01)  
532 [106, 107]. Starvation induced genes were defined as the average value of RPKM of gene  
533 expression from starved samples > 1 and the average value of RPKM of gene expression  
534 from vegetative samples < 0.1. Mating type-specific transcripts were defined as the  
535 average value of RPKM of gene expression from starved samples > 5 and the average  
536 value of RPKM of gene expression from vegetative samples < 0.1. Weighted gene co-  
537 expression eigengene network analysis was performed by WGCNA [108]. Gene Ontology  
538 (GO) term enrichment analysis was performed by using BiNGO v3.0.3 (p.adjust < 0.05),  
539 which was integrated in Cytoscape v3.4.0, and the plot was generated by the R package,  
540 ggplot2 [109-111].

541

### 542 Homolog detection of pheromone genes and environmental stress-related genes

543 Homologous pheromone gene sequences in *E. vannus* were acquired by using BLAST+  
544 v2.3.0 (E-value cutoff = 1e-5), according to the pheromone sequences of *E. crassus* [49,  
545 50]. Genomic DNA samples were harvested from vegetative cells of six mating types of *E.*  
546 *vannus*. Type-II pheromone loci in MAC were amplified using Q5 High-Fidelity 2X Master  
547 Mix (NEB, #M0492S, US) with 10 cells of each mating type and genotyping primers (PCR  
548 annealing temperature was 64.5 °C, sequences of genotyping primers see supplementary  
549 information, Table S9).

550 Homologous HSP70 gene sequences in *E. vannus* was acquired by using BLAST+  
551 v2.3.0 (E-value cutoff = 1e-5), according to the Hsp70 protein sequences of *E. focardii* and  
552 *E. nobilii* from the previous studies (GenBank accession number: AAP51165 and  
553 ABI23727, respectively) [14, 16]. The complete sequences of the *E. focardii* and *E. nobilii*  
554 HSP70 genes are available at NCBI with the accession numbers AY295877 and  
555 DQ866998, according to the previous studies [15, 16]. The essential amino acid positions  
556 of Hsp70 were reported in previous studies [112, 113]. The consensus amino acids  
557 sequence of Hsp70 was according to the previous reports [15, 16].

558

### 559 Phylogenetic analysis

560 The DNA and amino acid sequences of *Euplotes* pheromones homologous genes were  
561 acquired from NCBI, according to the previous work [50], and aligned by MUSCLE v3.8.31  
562 and ClustalW v2.1, respectively [114, 115]. Maximum Likelihood tree based on amino acid  
563 sequences was reconstructed by MEGA v7.0.20, using the LG model of amino acid  
564 substitution, 500 bootstrap replicates [116, 117].

565 For phylogenomic analysis by supertree approach, predicted protein sequences of  
566 *Euplotes vannus* by us and other 31 ciliates from previous works or transcriptome  
567 sequencing by the Marine Microbial Eukaryote Transcriptome Sequencing Project (data  
568 available on iMicrobe: <http://imicrobe.us/>, accession number and gene ID see  
569 supplementary information, Table S10) [95, 118-121] were used to generate the  
570 concatenated dataset. Maximum Likelihood tree based on the concatenated dataset  
571 covering 157 genes was reconstructed by using GPSit v1.0 (relaxed masking, E-value  
572 cutoff = 1e-10, sequence identity cutoff = 50%) [58] and RAXML-HPC2 v8.2.9 (on CIPRES  
573 Science Gateway, LG model of amino acid substitution +  $\Gamma$  distribution + F, four rate  
574 categories, 500 bootstrap replicates) [122]. Trees were visualized by MEGA version 7.0.20  
575 [116].

576

577

### 578 Additional files

579 **Figure S1.** K-mer analysis of *Euplotes vannus* mating types to estimated genome size.

580 **Figure S2.** Genome assembly completeness evaluation of ciliates by BUSCO.

581 **Figure S3.** Venn diagram shows the genes annotated by BLASTX and Interproscan.

582 **Figure S4.** Schematic representation of the exon/intron boundaries with WebLogo in all  
583 78661 introns in *E. vannus* mating type EVJ. The GTR and YAG motifs are well conserved.

584 **Figure S5.** A schema illustrates the criteria for detecting +1 frameshifting events. Blue  
585 boxes indicate the different BLASTX hits of a CDS region to a same target protein  
586 sequence (E-value cutoff =  $1e-5$ ). Grey boxes indicate the adjacent region between two  
587 BLASTX hits of a CDS region (inner distance cutoff = 10 nt). The brackets above denote  
588 the 0-frame codons and the brackets underneath denote the +1-frame codons. Yellow dots  
589 denote the nucleotides while the red ones denote the slippery site where frameshifting  
590 events occur.

591 **Figure S6.** Sequence alignment of the reverse compliments of the MAC contigs containing  
592 Type-II pheromone coding genes in *E. vannus* and *E. crassus*. Blue and red boxes denote  
593 the start and stop codon of the coding region of pheromone genes, respectively.

594 **Figure S7.** Sequence alignment of the Type-I pheromone protein sequences in *E. vannus*  
595 and *E. crassus*. Identical residues are shadowed in black and similar residues are shaded  
596 in grey. Asterisks mark the positions of stop codons. Filled and light arrowheads indicate  
597 the extension positions of the pre- and pro-regions, respectively. Red dots denote the  
598 conserved cysteine residues in secreted region. Numbers indicate the progressive amino  
599 acid positions in the sequences.

600 **Figure S8.** Sequence alignment of the reverse compliments of the MAC contigs containing  
601 Type-I pheromone coding genes in *E. vannus* and *E. crassus*. Blue and red boxes denote  
602 the start and stop codon of the coding region of pheromone genes, respectively.

603 **Figure S9.** GBrowse snapshots of genomic and transcriptomic reads mapping on  
604 pheromone gene-related chromosomes in different mating types.

605 **Figure S10.** Overall gene expression level in starved or vegetative cells of different mating  
606 types.

607 **Figure S11.** Venn diagram shows a large part of mating type-specific transcripts is also  
608 starvation induced.

609 **Figure S12.** Species relationship and functional annotation of the mating type-specific and  
610 starvation induced transcripts.

611 **Figure S13.** Differential gene expression analysis under temperature, salinity and free  
612 ammonia stresses (relative to Figure 5). (A) PCA analysis on gene expression of mating  
613 type EVJ under different stresses. (B) Different environmental stresses activated or  
614 deactivated different gene groups.

615 **Figure S14.** Sequence alignment of Hsp70 protein sequences.

616 **Table S1.** Mating pattern observed when cultures of two mating types are mixed and  
617 genomic and transcriptomic (mRNA) data accessibility of six mating types of *E. vannus*.

618 **Table S2.** Genome assembly information of four mating types of *Euplotes vannus*.

619 **Table S3.** Genomic and transcriptomic reads mapping information.

620 **Table S4.** List of identified ncRNAs.

621 **Table S5.** Annotation information of repeats in the merged genome assembly of *E. vannus*.

622 **Table S6.** Expression and annotation information of *Euplotes vannus* genes.

623 **Table S7.** Homologs of mating-type loci in each mating type.

624 **Table S8.** Homologous search results by BLASTN reveal the relationship between coding  
625 regions of four pheromone genes in MAC genome and the corresponding MDS regions in  
626 MIC genome of *E. vannus*.

627 **Table S9.** PCR primers for genotyping of Type-II pheromone genes in *E. vannus* and  
628 determine mating types.

629 **Table S10.** Information of accession of genome/transcriptome assemblies of 32 ciliates.

630

## 631 **Abbreviations**

632 Ev: *Euplotes vannus*; Ec: *Euplotes crassus*; Eo: *Euplotes octocarinatus*; En: *Euplotes*  
633 *nobili*; Er: *Euplotes raikovi*; IES: internal eliminated sequence; MAC: macronucleus; MIC:  
634 micronucleus; MDS: macronucleus destined sequence; ML: maximum likelihood; MTD:  
635 mating type determination; PRF: programmed ribosomal frameshifting.

636

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657

## 658 **Availability of data and materials**

659 *Euplotes vannus* MAC genome assembly and gene annotation data including coding  
660 regions and predicted protein sequences are available at *Euplotes vannus* DB (EVDB,  
661 <http://evan.ciliate.org>).

662

## 663 **Authors' contributions**

664 XC and FG conceived the study; YHJ and WBZ provided the biological materials; XC  
665 designed the experiments; YHJ performed the experiments; XC performed computational  
666 and experimental analysis for all figures and tables; XC, FG, CL, LK and WS interpreted  
667 the data; TK and NS constructed the genome database website; XC wrote the paper with  
668 contribution from all authors. All authors read and approved the final manuscript.

669

## 670 **Ethics approval and consent to participate**

671 Not applicable.

672

## 673 **Consent for publication**

674 Not applicable.

675

## 676 **Competing interests**

677 The authors declare that they have no competing interests.

678

679

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1001 **Table 1.** MAC genome assembly and transcriptome-improved gene annotation of *Euplotes*  
1002 *vannus* in comparison with that of other euplotids.

	<i>E. vannus</i>	<i>E. crassus</i>	<i>E. octocarinatus</i>
Genome size (Mb)	85.1	58.6	88.9
%GC	37.0	38.7	28.2
Contig #	38245	56587	41980
Contig N50 (bp)	2685	1581	2947
2-telomere contig #	25519	13783	29532
1-telomere contig #	7835	20646	4842
0-telomere contig #	4890	22158	7606
2-telomere contig percentage (%)	66.7	24.4	70.3
Genome size (with telomere) (Mb)	52.5	44.9	83.1
Scaffold (with telomere) #	33354	34429	34374
%Scaffold (with telomere)	87.2	60.8	81.9
Scaffold N50 (bp)	2714	1830	2999
Gene #	32755	-	29076
Exon #	175735	-	96843
Transcript #	43040	-	29076

1003

1004

1005 **Table 2.** MIC genome assembly information of *Euplotes vannus* and recognition of MDS-  
1006 containing contigs and those contain multiple MDSs.

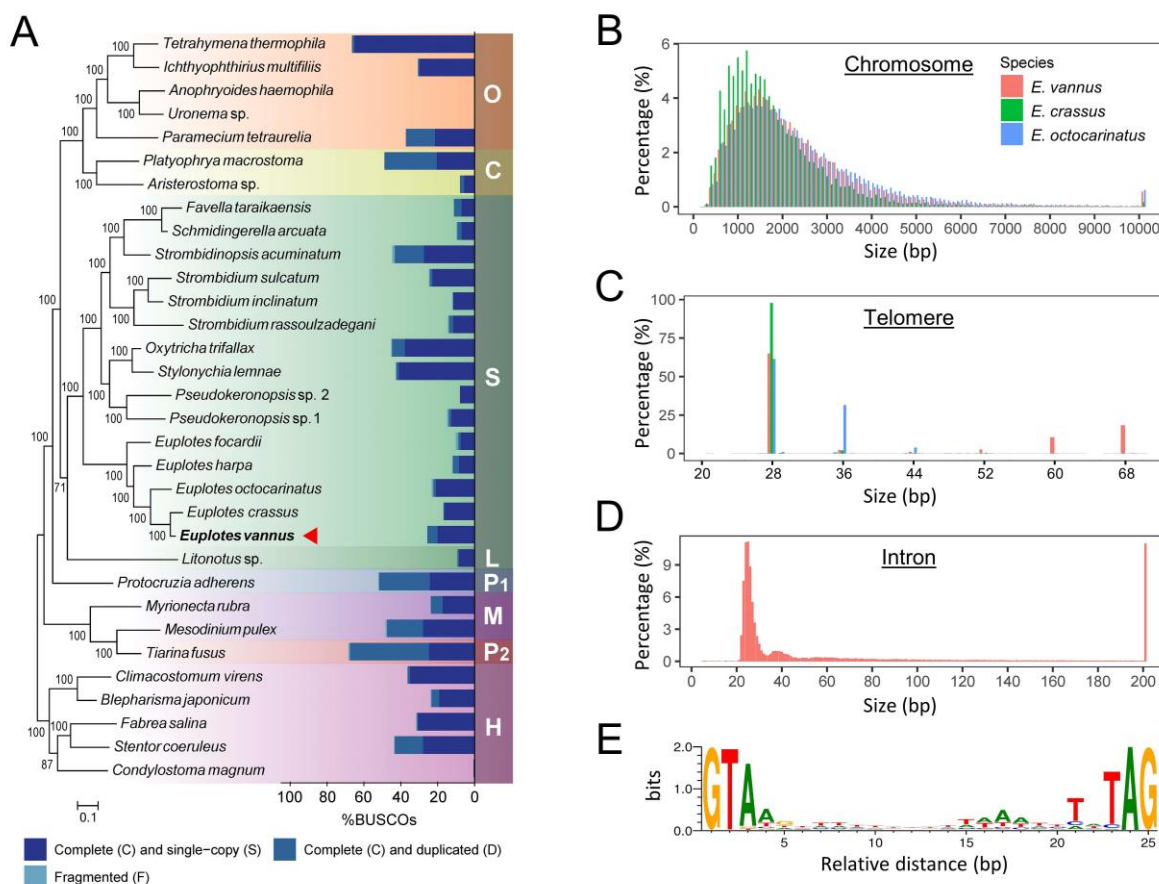
<b>MIC genome</b>	<b>Total</b>	<b>With MDS</b>	<b>Multi-MDS</b>
Genome size (Mb)	120.0	49.8	31.8
%GC	36.0	35.7	35.9
Contig #	104988	13140	5166
Contig N50 (bp)	1953	5597	7718

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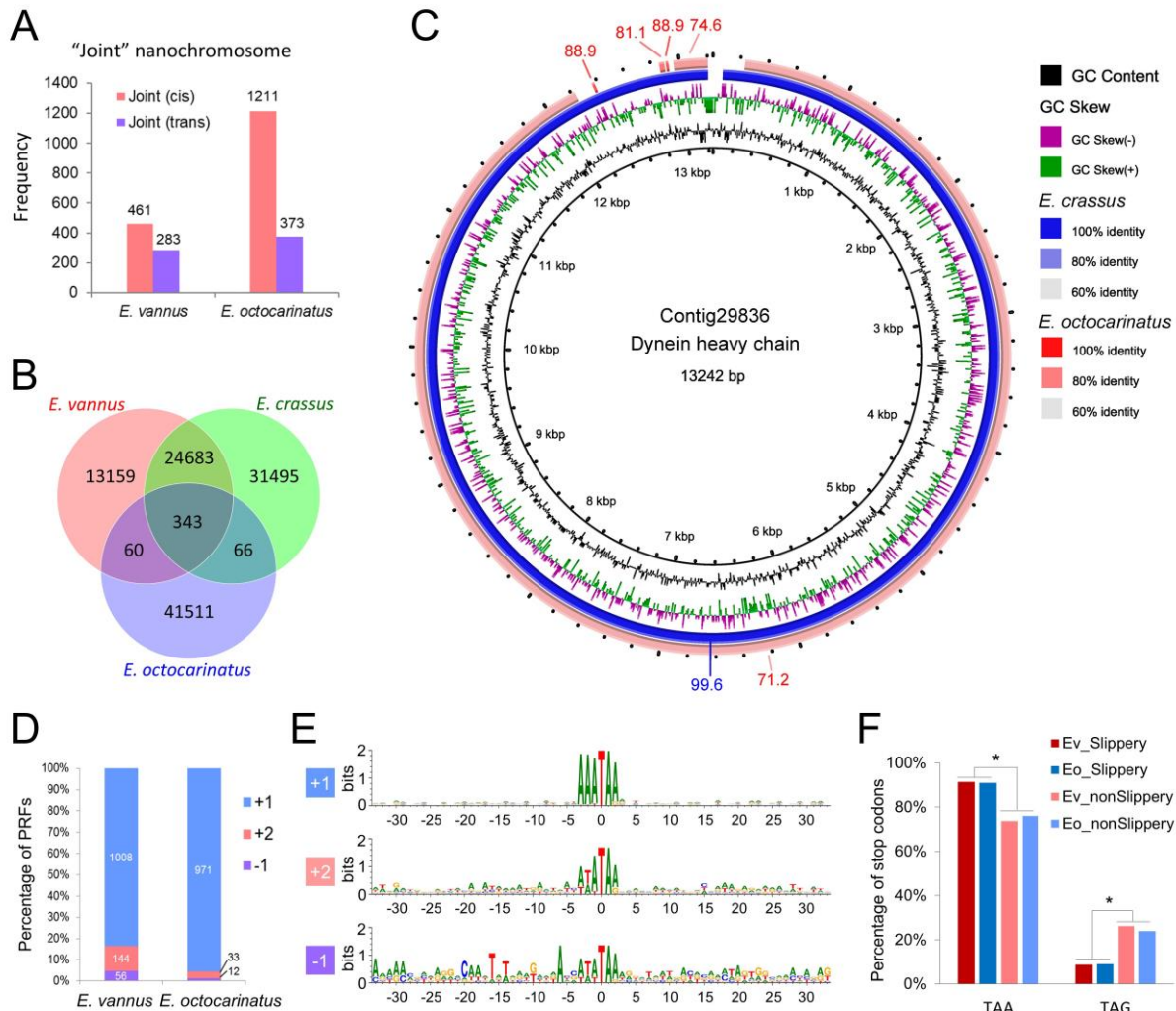
1009 **Figure captions**



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1011 **Figure 1. Genome assembly of *E. vannus*.** (A) Maximum likelihood phylogenetic tree by  
 1012 supermatrix approach and assembly completeness evaluation of ciliate  
 1013 genomes/transcriptomes by BUSCO. Dark blue blocks represent the percentage  
 1014 of complete and single-copy genes among protists, and the steel-blue blocks  
 1015 represent that of complete and duplicated genes in each species. Genomic data  
 1016 of *Euplotes vannus* sequenced in the current work is marked by the red triangle.  
 1017 S: class Spirotrichea. L: class Litostomatea. O: class Oligohymenophorea. C:  
 1018 class Colpodea. P1: class Protocruzia. M: class Mesodiniea. P2: class  
 1019 Prostomatea. H: class Heterotrichea. The scale bar corresponds to 10  
 1020 substitutions per 100 nucleotide positions. (B) Size distribution of 2-telomere  
 1021 scaffolds of *E. vannus*, *E. crassus* and *E. octocarinatus*. (C) Size distribution  
 1022 of telomeres of *E. vannus*, *E. crassus* and *E. octocarinatus*. (D) Size distribution  
 1023 of introns in *E. vannus* mating type EVJ. (E) Sequence motif of 8792 tiny introns  
 1024 with the size of 25 nt. Weblogo was generated and normalized to neutral base  
 1025 frequencies in intergenic regions.

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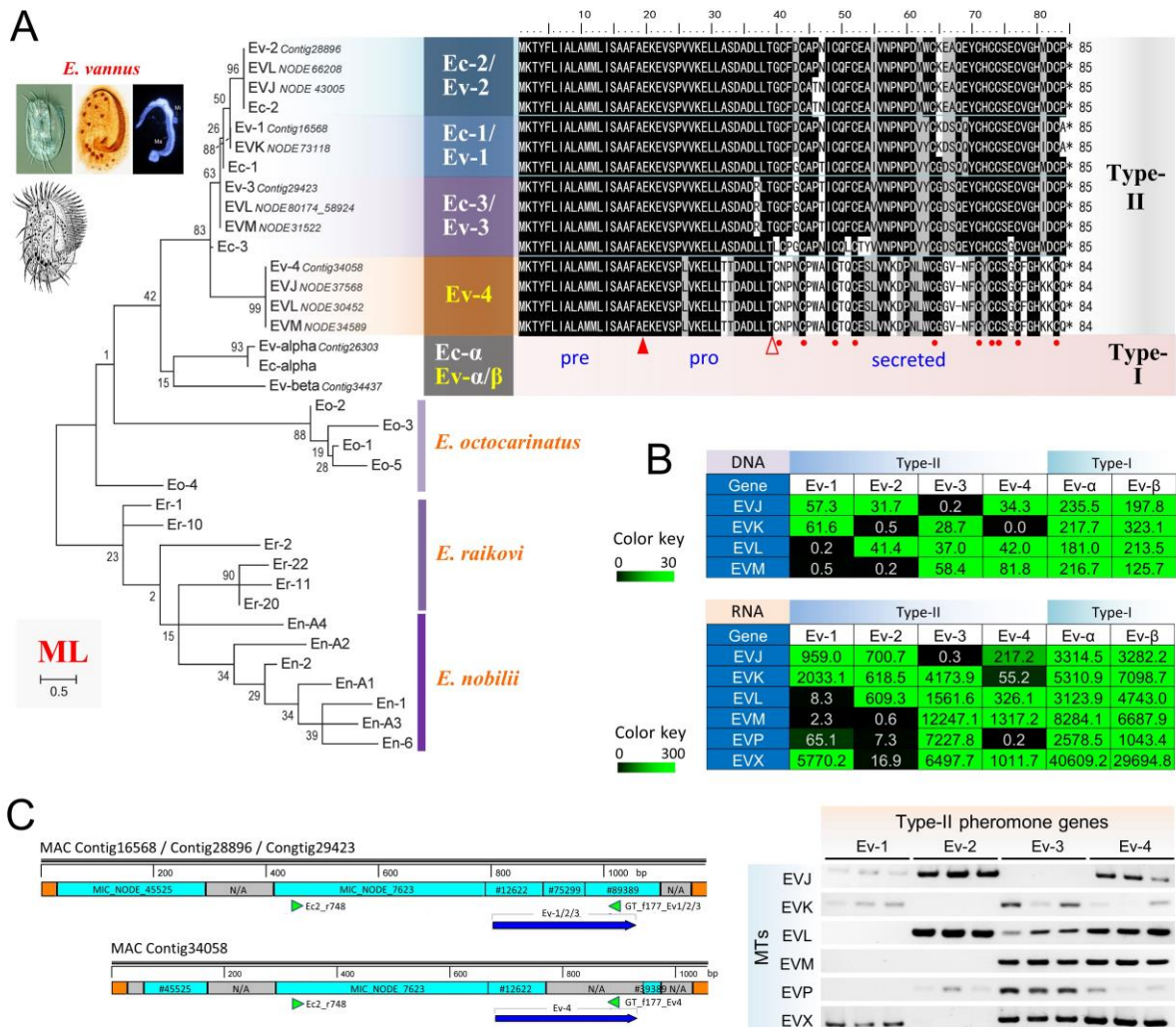
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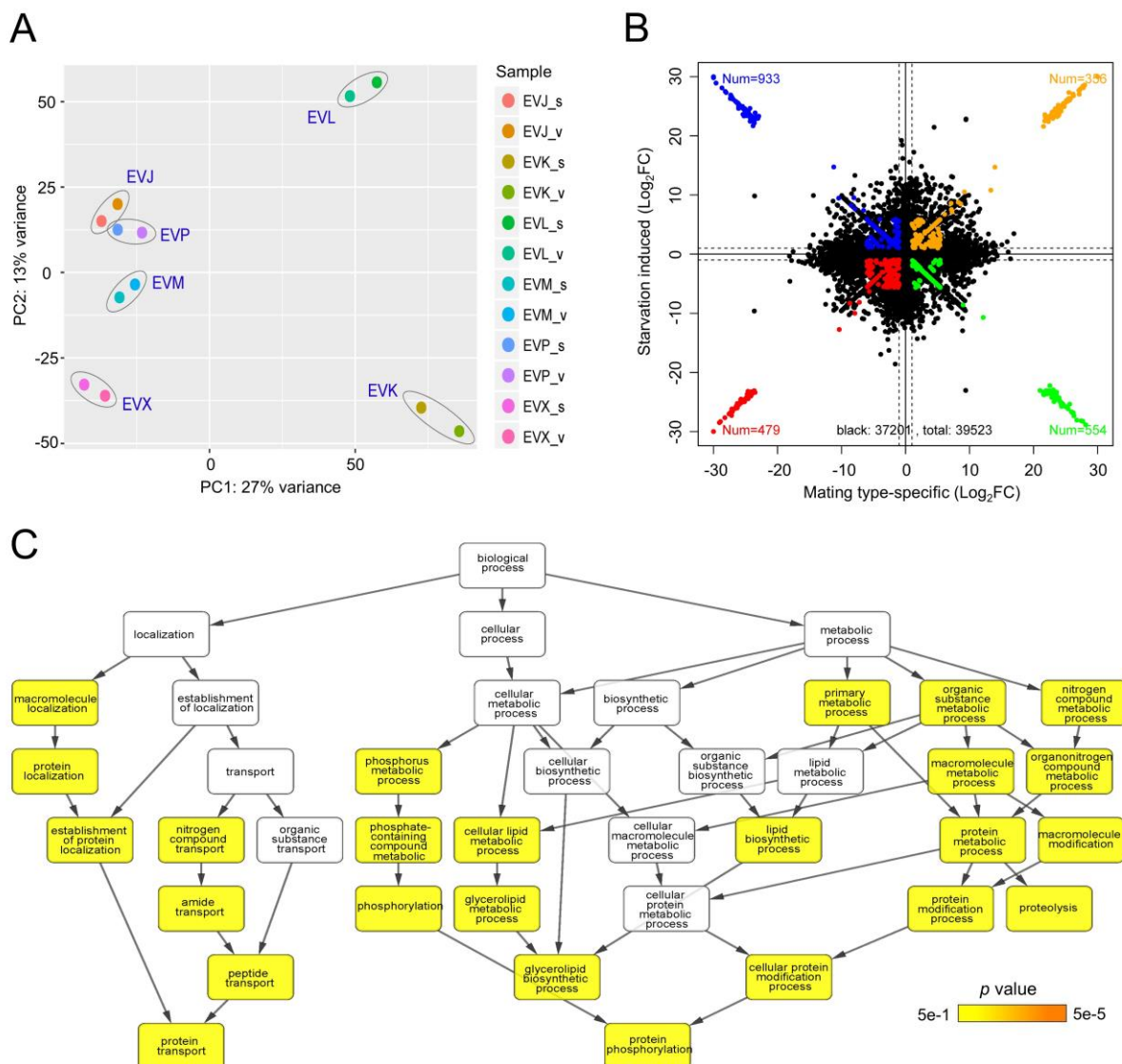
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**Figure 2. Evolution and synteny/comparative genomic analyses.** (A) "Joint" nanochromosome detection in *E. vannus* and *E. octocarinatus*. Red bars denote the frequency of joint nanochromosomes containing genes in a same transcription direction (cis) and purple bars denote that of joint nanochromosomes containing genes in opposite transcription directions (trans). (B) Closely related contigs among three euplotids. (C) Homologous comparison of the contigs containing dynein heavy chain coding gene among *E. vannus* (as reference), *E. crassus* (blue) and *E. octocarinatus* (red). (D) Frameshifting detection & comparison with *E. octocarinatus*. (E) Conserved sequence motif associated with frameshift sites. Sizes of letters denote information content, or sequence conservation, at each position. The analysis is based on the alignment of 30 bp upstream and downstream the frameshifting motif from 1236 predicted +1 frameshifting events that involves stop codon TAA or TAG. Note the canonical motif 5'-AAA-TAR-3' (R = A or G) in +1 PRF and noncanonical motif 5'-WWW-TAR-3' (W = A or T) in +2 and -1 PRF. (F) Stop codon usage in slippery and non-slippery transcripts of *E. vannus* (Ev) and *E. octocarinatus* (Eo). Asterisks denote the significant difference ( $p < 0.01$ ).



**Figure 3. Genomic investigation revealed new pheromone loci Ev-4 and Ev-beta and a mating type-specific combination of these loci in *E. vannus*.** (A) Phylogenetic analysis of *Euplotes* pheromones and sequence alignment of Type-II pheromones of *E. vannus* and *E. crassus*. Identical residues are shadowed in black and similar residues are shaded in grey. Asterisks mark the positions of stop codons. Filled and light arrowheads indicate the extension positions of the pre- and pro-regions, respectively. Red dots denote the 10 conserved cysteine residues in secreted region. Numbers indicate the progressive amino acid positions in the sequences. (B) Chromatin and gene expression profiling based on the RPKM of pheromone loci in each mating type of *E. vannus* by genome and transcriptome sequencing. The tables are colored by RPKM values. (C) The primer design (left) and PCR amplification results (right) of *E. vannus* Type-II pheromone genes. Boxes in orange, blue and grey denote the telomeres of the MAC contigs containing the pheromone genes, the MDS regions and regions those loci in MIC genome still unknown (N/A), respectively. Arrow heads in green denote the positions of the primers. Arrows in blue denote the coding regions of pheromone genes. PCR amplifications of each pheromone gene in each mating type are conducted with three biological replicates.

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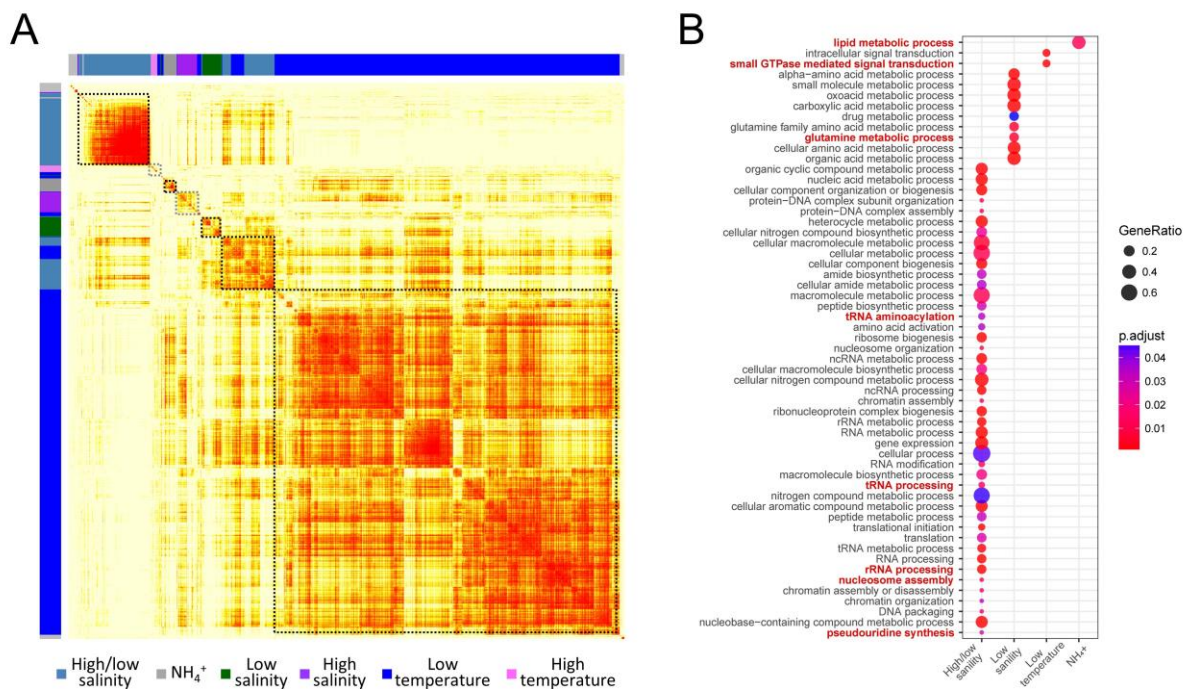
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**Figure 4. Gene function analysis of mating type-specific transcripts of *E. vannus*.** (A) PCA analysis on transcript enrichment of different mating types. (B) Cross plot of differential expression of mating type-specific and starvation-regulated transcripts. (C) GO enrichment analysis of mating type-specific and starvation induced transcripts.



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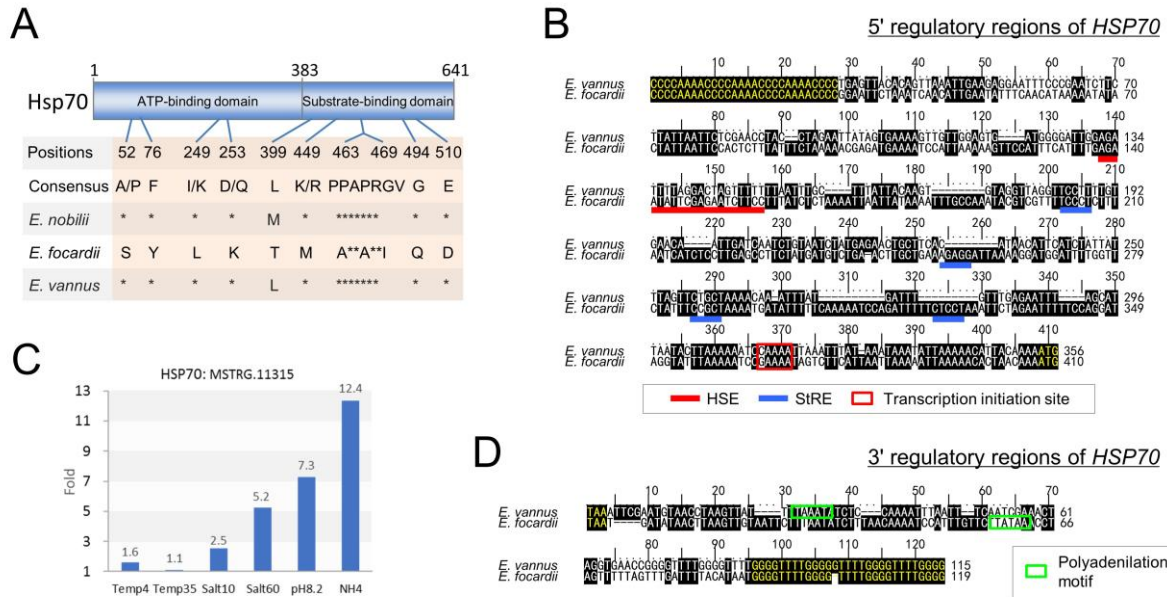
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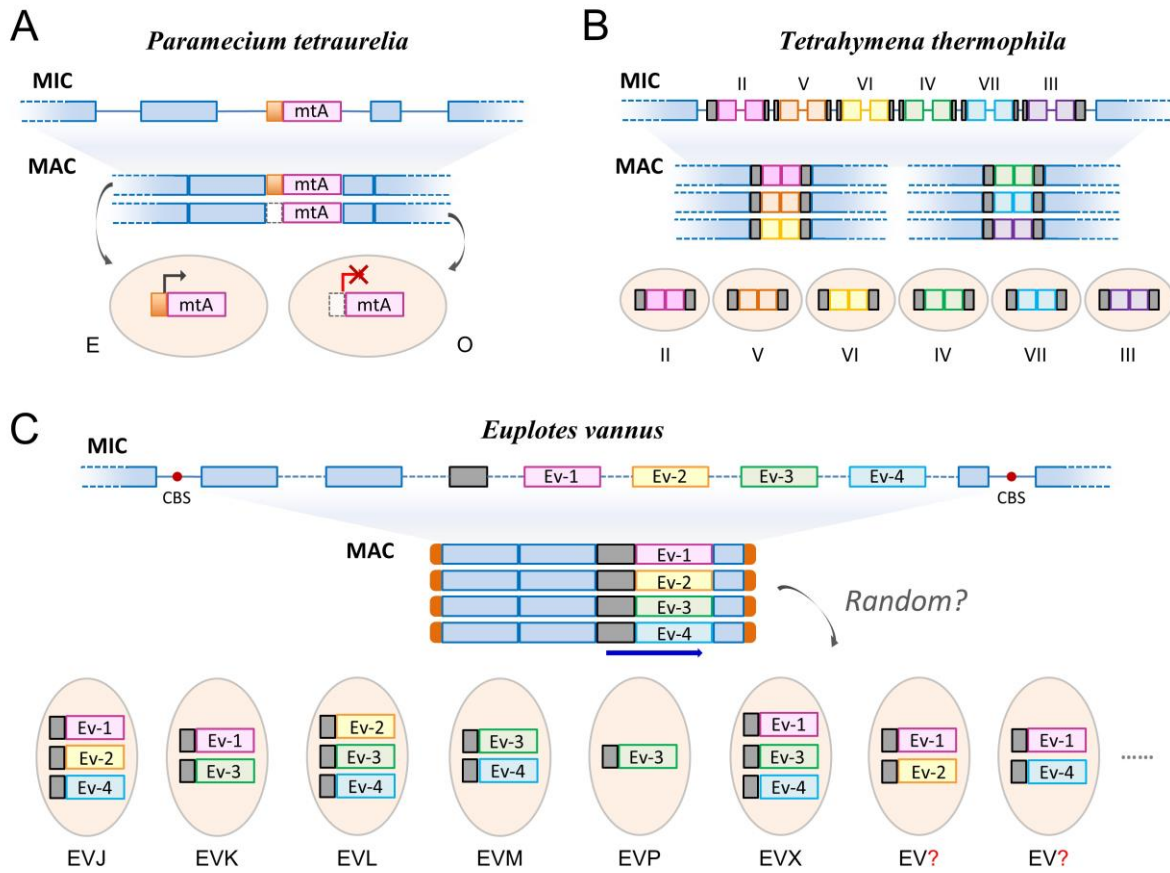
**Figure 5. Differential gene expression analysis under temperature, salinity and free ammonia stresses.** (A) Heatmap of weighted gene co-expression network, in accordance with different stress-response gene groups. (B) GO term enrichment analysis on different stress-response gene groups.



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1080 **Figure 6. HSP70 gene expression analysis under temperature, salinity and free**  
 1081 **ammonia stresses.** (A) Amino acid substitutions that occur in *E. focardii* at the  
 1082 level of its HSP70 ATP- and substrate-binding domains and are unique with  
 1083 respect to *E. nobilii* and other organisms. Asterisks denote identities. Numbers  
 1084 indicate essential amino acid positions of Hsp70. (B) Up-regulation folds in cells  
 1085 under different environmental stresses with respect to the control (25°C, 35 psu  
 1086 and pH 7.8). (C) Nucleotide sequence alignment of the 5' regulatory regions of  
 1087 the *E. vannus* (gene "MSTRG.11315" on contig "Contig21532") and *E. focardii*  
 1088 HSP70 genes. The identities are shaded; the telomeric C4A4 repeats and  
 1089 transcription initiation ATG codons are in yellow; putative sites for the  
 1090 transcription initiation are boxed; sequence motifs bearing agreement with HSE  
 1091 and StRE elements are indicated by red and blue bars, respectively. (D)  
 1092 Nucleotide sequence alignment of the 3' regulatory regions of the *E. vannus* and  
 1093 *E. focardii* HSP70 genes. The identities are shaded; the telomeric G4T4 repeats  
 1094 and stop TAA codons are in yellow; putative polyadenylation motifs are boxed.

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**Figure 7. Current models (simplified) for mating type determination in *Paramecium tetraurelia*, *Tetrahymena thermophila* and *Euplotes vannus*.** (A) One of the two *P. tetraurelia* mating types, mating type E depends on expression of the mtA gene during sexual reactivity. The other mating type O is determined during macronuclear development by excision of the mtA promoter (box in orange) as an internal eliminated sequence (IES), preventing expression of the gene. Adapted from Extended Data Figure 10 of Reference [41]. (B) Mating type gene pairs in *Tetrahymena thermophila* macronuclear are assembled by joining mating type-specific macronucleus destined sequence (MDS) from micronuclear to reproduce six mating types. Segments filled with grey represent conserved transmembrane regions. Adapted from Figure 3 of Reference [42]. (C) *E. vannus* shows a mating type-specific feature on the combination of different pheromone genes in MAC. Red dots denote chromosome breakage site (CBS). Segments filled with solid orange, light colors and grey denote telomeres, MDSs and conserved transmembrane regions, respectively. Dashed lines denote putative IESs.