Title
Distribution of the Sex-Determining Gene MID and Molecular Correspondence of Mating Types within the Isogamous Genus Gonium (Volvocales, Chlorophyta).

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Isogamy is a mode of sexual reproduction involving the agglutination and fusion of two gametes that are essentially identical in size and shape. Isogamous organisms are widespread in eukaryotes such as yeasts and algae. The genus *Gonium* comprises colonial volvocalean green algae consisting of 8-, 16- or 32-cells in the form of a curved plate; the isogametes of *plus* and *minus* of most *Gonium* species form tubular mating structures (TMS) at the base of the two flagella [1]. Nozaki [2] called this mode of TMS formation “bilateral mating papilla.” *G. multicoccum* gametes do not have any TMS [3].

*Chlamydomonas reinhardtii*, an isogamous single-celled green alga, has two genetically determined, heterothallic mating types: *plus* and *minus* [4]. It has been used to study molecular and cellular mechanisms of sexual development for over half a century. Although the gametic cell sizes of both mating types are similar, a *plus* gamete has a TMS or “fertilization tubule” filled with actin filaments, while a *minus* does not [5–8]. Such a mode of TMS formation is called “unilateral mating papilla” [2]. Recently, Mogi et al [9] immunostained actin localized to the TMS of activated gametes from both mating types in *G. pectorale*, suggesting a common subcellular architecture among the TMS of the unilateral and bilateral mating papilla.

Unilateral mating papilla may enable cytological determination of corresponding mating types across species, while bilateral mating papillae do not because they do not show any cytological gametic difference between the two. In the colonial Volvocales, more than one sexually isolated group or syngen is recognized in various morphological or taxonomic species (e.g. *Pandorina morum* [10]; *Gonium viridistellatum* [11]); correspondence based on crossing experiment is not definable even within a single species with bilateral mating papillae. Currently reported “mating types” of
Gonium strains have been determined based on crossing examinations within species, although their designations as “plus” or “minus” are arbitrary and do not necessarily correspond to those of the other species. To solve this lack of conformity, an objective and easily accessible molecular marker should be established. 

Such a marker should correspond to a conserved domain among lineages and cosegregate with one of the mating types. The C. reinhardtii mating type determining protein, minus dominance (MID), dominantly determines mating type minus as a transcription factor with a conserved putative DNA-binding RWP-RK domain [12–14], which served as a candidate sequence for designing degenerate primers for identification of homologs in colonial volvocalean algae, including two Gonium species [15–18]. MID homologs in reported organisms cosegregate with mating types or sexes, suggesting a conserved mechanism in sex determination/homologs. Thus,MID is an outstanding candidate for a molecular correspondence of mating types over species with bilateral mating papilla.

Here we propose a novel set of objective mating types in the genus Gonium, based on molecular identification of MID homologs. Nine strains of four Gonium species were examined. Quite interestingly, not only heterothallic strains but also a homothallic strain (G. multicoccum NIES-1708 [19]) retain a MID homolog.

Identification of MID homologs

Nested PCR with degenerate primers amplified partial regions of MID genes, based on which sequence-specific primers were designed for inverse PCR [21] or thermal asymmetric interlaced (TAIL) PCR [22] to sequence flanking regions (details are summarized in Text S1; primers are listed in Table 2).

Phylogenetic and molecular evolutionary analyses

Phylogenetic analyses were performed using two data sets. One consists of ClustalX 2.0 [24] aligned entire protein sequences of eleven MID homologs of the Volvocales (Fig. S1). The other alignment is composed of amino acid sequences (47 aa, Fig. S2) of RWP-RK domains (the 25 RWP-RK containing proteins recognized in C. reinhardtii and Volvox carteri genome databases, http://www.phytozome.net/ Phytozome v8.0, Joint Genome Institute, Walnut Creek, CA, USA [25,26], and the eleven MID homologs). Maximum likelihood (ML) method, based on Whelan and Goldman model (WAG) by PhyML 3.0 [27,28], and ML and neighbor joining method, using Jones-Taylor-Thornton model by MEGA version 5, were carried out with bootstrap values from 1000 replications [29–32].

A molecular evolutionary analysis of non-synonymous and synonymous substitutions was performed by YN00 in the PAML package [33,34].

Table 1. List of Gonium strains mentioned here.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Mating type designation</th>
<th>MID Acc. No.</th>
<th>MID/non-MID mating type (if heterothallic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G. maiaprilis</td>
<td>NIES-2455 (Asa041901,</td>
<td>plus</td>
<td>–</td>
<td>non-MID mating type</td>
</tr>
<tr>
<td></td>
<td>sampled 2004)</td>
<td></td>
<td>AB623044</td>
<td>M ID mating type</td>
</tr>
<tr>
<td>G. maiaprilis</td>
<td>NIES-2457 (Asa041903,</td>
<td>minus</td>
<td>AB774225 (this study)</td>
<td>M ID mating type</td>
</tr>
<tr>
<td></td>
<td>sampled 2004)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G. multicoccum</td>
<td>NIES-1038 (GQ-M-Tx-1</td>
<td>No designation</td>
<td>AB774226 (this study)</td>
<td>M ID mating type</td>
</tr>
<tr>
<td></td>
<td>*)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G. multicoccum</td>
<td>NIES-1039 (GQ-M-Tx-2</td>
<td>No designation</td>
<td>–</td>
<td>non-MID mating type</td>
</tr>
<tr>
<td></td>
<td>*)</td>
<td></td>
<td>AB774226</td>
<td>(homothallic)</td>
</tr>
<tr>
<td>G. octonarium</td>
<td>NIES-851 (GO-LC-1+</td>
<td>plus</td>
<td>–</td>
<td>non-MID mating type</td>
</tr>
<tr>
<td></td>
<td>*)</td>
<td></td>
<td>AB774227</td>
<td>M ID mating type</td>
</tr>
<tr>
<td>G. octonarium</td>
<td>NIES-852 (GO-LC-3–</td>
<td>minus</td>
<td>AB353340</td>
<td>M ID mating type</td>
</tr>
<tr>
<td></td>
<td>*)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G. pectorale</td>
<td>NIES-1710 (Kaneko3,</td>
<td>minus</td>
<td>–</td>
<td>non-MID mating type</td>
</tr>
<tr>
<td></td>
<td>sampled 2000)</td>
<td></td>
<td>AB774228</td>
<td>M ID mating type</td>
</tr>
<tr>
<td>G. pectorale</td>
<td>NIES-1711 (Kaneko4,</td>
<td>plus</td>
<td>–</td>
<td>non-MID mating type</td>
</tr>
<tr>
<td></td>
<td>sampled 2000)</td>
<td></td>
<td>AB774226</td>
<td>M ID mating type</td>
</tr>
<tr>
<td>G. quadratum</td>
<td>NIES-652 (90-423-3,</td>
<td>minus</td>
<td>–</td>
<td>non-MID mating type</td>
</tr>
<tr>
<td></td>
<td>sampled 1989)</td>
<td></td>
<td>AB353340</td>
<td>M ID mating type</td>
</tr>
<tr>
<td>G. quadratum</td>
<td>NIES-653 (90-423-2,</td>
<td>plus</td>
<td>–</td>
<td>non-MID mating type</td>
</tr>
<tr>
<td></td>
<td>sampled 1989)</td>
<td></td>
<td>AB774224</td>
<td>M ID mating type</td>
</tr>
<tr>
<td>G. viridistellatum</td>
<td>NIES-654 (KY-4 (+),</td>
<td>plus</td>
<td>–</td>
<td>non-MID mating type</td>
</tr>
<tr>
<td></td>
<td>UTEX 2519, sampled 1980</td>
<td></td>
<td>AB774224</td>
<td>M ID mating type</td>
</tr>
<tr>
<td>G. viridistellatum</td>
<td>NIES-655 (KY-7 (-),</td>
<td>minus</td>
<td>–</td>
<td>non-MID mating type</td>
</tr>
<tr>
<td></td>
<td>UTEX 2520, sampled 1980</td>
<td></td>
<td>AB774226</td>
<td>M ID mating type</td>
</tr>
</tbody>
</table>

*These four strains were kindly provided by Dr. Richard C. Starr (The Culture Collection of Algae at the University of Texas at Austin) in 1994.
**These two strains are heterothallic and formed zygotes when mixed (Nozaki, unpublished data).

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Materials and Methods

Strains and culture conditions

Strains were obtained from the Microbial Culture Collection at the National Institute of Environmental Studies (NIES) [20] as summarized in Table 1. Culture conditions were essentially the same as described previously [16].

Identification of MID homologs

In our degenerate PCR-based approach, the MID homolog from every species of Gonium was obtained (Table 1). The primary data were genomic sequences, so the exon-intron structures of MID homologs were manually predicted based on MID genes of G. pectorale and G. maiaprilis (Figure 1 [16,18]). The intron sites are

Results and Discussion

In our degenerate PCR-based approach, the MID homolog from every species of Gonium was obtained (Table 1). The primary data were genomic sequences, so the exon-intron structures of MID homologs were manually predicted based on MID genes of G. pectorale and G. maiaprilis (Figure 1 [16,18]). The intron sites are
exactly the same among the *Gonium* species; there are several insertion/deletion sites in the CDS.

*MID* orthologs of *Gonium* have moderate %GC which is a common feature of *MID* (intron length and %GC summarized in Table S1). The putative DNA-binding RWP-RK domain-containing C terminus region of *MID* is well conserved within the genus *Gonium*, while the N terminus region is relatively more varied, consistent with earlier *MID* gene comparisons [13].

As summarized in Table 1, the mating type denotations of *G. viridistellatum* turned out to be “inverted” in terms of *MID* distribution: only the “plus” strain, *G. viridistellatum* NIES-654, showed *MID* PCR signal (Figure 2). Phylogenetic analyses (Figures 3, 4) show that identified *MID* homologs are orthologous to one another among the RWP-RK domain-containing gene models recognized in *C. reinhardtii* and *V. carteri* genome databases [25,26]. Of all the species studied here, none of the *MID* flanking regions sequenced by inverse PCR or TAIL-PCR detected an *MTD1* homolog, which is encoded closely flanking *GpMID* in *G. pectorale* [35].

Methods for PCR-based mating type identification of *C. reinhardtii* strains [36–38] utilized specific primers not only for minus but also for plus including *FUS1*, a plus specific glycoprotein-coding gene [8,39]. Although this scheme can distinguish plus and minus reciprocally within a species, there is no *FUS1* homolog reported in the genus *Gonium* so far; *FUS1* homologs may have evolved too rapidly to be identified by degenerate primers [13].

Our genus-wide *MID* identification is not a “one-shot” identification of mutually exclusive mating types but establishes a correspondence among the different species, mating types of which have been distinguished within each morphological species.
Figure 1. Alignment of seven MID homologs from *Gonium pectorale* NIES-1710, *G. maiaprilis* NIES-2457, *G. viridistellatum* NIES-654, *G. quadratum* NIES-652, *G. multicoccum* NIES-1038 (heterothallic), NIES-1708 (homothallic), and *G. octonarium* NIES-852. Solid and shaded backgrounds indicate identity or similarity over 80% of the sequences aligned, respectively. Triangles indicate intron sites and the numbers the positions in the codons unless between codons.

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Figure 2. PCR assays for MID homolog distribution in four *Gonium* species. As a control experiment, amplification of the rDNA internal transcribed spacer region (ITS) is shown for each strain. Note that in *G. viridistellatum*, the plus strain is the MID containing strain, opposite the designation for the other *Gonium* species. N.D.: no designation.

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Figure 3. Maximum-likelihood (ML) tree (based on WAG model) of the full-length sequence of eleven MID proteins. Branch lengths are proportional to the estimated amino acid substitutions, which are indicated by the scale bar below the tree. Numbers over and below branch points indicate bootstrap values of the ML and neighbor-joining (NJ; based on the JTT model), analyses, respectively. MID homologs with asterisks (*) are reported in this study; a filled triangle indicates the homothallic strain.

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Figure 4. Maximum-likelihood tree (based on WAG model) of RWP-RK domains from eleven MID proteins and 25 RWP-RK domains from *C. reinhardtii* (Cr) and *V. carteri* (Vc) genome databases. Branch lengths are proportional to the estimated amino acid substitutions, which are indicated by the scale bar above the tree. Numbers over and below branch points indicate bootstrap values of the ML and NJ (based on the JTT model), analyses, respectively. MID homologs with asterisks (*) are reported in this study; a filled triangle indicates the homothallic strain.

doi:10.1371/journal.pone.0064385.g004

Table 3. Non-synonymous/synonymous substitution ratio among *Gonium* MID genes.

<table>
<thead>
<tr>
<th></th>
<th>G. pectorale</th>
<th>G. multicocum NIES-1038</th>
<th>G. multicocum NIES-1708</th>
<th>G. quadratum</th>
<th>G. viridistellatum</th>
<th>G. octonarium</th>
</tr>
</thead>
<tbody>
<tr>
<td>G. pectorale</td>
<td>0.027</td>
<td>0.0522</td>
<td>0.0441</td>
<td>0.0331</td>
<td>0.0407</td>
<td>0.0938</td>
</tr>
<tr>
<td>G. maiaprilis</td>
<td>0.051</td>
<td>0.0501</td>
<td>0.0476</td>
<td>0.0533</td>
<td>0.103</td>
<td></td>
</tr>
<tr>
<td>G. multicocum NIES-1038</td>
<td>0.0461</td>
<td>0.0414</td>
<td>0.0406</td>
<td>0.0533</td>
<td>0.103</td>
<td></td>
</tr>
<tr>
<td>G. multicocum NIES-1708</td>
<td>0.0465</td>
<td>0.0274</td>
<td>0.0789</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G. quadratum</td>
<td></td>
<td></td>
<td></td>
<td>0.0322</td>
<td>0.0828</td>
<td></td>
</tr>
<tr>
<td>G. viridistellatum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.1247</td>
<td></td>
</tr>
</tbody>
</table>

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behavior even under the sex-inducing conditions [40,41]. Current collections of 
Gonium strains originated decades ago (Table 1). Thus, PCR-based mating type identification is sine qua non for 
many cultures in the volvocine lineage.

MID homologs have also been identified from male strains of 
two anisogamous/oogamous colonial green algae Pleodorina starrii 
and V. carteri [15,17], indicating that isogamous minus and 
isogamous/oogamous male share a homologous mating genotype 
or sex. Similarly, presence and absence of MID homologs 
may connect isogamous species with bilateral mating papilla 
those that are unilateral. Unfortunately, other mating type-specific 
coding genes such as FUS1 or MTDI in C. reinhardtii or V. carteri 
either do not have homologs or exhibit weak homology, unlike 
MID [17,55,42]. Our co-ordination framework as presence/ 
brightness of the MID homolog can basically be applied to other 
volvocine isogamous species with bilateral mating papilla such as 
Pandorina or Yamaishigelia. Additionally, uniparental inheritance of 
organelar genomes changed in the course of evolution from 
isogamy to oogamy; in isogamous C. reinhardtii, G. pectorale and G. 
monatrophila, chloroplast DNA from plus and mitochondrial from 
minus are inherited by the F1 progeny; in oogamous V. carteri, on 
the other hand, both chloroplast and mitochondrial DNA are 
herited by the F1 progeny from female or plus [16,18,43,44]. In 
addition, there is very limited data on whether TMS-forming 
protein Ss of the organisms with unilateral papilla would be 
robustly associated with the non-MID mating type and hence might 
prove to be an uncertain indicator for sex; the mating structure of 
C. globosa, only a MID mating type of which is known, resembles that 
of C. reinhardtii minus [13]. Mating type/sex correspondence is the 
position on which to elucidate the transitions of uniparental 
inheritance and mating structures.

So far, searches for MID homologs have been reported only in 
heterothallic strains. Present results clearly show that a homothallic G. multicoccum NIES-1708 strain [19] also has the MID homolog (Table 1 and Figure 3). When compared, non-synonymous/ 
synonymous substitution ratios of MID genes from homothallic 
and heterothallic strains of G. multicoccum to those of the other 
species are below 0.2 (Table 3), indicating strong functional 
constrain of the genes. It seems that heterothallism in volvocine 
algae is ancestral; homothallism has multiple independent origins 
such as some strains from G. multicoccum, G. pectorale ("Russia’ 
strain [45], Pl. japonica [46], multiple Eudorina species [47], 
Pandorina multico mar [48], and several Volvox species, including most of 
Volvox sect. Volvox (Eudorina) [48]. Gene regulatory mechanisms in 
heterothallic strains remain unknown. In a homothallic organism, a 
strain established from only one vegetative cell differentiates into 
both gametes of sexual dimorphism, as demonstrated for the 

homothallic alga Chlamydomonas monocist [49]. The C. reinhardtii iso1 
mt mutant exhibited within a single strain an “isoagglutinating” 
phenotype [50] which is essentially a “partially homothallic” mode 
with an intact MID gene [51] but without any FUS1 gene. The 
identification of a G. multicoccum NIES-1700 MID homolog suggests a 
MID-dependent mechanism is involved in the sexual develop- 
mental program of homothallic wildtype organisms. However, the 
homothallic strain G. multicoccum NIES-1708 does not show sexual 
activity in nitrogen-deficient medium now [unpublished data], 
possibly because mating efficiency has declined in long-term 
culture. Investigating expression patterns of genes homologous to 
mating type differentiation factors (including MID) requires strains 
newly isolated from wild samples.

Supporting Information

Figure S1 Multiple alignments of MID orthologs. Background 
colors of residues are assigned by eBioX [http://www. 
ebioinformatics.org/index.html].

(TIF)

Figure S2 Multiple alignments of amino-acid sequences of 
RWP-RK domains from volvocine algae. The prefix Cr represents 
genes or gene models of Chlamydomonas reinhardtii, while Vc Volvox 
carteri and the numbers indicate their protein IDs in the genome 
database. C. globosa MID is formerly identified as C. inerita MID 
and renamed due to taxonomic re-identification [52]. Background 
colors of residues are assigned by eBioX [http://www. 
ebioinformatics.org/index.html].

(TIF)

Table S1 The%GC and exon-intron structure in coding 
sequences of Gonium MID orthologs identified in this study. 

(DOC)

Text S1 Supplementary methods. 

(DOC)

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Author Contributions

Conceived and designed the experiments: TH HN. Performed the 
experiments: TH JN YN HN. Analyzed the data: TH PJF HN. Contributed reagents/materials/analysis tools: TH JN YN HN. Wrote 
the paper: TH JFJ HN.

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present on the mating type plus fusion organelle and required for a critical 
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Mating Types of Gonium


