Identification and Functional Analysis of Delta-9 Desaturase, a Key Enzyme in PUFA Synthesis, Isolated from the Oleaginous Diatom *Fistulifera*

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**Abstract**

Oleaginous microalgae are one of the promising resource of nonedible biodiesel fuel (BDF) feed stock alternatives. Now a challenge task is the decrease of the long-chain polyunsaturated fatty acids (PUFAs) content affecting on the BDF oxidative stability by using gene manipulation techniques. However, only the limited knowledge has been available concerning the fatty acid and PUFAs synthesis pathways in microalgae. Especially, the function of Delta-9 desaturase, which is a key enzyme in PUFAs synthesis pathway, has not been determined in diatom. In this study, 4 Delta-9 desaturase genes (fD9desA, fD9desB, fD9desC and fD9desD) from the oleaginous diatom *Fistulifera* were newly isolated and functionally characterized. The putative Delta-9 acyl-CoA desaturases in the endoplasmic reticulum (ER) showed 3 histidine clusters that are well-conserved motifs in the typical Delta-9 desaturase. Furthermore, the function of these Delta-9 desaturases was confirmed in the *Saccharomyces cerevisiae ole1* gene deletion mutant (Jole1). All the putative Delta-9 acyl-CoA desaturases showed Delta-9 desaturation activity for C16:0 fatty acids; fD9desA and fD9desB also showed desaturation activity for C18:0 fatty acids. This study represents the first functional analysis of Delta-9 desaturases from oleaginous microalgae and from diatoms as the first enzyme to introduce a double bond in saturated fatty acids during PUFAs synthesis. The findings will provide beneficial insights into applying metabolic engineering processes to suppressing PUFAs synthesis in this oleaginous microalgal strain.

**Introduction**

Biodiesel fuel (BDF) has attracted considerable attention over the past decade as a renewable and biodegradable fuel alternative to fossil fuels. Commercially available BDFs are produced from a variety of terrestrial plants, including soybean, rapeseed, sunflower, castor seed, jatropha and palm oil. Terrestrial plants potentially have a negative impact on food supply. Furthermore, they have lower oil yield per area than oleaginous microalgae [1,2,3]. Based on the reasons, recently, oleaginous microalgae have been intensively studied as non-food biomass and high-triacylglyceride (TAG) producer for efficient BDF production [1,4].

BDF is a series of fatty acid methyl esters (FAMEs) generated by transesterification of TAG from feedstocks [5]. The physical and chemical properties of FAMEs are determined by its acyl composition, with respect to both carbon chain length and the number of double bonds. As the degree of unsaturation of fatty acids in FAMEs particularly affects the oxidative stability of BDF [6], the unsaturated fatty acid content in BDF is a primary limitation to its commercial use [7]. BDFs from soybean, sunflower and grape seed contain high levels of polyunsaturated fatty acids (PUFAs), resulting in poor oxidative stability [8,9]. On the other hand, BDFs from rapeseed, olive, corn, almond and high oleic sunflower oils show superior BDF properties because of their high content of monounsaturated [8]. Microalgal TAG mainly consists of short and saturated fatty acids, however, non-negligible quantities of long-chain PUFAs, such as methyl linolenate (C18:3), eicosapentaenoic acid (EPA; C20:5) or docosahexaenoic acid (DHA; C22:6) are also involved [10,11].

Toward addressing the above issue, breeding efforts have been done in terrestrial plants. The PUFA contents of TAG have been successfully reduced by the suppression of desaturase gene expression using RNA interference (RNAi) system in soybean, cotton seed and brassica seed [12,13,14]. By contrast, in microalgae, genetic modifications of FAME profiles have been hampered by the limited knowledge available concerning the fatty acid synthesis pathway (including PUFAs synthesis) and/or by difficulties in the genetic engineering approach [15,16]. Among eukaryotic microalgal groups, diatoms are well-established in terms of genomic and transgenic capabilities. Furthermore, the enzymes involved in fatty acid synthesis have been primarily identified in a model diatom, *Phaeodactylum tricornutum* [17,18,19].
In *P. tricornutum*, α3, Δ5, Δ6 and Δ12 desaturases were responsible for PUFA synthesis [18,19]. The end-product of the pathway is EPA. However, among various desaturases, the function of Δ9 desaturase from diatom has not been determined, although the enzyme plays a key role in PUFA synthesis as the first enzyme to introduce a double bond into saturated fatty acids [19].

A marine oleaginous diatom, *Fistulifera* sp. used in this study, has been recognized as a potential candidate for BDF production [20] because of its exceedingly high levels of intracellular TAGs (60% w/w) and its rapid growth. High-cell-density cultivation and outdoor mass cultivation of *Fistulifera* sp. have been demonstrated in flat-type photobioreactors [21], and column-type and raceway-type bioreactors [22]. In this strain, the major fatty acids are palmitate (C16:0; 30–40% of total fatty acids), palmitoleate (C16:1; 40–50%) and eicosapentaenoic acid (EPA, C20:5; 4–20%) as a PUFA. Recently, genetic transformation for this strain [23] was performed [23]. Metabolic engineering with the gene manipulation technique is a promising approach to decrease the PUFA content in TAG. One of the targets for genetic transformation was Δ9 desaturase because they may play a key role in fatty acid (and subsequent TAG) synthesis [12,13,14].

In this study, we report the screening of Δ9 desaturase genes in the oleaginous diatom *Fistulifera* and their functional characterization by expression in the yeast *A. lays* mutant. Through the comparison of the isolated Δ9 desaturases with those from other diatoms, unique features of Δ9 desaturase genes in *Fistulifera* sp. were determined. To our knowledge, this is the first study to confirm the function of Δ9 desaturases in diatoms and also in oleaginous microalgae.

### Materials and Methods

#### Strains and Growth Conditions

The marine pennate diatom *Fistulifera* sp. was grown in half-strength Guillard’s “f” solution (f/2) [24] dissolved in artificial seawater (Tomita Pharmaceutical Co., Ltd., Narano, Japan). Cultures were grown at 25°C under continuous and cool-white fluorescent lights at 140 μmol·m⁻²·s⁻¹ with aeration. Genes were cloned in *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA, USA) or *E. coli* DH5α (BioDynamics Laboratory Inc., Tokyo, Japan) cultured in Luria broth (Merck, Darmstadt, Germany) containing 50 μg/ml kanamycin or ampicillin at 37°C.

Putative Δ9 desaturase genes were expressed in *Saccharomyces cerevisiae* INVSc-1 (MATa/MATα, his3Δ1/α, ura3Δ0/0, leu2Δ0/0, trip1-289/trip1-289, and ura3-52/ura3-52) [Invitrogen] or the yeast *A. lays* mutant (MATα, his3Δ1, leu2Δ0, ura3Δ0, and ole1Δ::kanMX4) [25]. The yeast *A. lays* mutant (MATα, his3Δ1, leu2Δ0, ura3Δ0, and ole1Δ::kanMX4) was generated via the sporulation of the S. cerevisiae YGL055W/YV4743 heterozygous strain (MATα/MATα, his3Δ1/α, ura3Δ0/0, ura3Δ0/0, and ole1Δ::kanMX4) (ATCC number: 4024422).

#### Isolation of Δ9 desaturase Genes from *Fistulifera* sp.

To obtain the putative Δ9 desaturase genes of *Fistulifera* sp., a homology search using BlastX was performed with reference to

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**Figure 1. Phylogenetic tree of Δ9 desaturases from various organisms.** Six putative Δ9 desaturase sequences from *Fistulifera* sp. were highlighted. The phylogenetic tree was constructed by the neighbor-joining method using MEGA4 with bootstrap values based on the sequence alignments by the ClustalW program. The query sequences of the representative Δ9 desaturase sequences are *P. tricornutum* (GenBank accession number: EEC47008.1), *A. lays* (AAK25797.1), Chlamydomonas reinhardtii (CA830008.1), Homo sapiens (AAD29870.1), Mus musculus (AAA40103.1), Rattus norvegicus (AAAM34745.1), Synechocystis sp. (AB356215.1), Synechococcus sp. (AA861533.1), Nostoc sp. 36 (desC1) (CA819823.1), Gloeobacter violaceus PPC 7421 (BAC90807.1), Nostoc sp. 36 (desC2) (CA819826.1), *Thermosynechococcus elongates* BP-1 (AAD00699.1), Asclepias syriaca (AAC49791.1), Pelargonium x hortorum (AAC49421.1), Ricus communis (CA39859.1), and Carthamus tinctorius (AA33021.1).

doi:10.1371/journal.pone.0073507.g001
the 19,859 genes from the draft genome sequence of *Fistulifera* sp. [26]. The full-length cDNAs of putative Δ⁹ desaturase genes were obtained by 5′- and 3′-RACE using a Smarter RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA). Partial sequences of these genes predicted by the AUGUSTUS program were used for designing gene-specific primers to amplify the 5′ and 3′ ends of the target genes (Table S1). The PCR products were cloned into the pCR-Blunt II-Topo vector (Invitrogen). The full-length cDNA sequences were assembled based on the 5′- and 3′-RACE fragments.

### Table 1. Comparison of conserved motifs in Δ⁹ desaturases from diatoms.

| Organism                  | Gene name | Accession No. | Conserved histidine sequences | Cytochrome b₆’s binding domain | Number of predicted TMHs |
|---------------------------|-----------|---------------|-------------------------------|--------------------------------|--------------------------|
|                           |           |               | First | Second | Third | TMHMM | HMMTOP |
| Diatom                    |           |               |       |        |       |       |        |
| *Fistulifera* sp.         | fD9desA   | AB831011      | HRLWAH | HRVHH  | HNWHH | Not observed | 2      | 5      |
|                           | fD9desB   | AB831012      | HRLWSH | HRVHH  | HNWHH | Not observed | 2      | 5      |
|                           | fD9desC   | AB831013      | HRLWAH | HRVHH  | HNWHH | Not observed | 2      | 5      |
|                           | fD9desD   | AB831014      | HRLWSH | HRVHH  | HNWHH | Not observed | 2      | 5      |
| *Phaeodactylum tricornutum* |          | EEC47008      | HRLWSH | HRVHH  | HNWHH | Not observed | 4      | 5      |
| *Thalassiosira pseudonana* |          | EED91785      | HRLWSH | HRVHH  | HNWHH | Not observed | 3      | 5      |
|                           |          | EED86245      | HRLWSH | HRVHH  | HNWHH | Not observed | 3      | 5      |

The conserved amino acids are on gray backgrounds. The 3 histidine clusters are framed.
sequences were also investigated to determine whether the protein has N-terminal signal peptides; SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/) [20], TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/) [29], and HECTAR (http://www.sb-roscoff.fr/hecitar/) [30] were used for this analysis. TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) [31] and TMHTOP 2.0 (http://www.enzim.hu/hmmtop/index.php) [32] were used for the prediction of transmembrane domains.

Functional Characterization of Δ9 Desaturases in the Yeast Aole1 Mutant

For functional characterization, 4 Δ9 desaturase genes (fD9desA, fD9desB, fD9desC, and fD9desD) with the Kozak sequence [33] in front of the start codon were cloned into yeast vectors, and transformed into yeast INVSc-1 harboring the control pYES2.1/V5-His/lacZ. Each transformant harboring the plasmid for the expression of Δ9 desaturases was suspended in distilled water and adjusted to an OD of 1. The resulting supplements were spotted on an SD agar plate lacking uracil but containing 2% galactose and (a) no fatty acids; (b) 500 µM C16:1 or C18:1 fatty acids was inoculated with the pYES2.1/V5-His/lacZ transformed and grown at 20°C for 96 h in a water bath shaker. Cell pellets were sequentially washed with 1% Tergitol Type NP-40 and 0.5% Tergitol Type NP-40, freeze-dried, and subject to fatty acid analysis.

Fatty Acid Analysis Using GC/MS

The freeze-dried yeast cells were directly transmethyalted with 1.25 M hydrochloric acid in methanol (1 h at 100°C) to prepare the FAMEs. The FAMEs were extracted in n-hexane and analyzed using GC/MS.

### Table 2. Substrate specificity analysis of Δ9 desaturases (fD9desA, fD9desB, fD9desC, and fD9desD) from Fistulifera sp. on the basis of expression in the yeast Aole1 mutant (n = 3).

| Transformant          | Supplementation of C18:1 | Supplementation of C16:1 |
|-----------------------|--------------------------|--------------------------|
|                       | C16:0 (%)*               | C16:1 (%)*               | C18:0 (%)* | C18:1 (%)* |
| INVSc-1+ control vector | 27.2±3.1                 | 57.2±7.4                 | 26.4±1.9   | 7.9±0.6    |
| Aole1+ control vector  | 65.6±0.4                 | 0                        | 31.7±1.8   | 0          |
| Aole1+ fD9desA         | 72.1±2.4                 | 0.6±0.2                  | 24.7±0.7   | 1.8±0.2    |
| Aole1+ fD9desC         | 70.4±3.1                 | 0.9±0.3                  | 27.5±2.2   | 1.6±0.3    |
| Aole1+ fD9desB         | 72.4±3.2                 | 0.5±0.5                  | 27.7±0.9   | 0          |
| Aole1+ fD9desD         | 69.6±2.1                 | 0.2±0.1                  | 28.5±1.7   | 0          |

The yeast strain INVSc-1 transformed with the control vector pYES2.1/V5-His/lacZ (Control vector) served as the positive control. The yeast Aole1 mutant transformed with pYES2.1/V5-His/lacZ served as the negative control.

The relative amount of each fatty acid was expressed as a percentage of total fatty acids (± SD) after subtracting the amount of the supplemented fatty acid from the total.

doi:10.1371/journal.pone.0073507.t002

Figure 3. Complementation assay of four Δ9 desaturases from Fistulifera sp. in the yeast Aole1 mutant. A dilution series of each yeast sample solution was spotted on SD medium-based agar plates containing galactose and (1) no unsaturated fatty acids (UFAs), (2) 500 µM of the C16:1 fatty acid, or (3) 500 µM of the C18:1 fatty acid. (a), the yeast strain INVSc-1 transformed with the control vector pYES2.1/V5-His/lacZ served as a positive control; (b), the yeast Aole1 mutant transformed with pYES2.1/V5-His/lacZ served as a negative control; (c–f), the yeast Aole1 mutant transformed with pYES2.1/V5-His/lacZ contained fD9desA, fD9desB, fD9desC, or fD9desD genes.

doi:10.1371/journal.pone.0073507.g003
by GC/MS (QP2010 Plus; Shimadzu, Kyoto, Japan) with FAMEWAX (RESTEK, Bellefonte, PA, USA) in the electron impact mode. FAMES were identified using the F.A.M.E. Mix, C4–C24 Unsaturates (Sigma-Aldrich, Dorset, UK). Each sample was analyzed in 3 independent experiments.

Results

Sequence Analysis of Putative Δ⁹ Desaturase Genes from Fistulifera sp.

The 6 Δ⁹ desaturase candidate genes (Gene ID: g5394, g10778, g10781, g12958, g19483 and g19496) were identified with the BlastX algorithm from all 19,859 genes of this strain as the query sequence from a non-redundant protein sequences database [Manuscript in preparation]. Because the predicted Δ⁹ desaturases from draft genome sequence seemed to be partial ORFs due to the lack of the conserved motifs of histidine box and cytochrome b₅ domain, the full-length sequences of putative acyl-CoA Δ⁹ desaturase cDNA were sequenced from the products obtained by rapid amplification of cDNA ends (RACE) PCR. The cDNAs containing predicted gene regions were verified to be 996 bp for g10778 and g19483 and 1,002 bp for g10781 and g19486; these were designated as fD9desA, fD9desB, fD9desC, and fD9desD, respectively (Fig. 1). The fD9desA nucleotide sequences exhibit high identity with the fD9desB (96%), and the fD9desC had 93% identity with the fD9desD. The amino acid sequences of fD9desA and fD9desB were identical, while fD9desC and fD9desD showed the differences of 2 amino acid residues. The 2 proteins encoded by g5394 and g12958 had 49% and 47% identity, respectively, with Δ⁹ desaturase from the plant Acselplus syrica (GenBank accession no. AAC49719.1) [35]. The 4 proteins encoded by g10778, g10781, g19483 and g19486 showed 78%, 69%, 72% and 71% identity, respectively, with the putative Δ⁹ desaturase from a diatom, P. tricornutum (EEC47008.1). A phylogenetic tree of Δ⁹ desaturase amino acid sequences from different organisms, prepared using ClustalW, showed that the g5394 and g12958 genes appeared in the amino acid sequences without the cytochrome b₅ domain. The findings of truncation and disruption experiments for the cytochrome b₅ domain Δ⁹ desaturases have suggested that this domain is not strictly required for fatty acid desaturation [39].

Localization of general Δ⁹ desaturases in the ER membrane are predicted from N-terminal amino acid sequences [19]. Three algorithms for subcellular targeting prediction, TargetP 1.1, SignalP 4.0 and HECTAR, were used in this study. The Δ⁹ desaturase candidate genes were predicted to have neither N-terminal signal sequences nor internal cleavable signal sequences. All 4 Δ⁹ desaturases were expected to possess 2–5 transmembrane domains, according to a prediction by TMHMM 2.0 and HMMTOP 2.0 (Table 1). This prediction supports the existence of transmembrane helices in Δ⁹ desaturases from Fistulifera sp., although further analysis is needed to confirm the number of transmembrane regions.

Functional Characterization of Putative Δ⁹ Desaturases in the Yeast Aole1 Mutant

In order to confirm the function of Δ⁹ desaturases, 4 putative genes were cloned in the protein expression vector, pYES2.1/V5-HisTOPO, and transformed in the yeast Aole1 mutant. Aole1 is a Δ⁹ desaturase knockout mutant that requires supplementation with C16:1 or C18:1 fatty acids to grow. When the putative Δ⁹ desaturases work properly in the synthesis of monounsaturated C16:1 and/or C18:1 fatty acids, the transformants can grow on an agar plate without supplementation with exogenous unsaturated fatty acids. The transformed yeast Aole1 mutant with the control vector (in the absence of any desaturase gene), serving as a negative control, did not grow in the absence of fatty acid supplementation (Fig. 3(a), no UFA). The INVSc-1 cells possessing the native ole1 gene in the genome, serving as a positive control, grew well in the absence of fatty acid supplementation (Fig. 3(b), no UFA) because the cell can generate these essential desaturated fatty acids endogenously. The fD9desA, fD9desB, fD9desC, and fD9desD genes failed to complement the yeast ole1 mutation in the absence of 16:1/18:1 fatty acid supplementation (Fig. 3(c)-(f), no UFA). Additionally, the growth of transformants with 16:1/18:1 fatty acid supplementation was also confirmed. In a previous study, a Δ⁹ desaturase (Δ⁹-3) from the fungus Mortierella alpina failed to complement the yeast Aole1 mutant when transformants were grown in the absence of monounsaturated fatty acids supplementation [40]. It is likely that the Δ⁹-3 desaturase from M. alpina and Δ⁹ desaturases from Fistulifera sp. did not have sufficient activity for the complementation in yeast, respectively.

Next, to further investigate the in vivo function and specificity of these Δ⁹ desaturase genes, the fatty acid profiles of the yeast Aole1 transformant-carrying gene expression vector with the Δ⁹ desaturase genes were evaluated by gas chromatography and mass spectrometry (GC/MS) analysis (Table 2). Two Δ⁹ desaturases from Fistulifera sp., fD9desC and fD9desD, showed activity for C16:0 as a substrate (0.6% and 0.3% desaturation to C16:1, respectively) (100 × product/substrate+product) during growth with C18:1 supplementation (Table 2) but did not show activity for C18:0. The remaining 2 Δ⁹ desaturases, fD9desA and fD9desB, showed similar activities to each another with C16:0 as a substrate (0.8% and 0.4% desaturation to C16:1, respectively) (100 × product/substrate+product). However, the ideal substrate of fD9desA and fD9desB was C18:0 converted to C18:1 (6.9% and 5.3% desaturation to C18:1). The desaturation efficiencies of fD9desA and fD9desB for C18:0 were significantly higher than those for C16:0. The detected value of desaturation by Δ⁹ desaturases from Fistulifera sp in yeast was similar to that of house cricket (Acheta domestica) (5% desaturation to 18:1) [41]. These heterogeneous gene expressions and functional analyses in the model organism may cause the negative effect for the activity, exhibiting these relatively low activities.
Discussion

Six Δ⁹ desaturase genes were identified in Fistulifera sp. by bioinformatic analysis. These genes were categorized as 4 ER acyl-CoA desaturases (fD9desA, fD9desB, fD9desC, and fD9desD) and 2 plastidial acyl-ACP desaturases (g5394 and g12958). By way of comparison, P. tricornutum has only 1 ER acyl-CoA desaturase and 1 plastidial acyl-ACP desaturase, and T. pseudonana has 2 ER acyl-CoA desaturases and 1 plastidial acyl-ACP desaturase (Table 1). These results indicate that Fistulifera sp. has more Δ⁹ desaturase genes than other diatoms do. The higher number of Δ⁹ desaturase genes in Fistulifera sp. suggests that the oleaginous strain may have well-developed genome organization for fatty acid metabolism to enable considerable accumulation of TAG endogenously. The identified ER acyl-CoA desaturases show different substrate specificities (Table 2). Two Δ⁹ desaturases (fD9desA and fD9desB) converted both C16:0 and C18:0 to C16:1 and C18:1, respectively, and the other 2 Δ⁹ desaturases (fD9desC and fD9desD) converted C16:0 to C16:1. The sequences of the Δ⁹ desaturases showed the presence of highly conserved histidine cluster motifs [48] (Fig. 2), and a difference of 1 amino acid residue in the first histidine motif box was observed among these 4 Δ⁹ desaturases. The former group of Δ⁹ desaturases (fD9desA and fD9desB) has the HRLW[VH sequence, and the latter group of Δ⁹ desaturases (fD9desC and fD9desD) has the HRLV[AH sequence, with the serine substituted with alanine. In other words, Δ⁹ desaturase pairs from Fistulifera sp. have the same histidine motifs and desaturation specificities (Table 2). In the oil-producing fungus M. alpina, a different amino acid sequence in the first histidine box was found in 3 Δ⁹ desaturases. Different Δ⁹ desaturases with different sequences in the first histidine box showed varying specificities for fatty acid desaturation [49]. Therefore, the difference in amino acid sequences is considered to reflect the substrate specificity of Δ⁹ desaturases from Fistulifera sp. P. tricornutum has only 1 ER acyl-CoA desaturase with the amino acid sequence of HRLW[V in the first histidine motif, while T. pseudonana has 2 ER acyl-CoA desaturases with the same amino acid sequence (HRLW[V)

Table 1. On the basis of these results, we hypothesized that all the Δ⁹ desaturases in both diatoms possess the same substrate specificity, although a detailed functional analysis should be performed to confirm this hypothesis. If it is confirmed, this would indicate that the varying specificities of Δ⁹ desaturases from Fistulifera sp. may be related to the specific fatty acid metabolism in this strain. In addition, the Δ⁹ desaturation activity was assayed for the substrates of 16:0 and 18:0. In the case of mouse Δ⁹ desaturase, for wide range of saturated fatty acids from 12:0 to 19:0, the desaturation activity was detected [48]. To fully confirm the functional specificity of Δ⁹ desaturase from Fistulifera sp. for various substrates, further analysis should be provided in the future.

In conclusion, 4 ER Δ⁹ acyl-CoA desaturase and 2 plastidial Δ⁹ acyl-ACP desaturase genes were identified from the oleaginous diatom Fistulifera sp., and gene homologs were also observed in other diatoms. Among the Δ⁹ desaturase genes, four Δ⁹ acyl-CoA desaturases showed desaturation activity of the saturated fatty acids of C16:0 and/or C18:0 in complementation assays using the yeast Δole1 mutant. This study is the first functional confirmation of Δ⁹ desaturase from diatom and from oleaginous microalgae. While the in vivo function of the Δ⁹ desaturase genes in the Fistulifera sp. should be separately addressed, this study provides information that will help in the regulation of the fatty acid profiles in the oleaginous microalgae.

Supporting Information

Table S1 List of primers used for this study.

Author Contributions

Conceived and designed the experiments: M.Muto MT TY TT. Performed the experiments: M.Muto CK AS M.Matsumoto. Analyzed the data: M.Muto CK M.Matsumoto MT TY TT. Contributed reagents/materials/analysis tools: AS M.Muto. Wrote the paper: M.Muto MT TT.

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