A taxonomic study of Spirogyra and related genera (Zygnematophyceae, Streptophyta) based on morphological comparison and molecular phylogenetic analyses using cultured material

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論 文 内 容 の 要 旨

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専　攻　　生物学

論文題目

A taxonomic study of *Spirogyra* and related genera (Zygnematophyceae, Streptophyta) based on morphological comparison and molecular phylogenetic analyses using cultured material

（培養株を用いた形態比較と分子系統解析に基づくアオミドロ属及び近縁属（ストレプト植物門・ホシミドロ藻綱）の分類学的研究）

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The genus *Spirogyra* and its related genera *Sirogonium* and *Temnogyra* (hereafter referred to as *Spirogyra*-like algae, SLA) belong to streptophytes that include land plants. They are found mainly in freshwater, and there are over 570 species described from all over the world, with 84 species known from Japan. The SLA have a filamentous thallus consisting of a series of cylindrical cells with characteristic spiral ribbon-like chloroplasts. They are referred to as conjugating algae because of their characteristic sexual reproduction, in which gametes without flagella conjugate to form zygospores.

Sirogonium and *Temnogyra,* differ from *Spirogyra* in their conjugation characteristics: in *Spirogyra*, a progametangial cell divides to form two gametangia, whereas, in these two genera, a progametangial cell divides to form a gametangium and a sterile cell that does not conjugate. In *Temnogyra*, the gametangia are connected by conjugation tubes as in *Spirogyra*-type, and in *Sirogonium*, they are connected directly without conjugation tubes. Recent molecular phylogenetic analyses based on *rbcL* gene have shown that the SLA are divided into seven clades (Clade I-VII) and that three species of *Sirogonium* form a clade with some species of *Spirogyra*. However, the species of *Temnogyra* have not yet been analyzed. In addition, the phylogenetic relationship between the seven clades were not solved.

In the SLA classification, characteristics such as sexual reproduction and mature zygospores are significant for accurate species-level and genus-level identification. In previous taxonomic studies, species identification has been carried out using field-collected samples with mature zygospores. However, there is a wide range of seasonality in the occurrence of mature zygospores, and it is often difficult to collect samples with mature zygospores. Molecular phylogenetic studies of species identified based on field samples with sexual traits have been limited. Therefore, establishing a reproducible method to induce sexual reproduction in the SLA is necessary to develop a taxonomic study. Recently, the inducing conjugation method using agar plates has been established, and species identification of the SLA has been carried out based on the characteristics of the obtained mature zygospores.

In the present study, I improved the inducing conjugation method and identified more SLA species accurately based on the morphology of sexual reproduction. I also re-examined the taxonomy of the SLA by clarifying their phylogenetic relationships based on molecular data. Extensive field survey in Japan was conducted and 505 culture strains were established. I identified 51 species based on conjugating field samples and sexual morphology observed by inducing conjugation. Two species exhibiting *Temnogyra*-type conjugation and other two species of *Sirogonium*-type

conjugation were identified. Thirteen species were found in Japan for the first time, and three were undescribed species. Some of them were rare species with few reports. It was assumed that they seldom, or rarely, sexually reproduced in nature, so our cultivation procedure for inducing conjugation in vegetative cells proved useful for species identification (Chapter 1).

Sexual reproduction in zygnematophycean algae occurs either within a single clone (homothallic strains) or between different clones (heterothallic strains). The SLA are found in a wide range of freshwater environments, and the process of conjugation has been well studied. However, heterothallic strains of *Spirogyra* have not been reported because of experimental difficulties in inducing conjugation. In this study, I detected heterothallic strains of *Spirogyra* by inducing conjugation for the first time. These strains were identified as *S. fluviatilis,* and two genetically different strains were required for conjugation. Crossbreeding experiments with these strains showed that sexuality was genetically determined and that the F1 strain germinated from a zygospore was only conjugated with one parent strain. Molecular phylogenetic analyses conducted in this study showed that these heterothallic strains formed a monophyletic group with several homothallic strains. Only one heterothallic strain was found in this study. More heterothallic strains will need to be discovered using the methods established in this study in order to solve homothallic and heterothallic transitions in *Spirogyra* (Chapter 2).

A total of 136 strains with different *rbcL* sequences (1094 bp) were obtained in Japan, and the maximum likelihood phylogenetic analysis was carried out using *rbcL* sequences. According to previous studies, the SLA was divided into seven clades (Clade I-VII), but 16 strains from Japan were not included in any clade. These strains included two species exhibiting *Temnogyra*-type conjugation (*S. corrugate* and *S. punctata*) and a strain with semi-replicate transverse walls. The latter was described as *S. tertia sp. nov.* The clades I, III, IV, V, and VII were monophyletic with high statistical support (98-99% bootstrap value), but Clade II and VI formed a monophyletic clade with lower statistical support (65% bootstrap value). The clade VI and its sister lineage *S. micropunctata* from Japan formed clade VI' with moderate statistical support (bootstrap value 85%). In addition, Clade III was divided into Clade IIIa and Clade IIIb.

To elucidate the phylogenetic relationships and evolutionary processes of morphological traits within the SLA, I sequenced whole chloroplast genomes of the selected 32 strains of the SLA and conducted molecular phylogenetic analyses using a multi-gene dataset. The molecular phylogenetic analyses, based on 73 chloroplast

genes, support the phylogenetic relationship between SLA clades (Clade I, II, IIIa, IIIb, IV, V, VI', VII) with high statistical support. One Japanese strain (*S. crassispina* chiA303), which was included in Clade II in the phylogenetic analysis using *rbcL*, was not included in Clade II and was separated from any of the other clades. Two species exhibiting *Temnogyra*-type conjugation and two other Japanese strains of unknown conjugation morphology form a monophyletic group (Clade T). Two species exhibiting *Sirogonium*-type conjugation species were included in Clade II as previous studies. The results of ancestral state reconstruction suggested that the ancestor of the SLA performed *Spirogyra*-type conjugation, and it was implied that *Sirogonium*-type and *Temnogyra*-type species were evolved from *Spirogyra*-type ancestor. The results of ancestral state reconstruction also suggested that the ancestor of the SLA was approximately 50 µm in a cell diameter and had multiple chloroplasts in each cell. In addition, the ancestor of the SLA had transverse walls with a folded structure. From their common ancestor, Species of Clade VII and Clade II evolved with enlarged cell diameters and loss of folded structures at transverse walls, respectively, while *S. crassispina* chiA303 retained its folded structure. In previous studies, all species with a folded structure were included in Clade I, but the strain with a folded structure not included in Clade I was found for the first time in this study. The common ancestor of the other lineages (Clade I, IIIa, IIIb, IV, V, VI', T, and *S. tertia sp. nov.* wak305) was estimated to have a cell diameter of about 30 µm, a single chloroplast per cell and a folded structure at the transverse walls. *S. tertia sp. nov.* wak305 was the sister lineage of the remaining lineages. This species has an incomplete folded structure (semi-replicate), and the phylogenetic position of the species with this structure was revealed for the first time. In the next diverged lineage, Clade I, some species had a folded structure and others do not, suggesting multiple losses within this clade. The common ancestor of Clade IIIa, IIIb, IV, V, VI', T was presumed to have lost the folded structure. The results of ancestral trait reconstruction implied that the conjugation pattern changed from *Spirogyra*-type to *Sirogonium*-type twice independently in Clade II and from *Spirogyra*-type to *Temnogyra*-type in the common ancestor of Clade T. (Chapter 3).

As a result of the establishment of new culture strains and the improvement of inducing conjugation, two *Temnogyra*-type species and two of *Sirogonium*-type species were identified, which were included in clades with *Spirogyra*-type strains. Therefore, I concluded that three genera of the SLA should be assigned to the genus *Spirogyra*. Further taxonomic studies using more strains, including the type species of the genera *Sirogonium* and *Temnogyra* from the type locality, are needed to revise the overall

taxonomic system of the SLA. The establishment of culture strains and inducing conjugation will play a significant role in future SLA classification methods. However, it is not easy to induce conjugation in all SLA species. Therefore, combining this with field collection with mature zygospores will be necessary. Finally, the large number of cultured strains and the knowledge of sexual reproduction established in this study are expected to be used in various future studies, such as the elucidation of the evolution of sex-determination systems in streptophytes using SLA species as a model.

（別紙１）　　論文審査の結果の要旨

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論文題目	A taxonomic study of *Spirogyra* and related genera (Zygnematophyceae, Streptophyta) based on morphological comparison and molecular phylogenetic analyses using cultured material（培養株を用いた形態比較と分子系統解析に基づくアオミドロ属及び近縁属（ストレプト植物門・ホシミドロ藻綱）の分類学的研究）		
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要　　旨

アオミドロ属（*Spirogyra*）とその近縁属であるシロゴニウム属（*Sirogonium*）とテムノギラ属（*Temnogyra*）（以下、アオミドロ類と呼ぶ）は陸上植物を含むストレプト植物に属する藻類であり、ホシミドロ藻綱・ホシミドロ科に分類される。アオミドロ類はおもに淡水域に広く生育し、世界各地から約 570 種が記載されており、日本では 84 種が知られている。本藻類は特徴的な螺旋を描くリボン状の葉緑体を持つ円筒形の細胞が連なった糸状の形態をしている。アオミドロ類が含まれるホシミドロ藻綱は、鞭毛をもたない不動配偶子による接合という特徴的な有性生殖を行うことから接合藻類と呼ばれることがあり、2 つの細胞が接合管を伸ばして接着し、鞭毛のない配偶子が一方のオス側からもう一方のメス側へ移動し接合胞子を形成する。

アオミドロ類の 3 属は有性生殖の特徴によって区別されている。アオミドロ属は全ての細胞が配偶子嚢に分化するのに対して、シロゴニウム属とテムノギラ属は配偶子嚢形成時の細胞分裂で接合に関与しない不稔細胞を生じる。近年の葉緑体 DNA 配列に基づく分子系統解析の結果、アオミドロ類は主に 7 つのクレードに分けられ、シロゴニウム属の 3 種だけがアオミドロ属の一部の種とクレードを形成することが示されたが、テムノギラ属の種は未解析であり、アオミドロ類の各クレード間の系統関係は不明であった。アオミドロ類の分類において、特に有性生殖や成熟した接合胞子の特徴が正確な種および属レベルの同定に必要である。これまでの分類学的研究では、野外から直接採集された成熟した接合胞子を持つ個体を用いて種同定が実施されていた。しかし、成熟した接合胞子の生じる時期には多様な季節性が知られており、成熟した接合胞子を持つ個体を採集することは困難な場合が多い。そのため、成熟個体に基づいて種同定された種の分子系統学的研究は非常に限られていた。したがって、アオミドロ類において有性生殖を誘導するための再現性のある方法の確立が分類学的体系の構築には必要である。近年、先行研究によってアオミドロ属の数株において寒天培地を用いた接合誘導法が確立され、得られた成熟接合子の特徴に基づいた種同定が行われている。

本研究において、学位申請者の高野智之氏は、接合誘導法を改良するとともに、より多くのアオミドロ類の種を有性生殖の形態に基づいて正確に種同定し、それらの系統関係を分子データに基づき明らかにすることによってアオミドロ類の分類学的再検討を実施した。日本において広範な野外調査を行い、505 株の培養株を新たに確立した。それらのうち、接合中の野外サンプルや接合誘導によって観察された接合形態に基づいて、51 種の種同定に成功した。そのうち 13 種は日本新産種であり、3 種については未記載種であることを発見した。（第 1 章）。

また、ホシミドロ藻綱における有性生殖には、単一のクローン内で接合胞子を形成するもの（ホモタリック株）と、異なるクローン間で接合胞子を形成するもの（ヘテロタリック株）が知られている。アオミドロ属は広範な淡水環境に生育しており、その接合過程に関してよく研究されている。しかしながら、アオミドロ属では接合誘導が実験的に困難であったため、ヘテロタリック株はこれまで報告されていなかった。本研究の中で、高野智之氏は接合誘導によってアオミドロ属のヘテロタリック株を初めて発見した。これらの株は *Spirogyra fluviatilis* と種同定され、接合には遺伝的に異なる 2 つの株が必要であり、これらの株を用いた掛け合わせ実験では、これらの株において性が遺伝的に決定されていること、および接合胞子から発芽した F1 株は一方の親株のみと接合することを明らかにした。さらに分子系統解析の結果、こ

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れらのヘテロタリック系統はいくつかのホモタリック系統と単系統群を形成することを示した（第２章）

さらに高野智之氏は、アオミドロ類の主要な系統群間の系統関係と形態形質の進化過程を明らかにするために、主要な系統群の葉緑体全ゲノム配列を決定し、多遺伝子に基づく分子系統解析を実施した。葉緑体 73 遺伝子に基づく分子系統解析の結果、アオミドロ類の各クレード間の系統関係は高い統計的支持率で支持され、シロゴニウム属とテムノギラ属は系統的に離れており、アオミドロ型の祖先種からそれぞれ進化したと推測した。（第３章）。

以上のように、本研究において高野智之氏は新たな培養株の確立と接合誘導の改良の結果、日本新産種 13 種と未記載種 3 種を含む 51 種の種同定をおこなった。また、アオミドロ類で初めてヘテロタリック系統株を発見した。多遺伝子を用いた系統解析の結果、シロゴニウム属はアオミドロ属の中で多系統群となり、テムノギラ属もアオミドロ属のクレードに含まれることを明らかにした。高野智之氏の研究は、今後、アオミドロ類全体の分類体系を改訂するためにシロゴニウム属とテムノギラ属のタイプ種を含むより多くの分類群を用いた同様の分類学的研究が必要であり、いくつかの課題も残されたが、本研究で確立された多くの培養株や有性生殖に関する知見は、今後、アオミドロ類をモデルに用いたストレプト植物における性決定システムの進化の解明等、様々な研究へ発展することが期待される。

本研究はアオミドロ類について、培養株を用いた形態比較と分子系統解析に基づき、詳細にその分類と系統関係を研究したものであり、アオミドロ類の分類体系、系統関係および形態進化について重要な知見を得たものとして価値ある集積であると認める。よって、学位申請者の高野智之氏は、博士（理学）の学位を得る資格があると認める。