

SYSTEMATICS OF THE GENUS *ZYGNEMA* (ZYGNETOPHYCEAE, CHAROPHYTA) FROM CALIFORNIAN WATERSHEDS¹

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Natural populations of *Zygnema* were collected from 80 stream sites across California, and eight species were identified and characterized morphologically. Generic and infrageneric concepts of *Zygnema* and *Zygogonium* were tested with *cox3* and *rbcL* gene sequence analysis. Strains of *Zygnema* were positioned in a single monophyletic clade sister to *Zygogonium tunetanium* Gauth.-Lièvre. In both the *rbcL* and *cox3* phylogenies, strains of *Zygnema* formed two major clades. The first clade contained species that have zygospores with a blue-colored mesospore or akinetes with a colorless mesospore. The second clade contained species that have a yellow or brown mesospore. The existing taxonomic concepts for *Zygnema* classification are not consistent with our molecular phylogeny and do not correspond to natural groups. We propose that mesospore color may be useful in the infrageneric classification of *Zygnema*. Newly described *Zygnema aplanosporum* sp. nov. and *Zygnema californicum* sp. nov. have zygospores with a blue mesospore formed in the conjugation tube and separated by a cellulosic sporangial wall. *Z. aplanosporum* also possessed a combination of vegetative and reproductive features characteristic of *Zygogonium*, such as presence of short branches, rhizoidal outgrowths, thickened vegetative cell walls, purple-colored cell content, small compressed-globular chloroplasts as well as predominant asexual reproduction. *Z. aplanosporum* and *Z. californicum* were deeply embedded in a larger clade of *Zygnema* both in *rbcL* and *cox3* analyses. Based on our observations, there are no features or combination of features that separate *Zygnema* and *Zygogonium*. Therefore, we conclude that *Zygogonium* is probably a synonym of *Zygnema*.

Key index words: California; generic and infrageneric concept; morphology; *rbcL* and *cox3* phylogenies; reproduction; sp. nov.; streams; *Zygnema*; Zygnematophyceae; *Zygogonium*

Abbreviations: *cox3*, cytochrome oxidase subunit 3; *rbcL*, RUBISCO LSU

The Zygnematophyceae are freshwater algae distributed in many lotic and lentic habitats worldwide. Members include the common filamentous genera *Mougeotia* C. Agardh, *Spirogyra* Link, and *Zygnema* C. Agardh as well as the unicellular desmids (Hoshaw and McCourt 1988, Simons 1994, Novis 2004). Molecular phylogenetic studies have shown that these algae have a complex evolutionary history: the smooth-walled unicellular Mesotaeniaceae and filamentous Zygnemataceae are not natural groups (Mccourt et al. 2000, Gontcharov et al. 2003, Hall et al. 2008). However, generic relationships remain obscure and many genera have not been thoroughly investigated using modern molecular phylogenetic methods. The vegetative filaments of *Zygogonium* Kütz. and *Zygnemopsis* (Skuja) Transeau are difficult to distinguish from *Zygnema* (Transeau 1951, Guiry and Guiry 2010). The genus *Zygogonium* has been considered synonymous with *Zygnema* in past studies (e.g., Czurda 1932). Molecular phylogenetic investigations determined that *Zygnemopsis* was only distantly related to *Zygnema*, whereas *Zygogonium tunetanium* Gauth.-Lièvre was more closely related to a clade of *Zygnema* spp. (Gontcharov et al. 2004, Hall et al. 2008). However, the identity of *Zygo. tunetanium* could not be confirmed (Hall et al. 2008). Strains of *Zygnema* form a well-supported monophyletic clade (Gontcharov et al. 2004, Hall et al. 2008). However, the genus *Zygnema* is species rich and structurally diverse. Most infrageneric relationships remain untested using confidently identified strains and molecular phylogenetic methods.

At least 137 species of *Zygnema* and 28 species of *Zygogonium* have been described (Kadłubowska 1984). These species are distinguished based on differences in spore wall ornamentation, sporangial shape, and, to a lesser degree, vegetative characteristics. Previous work on *Spirogyra* suggested that at

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least one factor, polyploidy, played a major role in producing morphological diversity (Hoshaw et al. 1985, Wang et al. 1986). Both polyploidy and hybridization between filaments of different species caused great variation in vegetative morphology, although the zygospore shape and wall structure remained unchanged and the most reliable feature for species identification (Allen 1958, McCourt et al. 1986, McCourt and Hoshaw 1990). Reproductive morphology is used extensively in identification of genera and species of the filamentous Zygnematophyceae. Sexual reproduction occurs by conjugation and fusion of nonflagellate gametes. The zygospore formed by conjugation secretes a multilayered wall composed of the exospore, mesospore, and endospore. The exospore and mesospore are of taxonomic importance. The exospore and endospore are of a thin hyaline nature and composed of cellulose and/or pectic substances. The mesospore usually consists of two layers; the inner one is thick, pigmented, sometimes sculptured, with a germination suture (Simons et al. 1982). The mesospore is known to contain the resistant sporopollenin (Ashraf and Godward 1980, Simons et al. 1982). Parthenospores are sometimes produced as a result of incomplete conjugation (Transeau 1951). Some taxa belonging to the genera *Zygnema* and *Zygogonium*, such as *Zygnema sterile* Transeau, *Z. subcylindricum* H. Krieg., and *Zygogonium capense* (Hodgetts) Transeau, are considered to reproduce asexually by specialized cells (akinetes or aplanospores), and sexual reproduction has never been recorded (Transeau 1951, Gauthier-Lièvre 1965, Kadłubowska 1984). In other *Zygnema* and *Zygogonium* species, sexual and asexual reproductive spores occur together and have identical spore wall structure and ornamentation (Transeau 1951, Kadłubowska 1984, Wei et al. 1989, Wei and Yung 2000).

In *Zygogonium*, a common mode of reproduction is by aplanospores, and sexual reproduction is less frequently seen than in *Zygnema* (Transeau 1951). When sexually reproductive, cytoplasmic residue is left in the gametangia after spore formation in *Zygogonium* but not in *Zygnema* (Transeau 1951, Kadłubowska 1984, Johnson 2002). The presence of a sporangial wall around developing zygospores that separates them from the gametangia and the occasional branching of filaments in *Zygogonium* are additional characters not typical for *Zygnema* (Transeau 1951, Kadłubowska 1984, Johnson 2002). However, a separate wall surrounding the zygospores was observed in a few *Zygnema* species and interpreted as a feature giving the species a position close to *Zygogonium* (Kadłubowska and Christensen 1979, Kadłubowska 1984, Rundina 1998).

There is little information on diversity and distribution of Zygnematophyceae—particularly filamentous Zygnematophyceae—in Californian watersheds compared to the eastern and central United States (Smith 1950, McCourt et al. 1986). To date, the

record of filamentous Zygnematophyceae from California consists of eight species of *Spirogyra*, four species of *Zygnema*, and three species of *Mougeotia*, as well as a few records not identified beyond genus (Collins 1909, 1912, 1918, Transeau 1951, Brown 1965, Goldman 1974, Vis and Sheath 1996).

Our study is based on material collected from streams across California as part of two large stream biomonitoring projects. Representatives of filamentous Zygnematophyceae frequently dominated the stream periphyton assemblages with sterile and reproductive filaments of *Spirogyra*, *Zygnema*, and *Mougeotia*. This article tests generic and infrageneric concepts of *Zygnema* and *Zygogonium* using a large sampling of *Zygnema* species in a molecular phylogenetic framework. We present detailed morphological characterization of the two newly described *Zygnema* species and discuss their vegetative and morphological features in relation to *Zygogonium*.

MATERIALS AND METHODS

This study was carried out on natural populations collected from perennial and nonperennial Californian streams in the spring, summer, and fall of 2007–2010 as part of projects sponsored by the California Water Board. More than 600 localities were sampled. Samples were collected using the modified sampling protocol of Environmental Monitoring and Assessment Program (Fetscher et al. 2009) and fixed in the field with 2.5% histological grade glutaraldehyde (Sheath and Cole 1992). Water temperature, conductivity, and pH were recorded for each site using field meters (OAKTON Instruments, Vernon Hills, IL, USA). For dissolved inorganic nutrients, such as total dissolved nitrogen and total dissolved phosphorus, stream water samples were filtered using 0.45 μm pore-size glass fiber filters (Millipore Ireland Ltd., Cork, Ireland). Total dissolved nitrogen and total dissolved phosphorus were measured after USGS I-2650-03 (Patton and Kryskalla 2003) at University of Georgia, Odum School of Ecology Analytical Chemistry Lab.

In addition to the fixed benthic algal samples, fresh qualitative samples were collected simultaneously at each stream site. Numerous stream sites were resampled to obtain reproducing *Zygnema* filaments. Filamentous algae were collected by hand and kept at 4°C until processed in the laboratory. Samples containing reproductive filaments of *Zygnema* were incubated in water from the habitat, filtered, and diluted with distilled water for further intervals to complete sexual or asexual reproduction and to get mature spores. The samples were placed in the northern window of the laboratory at room temperature (held constant at 20°C). The reproductive filaments were checked every 3 days and different stages of conjugation and spore development were documented by photomicrographs. Specimen observation and photomicrography were performed using an Olympus microscope BX41 with an attached Olympus MicroFire S99809 digital camera (Olympus Imaging America Inc., Center Valley, PA, USA). The size ranges given in the descriptions are based on a minimum of 20 measurements of specimens belonging to each population and were taken by Rincon image analysis software (Imaging Planet, Goleta, CA, USA). Photographed specimens were either living, fixed in 2.5% glutaraldehyde or treated with 10% KOH for distinguishing spore wall layers. Vegetative filaments from conjugating individuals were cultured in Bold's basal medium (Nichols and Bold 1965) on a 12:12 light:dark cycle at 12°C. Sexually mature fixed voucher specimens were deposited in

University Herbarium at University of California, Berkeley, USA. Additional strains used in this study were obtained from public culture collections or isolated from the wild in other states or countries. A subset of taxa from Hall et al. (2008) was used as an outgroup (Table S1 in the supplementary materials).

The chemical composition of the sporangial wall was examined using cytochemical techniques for the localization of cellulose (Calcofluor White, Krishnamurthy 1999) and for pectic substances (Ruthenium Red, Jensen 1962), chemicals from Fisher Scientific (Pittsburg, PA, USA). Preparation of spores for SEM was performed after Hull et al. (1985). The spores were gold coated and observed by FEI Quanta 600 FEG SEM (FEI; North America NanoPort, Hillsboro, OR, USA) and by Hitachi S-2700 SEM (Hitachi High Technologies America Inc., Pleasanton, CA, USA). The exospore was removed manually. The main taxonomic sources were Transeau (1951), Randhawa (1959), Gauthier-Lièvre (1965), Kadłubowska (1984), Rundina (1998), Johnson (2002), as well as publications by Wei et al. (1989), Kadłubowska and Langangen (1998), and Lewis and Entwisle (2007).

Genomic DNA was extracted from frozen material using the Nucleon PhytoPure DNA extraction kit (GE Healthcare, Pittsburgh, PA, USA). Portions of the mitochondrion-encoded *cox3* and chloroplast-encoded *rbcL* genes were amplified by PCR using previously described primers and temperature cycling protocols (Hall et al. 2008). We used GoTaq Green Master Mix (Promega, San Luis Obispo, CA, USA) for PCR. Gene sequences were determined at the University of Washington Genome Center (Seattle, WA, USA) or downloaded from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>). Sequencing reads were assembled and edited in Sequencher v. 4.10.1 (Gene Codes, Ann Arbor, MI, USA) and gene sequences were aligned in MacClade (Maddison and Maddison 2000). The *cox3* alignment was 591 nucleotides in length, although the last 14 nucleotides were excluded because of missing data. The *rbcL* alignment was 1,354 nucleotides long with the last 37 nucleotides excluded because of missing data. Data sets were not partitioned. The nucleotide alignments were analyzed in PAUP* v. 4 beta 10 (Swofford 2003) for parsimony and maximum likelihood. The most parsimonious tree was searched for using a heuristic search with 100 random taxon addition sequences. Support for relationships was estimated using 500 bootstrap (BS) pseudoreplicates under parsimony. A heuristic search with three random taxon addition sequences was employed under maximum likelihood using the GTR + I + Γ model as recommended by MrModel Test (Nylander 2004). Maximum-likelihood bootstrap support was estimated with RAxML v. 7.2.7 (Stamatakis et al. 2005, 2008) using the CIPRES Science Gateway v. 3.0 (<http://www.phylo.org/>). Two parallel Bayesian runs using (six substitution types plus a gamma distribution shape parameter plus invariant sites) were performed using MrBayes v. 3.1.2 (Ronquist and Huelsenbeck 2003) on the CIPRES cluster. Six chains were run for 4 million generations and sampled every 100 generations. Stationarity of the log likelihood scores was determined by graphing in Excel, and the first 2,501 trees were discarded as burnin.

RESULTS

Species of *Zygnema* were recorded in 13.3% of stream sites studied across California. Eight species of *Zygnema* were identified from studied streams, all of which were new records for the Californian algal flora, including two *Zygnema* species new to science. *Zygnema sterile* was the only species identifiable in the vegetative stage and frequently formed akinetes; thus, it was the most commonly recorded species

occurring in 4.6% of samples. The other *Zygnema* species were more rarely encountered. Nearly 3.4% of stream samples contained *Zygnema* filaments, reproducing by aplanospores or zygospores.

All eight *Zygnema* species were morphologically characterized and sequenced for both *rbcL* and *cox3*. Species descriptions are based on a combination of vegetative and reproductive features, and especially focused on zygospore (aplanospores or akinetes if present) shape and wall structure and ornamentation, as well as conjugation type, gametangium and sporangium characters. Figures are arranged as follows: Fig. 1, A–R, illustrates reproductive structures of all taxa for comparison; Fig. 2, A–R, shows the reproductive features of the two new species in more detail; Fig. 3, A–M shows the features of the vegetative filaments; and Fig. 4, A–C, presents scanning electron micrographs of aplanospores. The infrageneric classification of *Zygnema* follows Kadłubowska (1984).

Section *Pectinatum* (Czurda) H. Krieg.

Zygnema aplanosporum Stancheva, J. D. Hall et Sheath **sp. nov.** (Figs. 1, E–G; 2, A–I, O–Q; 3, A–I; and 4, A–C).

Cellulae vegetativae 24.1–33.2 μm latae, 12.5–124 μm longae; chloroplasti 2 vel 4, compressi, globulares, processibus brevibus praediti. Filamenta vegetativa interdum brevi-ramosa. Proliferationes rhizoidales uni- vel multi-cellulares seu ex cellulis basalibus seu cellulis intercalaribus geniculatis mucigenis orientes. Parietes vegetativi cellulares incrassati; in cellulis aliquibus septum et cytoplasma colore purpureo tincta. Conjugatio scalariformis; zygosporeae in tubo conjugationis valde ampliato insidentes, in gametangia extendentes. Zygosporeae globosae, subglobosae, oblongae, subcompressae, 26–42 μm latae, usque 53 μm longae. Aplanosporeae vulgares, globosae vel subglobosae, subcompressae vel irregulares, diam. 27.6–40.6 μm . Zygosporeae atque aplanosporeae ab gametangiis per parietes sporangii cellulosos separatae. Exospora levis, tenuis, sine colore; mesosporeae dense granulata, in sporis admodum maturis atrocaerulea, atrovirescens vel atrobrunnea; sutura prominens.

Vegetative cells 24.1–33.2 μm wide, 12.5–124 μm long; two or four chloroplasts, compressed, globular with short protrusions (Fig. 3, A and C). Vegetative filaments occasionally have short branches (Fig. 3F). Unicellular or multicellular rhizoidal outgrowths (Fig. 3, D, G–I) originate either from basal cells or from intercalary geniculate cells which produce mucilage (Fig. 3E). Vegetative cell walls thickened with septum and cytoplasm colored purple in some populations (Fig. 3B). Conjugation scalariform (Figs. 1G; 2, Q and R); zygospores in greatly enlarged conjugation tube and extending into gametangia. Zygospores globose, subglobose, oblong, slightly compressed, 26–42 μm wide, up to 53 μm in length (Fig. 2P). Aplanospore formation common. Aplanospores globose to subglobose, slightly compressed or irregular, 27.6–40.6 μm in diameter (Fig. 2, C and D). Both zygospores and aplanospores

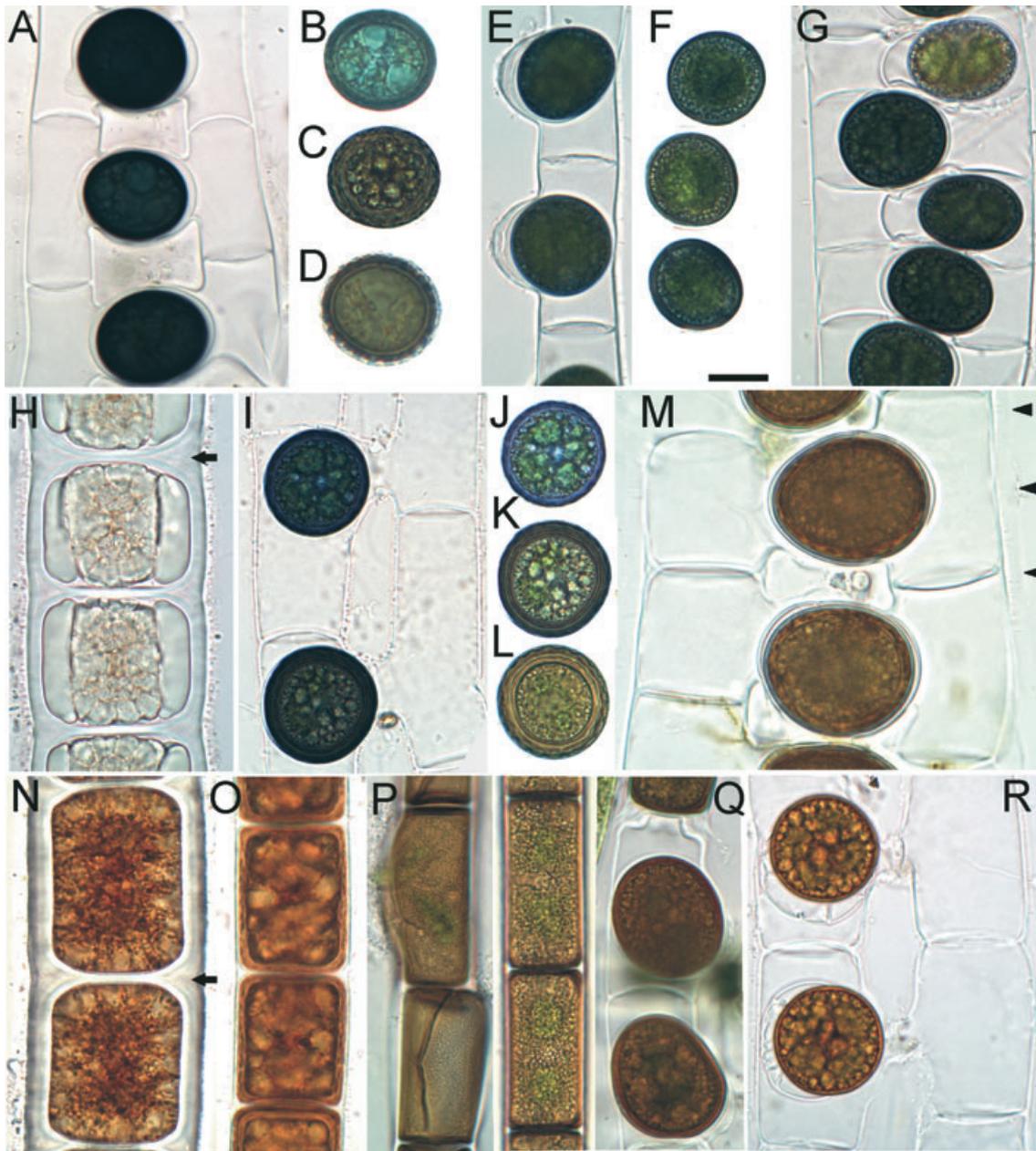


FIG. 1. Light microscopic images of the reproductive structures of *Zygnema* species from California included in phylogenetic analysis: (A) *Z. californicum* scalariform conjugation; (B–D) *Z. californicum* zygospores at successive stages of development; (E and F) *Z. applanosporum* aplanospores; (G) *Z. applanosporum* scalariform conjugation; (H) *Z. sterile* akinetes, note the colorless mesospore; (I) *Z. carinthiacum* scalariform conjugation; (J–L) *Z. carinthiacum* zygospores at successive stages of development; (M) *Z. giganteum* scalariform conjugation; (N) *Z. sterile* brown akinetes; (O) *Z. irregulare* akinetes with brown mesospore; (P) *Z. subcylindricum* akinetes with brown mesospore on different focus plane; (Q) *Z. subcylindricum* conjugation with zygospores in the gametangia; (R) *Z. argillarii* scalariform conjugation, note the expanded exospore. (D) zygospore KOH treated. Arrows show multilayered walls, arrowheads show pectic sheath that covers the filaments. Scale bar, 20 μm .

spores separated from gametangia by cellulosic sporangial wall (Fig. 4A). Exospore smooth, thin, colorless (Fig. 4B); mesospore densely granulate (Figs. 1F; 2, D–I, O; 4C), dark blue, dark bluish-green to dark brown in fully matured spores (Fig. 2, H, I, O); suture prominent (Fig. 2, G–I).

Holotype: Specimen UC 1966682 deposited in University Herbarium at University of California, Berkeley.

Type locality: Santa Ysabel Creek (33°12' N, 116°67' W) crossed by California state route 79 nearby Santa Ysabel Mission (23013 SR 79), San Dieguito watershed, San Diego County, California, USA, May 15, 2008.

Type strain: RS001, from culture.

Etymology: The epithet refers to the predominant reproduction by aplanospores.

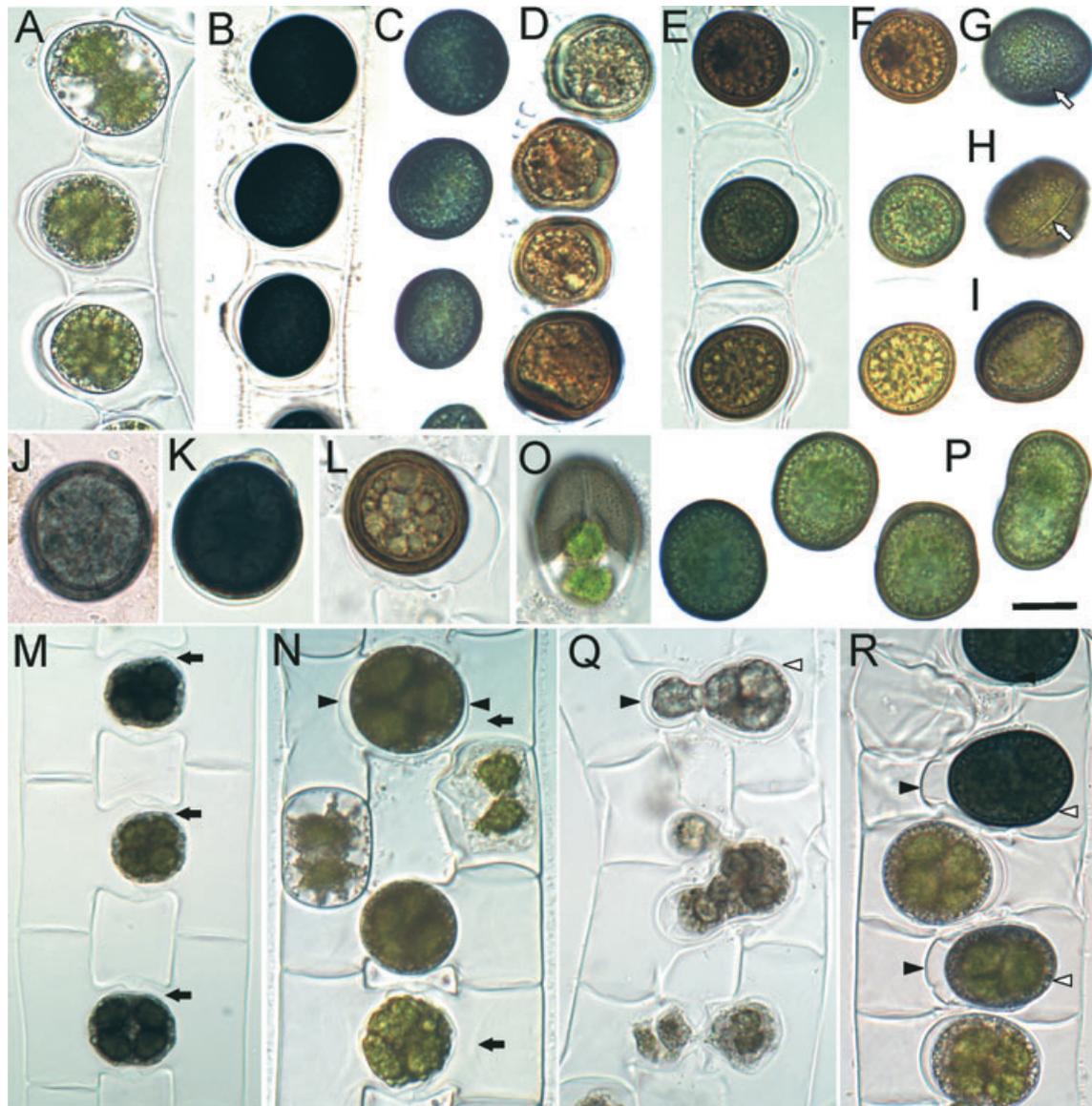


FIG. 2. Light microscopic images of the reproductive structures of the two new *Zygnema* species: (A–I) *Z. aplanosporum*. (A–F) filaments with aplanospores at successive stages of development, note the sporangial wall; (G) aplanospores with granulated blue mesospore and suture; (H and I) mature aplanospores with granulated brown mesospore and suture. (J–N) *Zygnema californicum*. (J) zygospore showed finely denticulate inner mesospore layer; (K) zygospore showed scrobiculate inner exospore layer; (L) liberation of mature zygospore; (M) early stages of scalariform conjugation with zygospores surrounded by pectic layer; (N) latter stages of scalariform conjugation with zygospores surrounded by pectic layer and sporangial wall, note the pectic substances in the gametangia; (O) *Z. aplanosporum* germinating aplanospore, note brown color of granulated mesospore; (P) *Z. aplanosporum* zygospores with blue granulated mesospore; (Q) *Z. aplanosporum* early stages of scalariform conjugation showed sporangial wall around the fusing gametes; (R) *Z. aplanosporum* scalariform conjugation showed sporangial wall around zygospores. Black arrows show pectic layers, white arrows show suture, black arrowheads show sporangial wall, white arrowheads show openings in sporangial wall. (D and K) spores KOH treated. Scale bar, 20 μ m.

Other strains examined: RS007: Indian Creek (32°90' N, 116°49' W), Tijuana River watershed, San Diego County, June 2, 2010; RS009: Tom Neal Creek (41°09' N, 122°20' W), Shasta County, August 3, 2010; RS006: Garcia River (38°92' N, 123°62' W), Mendocino County, June 28, 2010; RS015: Garcia River in Lamour Creek Subwatershed (38°89' N, 123°39' W), Mendocino County, June 14, 2010, California, USA. The selection of strains included populations from most southern and northern

stream locations across California to confirm their genetic identity.

Notes: Reproduction mostly by aplanospores (Figs. 1, E–F; 2, A–I). Aplanospores formed inside the vegetative cell which was inflated up to 55 μ m in diameter (Figs. 1E; 2, A, B, E). Aplanospores surrounded by colorless cellulosic sporangial wall, expanded, and thickened toward central inflation, and opened at the opposite side (Figs. 1E; 2, B and E; 4A). When aplanospores were liberated, the

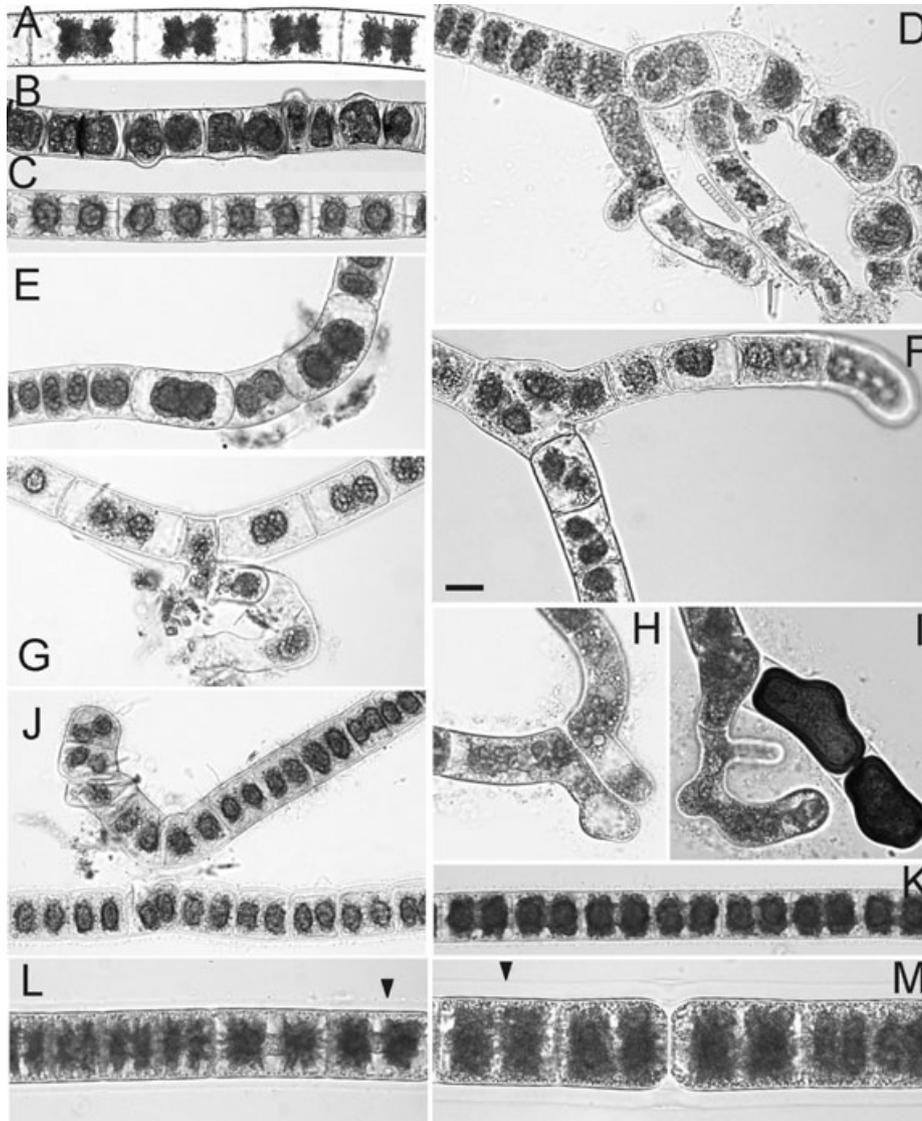


FIG. 3. Light microscopic images of the vegetative filaments of *Zygnema* species from California: (A–I) *Z. aplanosporum* vegetative filaments. (A) filament with compressed, globular chloroplast; (B) filament with thickened cell wall and purple colored cell content; (C) filament from culture; (D) filamentous rhizoids; (E) geniculate filament cells producing mucilage; (F) filament branch; (G) rhizoids; (H) rhizoidal outgrowths originating from geniculate filament cells, from culture; (I) akinetes and basal rhizoidal outgrowths from culture. (J and K) *Z. californicum* vegetative filaments, note the geniculate filament cells producing mucilage; (L) *Z. giganteum* vegetative filaments; (M) *Z. sterile* vegetative filaments. Arrowheads show pectic sheath that covers the filaments. Scale bar, 20 μ m.

sporangial wall detached from the aplanospores and remained in the filament. A similar sporangial wall separated zygosporangia from the gametangia. This wall was formed at the very early stages of conjugation during the fusion of gametes (Fig. 2Q). The sporangial wall was one-side expanded and thickened, and opened on the opposite side (Fig. 2, Q and R). The zygosporangia were discharged by the opening of the sporangial wall, which remained in the filament. Aplanospores and zygosporangia possessed walls identical in color, structure, and ornamentation. Akinetes not common, irregularly compressed-cylindrical, $28\text{--}29 \times 30\text{--}80 \mu\text{m}$ completely filling the cell, with similar wall structure (Fig. 3I). Parthenospores common, globose, up to $30 \mu\text{m}$ in diameter.

The Calcofluor White method revealed that the sporangial wall, which separated zygosporangia and aplanospores from gametangia, was composed of cellulose. The Ruthenium Red method showed that pectic substances were accumulated as a layer beneath a sporangial wall which completely enclosed the spores at the beginning of their formation.

Distribution and ecology. This species was recorded in 15 stream sites from eleven California counties (for details see Table S1). In all sites, vegetative filaments produced aplanospores. Sexual reproduction was observed only in 2009, in collections from the four most northern stream sites in Potato Creek and Garcia River. Sexually reproducing filaments

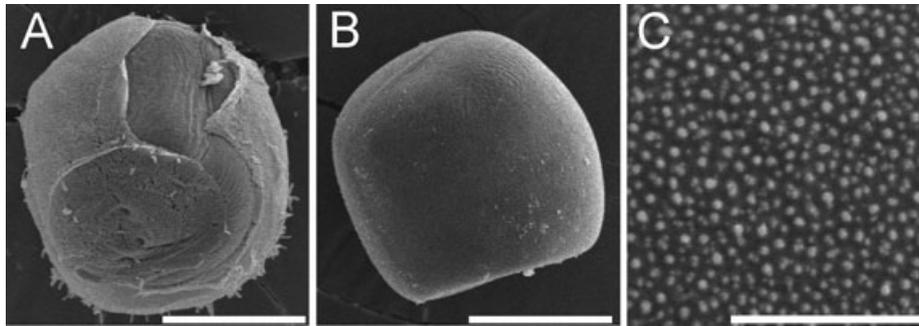


FIG. 4. Scanning electron microscopic images of *Z. aplanosporum* aplanospores: (A) aplanospores covered by sporangial wall partly disclosing the exospores, (B) aplanospore covered by exospores, (C) detail of densely granulate mesospore. Scale bars, (A) and (B) 20 µm; (C) 2 µm.

formed zygospores and aplanospores simultaneously. Akinetes were observed in two populations (Austin Creek and Independence Creek), as well as in laboratory cultures from the type collection.

Water chemistry parameters in the type locality measured on 15 May 2008 were pH, 8.3; temperature, 18°C; conductivity, 402 µS · cm⁻¹; total dissolved phosphorus, 26 µg · L⁻¹; and total dissolved nitrogen, 161.6 µg · L⁻¹. *Z. aplanosporum* was distributed in streams located exclusively in forest areas at elevations 109–2,109 m a. s. l. It was associated with *Nostoc verrucosum*, *Paralemanea catenata*, *Batrachospermum boryanum*, as well as with other zygnematalean algae.

Related species. A group of species hitherto classified as *Zygogonium*, that is, *Zygo. seuratii* Gauth.-Lièvre, *Zygo. stephensiae* Transeau, *Zygo. marocanum* Gauth.-Lièvre, and *Zygo. sudanense* Gauth.-Lièvre, are very similar in regard to conjugation and sporangial wall features, of which the first species completely resembles the shape and structure of *Zygnema aplanosporum* sporangial wall. However, *Z. aplanosporum* differed from the above species by a combination of the following characters: mesospore wall color and ornamentation (Table 1), as well as by constant formation of aplanospores and intercalary geniculate cells in the filament.

Zygnema californicum Stancheva, J. D. Hall et Sheath **sp. nov.** (Figs. 1, A–D; 2, J–N; and 3, J and K)

Cellulae vegetativae 23–29 µm latae, 32–89 µm longae; chloroplasti duo stellati, processibus brevibus praediti. Conjugatio scalariformis; zygosporeae in tubis conjugationis formatae, ovoidea vel globosae, subcompressae, 32–40 × 34–46 µm, ab gametangiis per parietem sporangii cellulorum separatae. Exospore sine colore; stratum exterius leve, interius scrobiculatum. Mesosporeae e stratis duobus compositae, in zygosporeis matures atro-caesia vel brunneae; stratum interius leniter denticulatum. Sutura in zygosporeis aliquibus visibilis.

Vegetative cells 23–29 µm wide; 32–89 µm long; two stellate chloroplasts with short protrusions (Fig. 3K). Conjugation scalariform; zygospores formed in the conjugation tubes, ovoid to globose,

slightly compressed, 32–40 × 34–46 µm (Figs. 1A; 2, M and N), separated from the gametangia by a cellulosic sporangial wall. Exospore colorless; outer layer smooth, inner layer scrobiculate (Fig. 2K). Mesospore of two layers; dark blue to brown in matured zygospores; outer layer scrobiculate, with deep pits 2–3 µm in diameter, spaced 2–3 µm apart (Fig. 1, C and D); inner layer finely denticulate (Fig. 2J). Suture visible in some zygospores.

Holotype: Specimen UC 1966681 deposited in University Herbarium at University of California, Berkeley.

Type locality: Matilija Creek (34°50' N, 119°37' W), California state route 58, 100 km west from interstate highway 5, Ventura River watershed, Ventura County, California, USA, June 9, 2010.

Type strain: RS010, from field material.

Etymology: The epithet refers to the U.S. state California, where *Z. californicum* was first observed.

Other strains examined: RS016: Matilija Creek (34°50' N, 119°37' W), Ventura County, August 3, 2010, from field material.

Notes: Some intercalary cells in the vegetative filament were geniculate. They produced mucilage, which united a few filaments at their basal part and attached them to the substratum (Fig. 3J). The Ruthenium Red method revealed that at the beginning of reproduction zygospores were surrounded by a pectic layer 2–5 µm thick, and a smooth pectic material was accumulated in the gametangia (Fig. 2, M and N). The Calcofluor White method showed that matured zygospores were enclosed in a smooth cellulosic colorless sporangial wall, which remained in the filaments when zygospores were released (Figs. 1A; 2, N and L). The spores were liberated by the breaking of an equatorial suture in the sporangial wall (Fig. 2L).

Distribution and ecology. This species is known only from the type locality where it was multiple times collected from 2008 to 2010. Water chemistry parameters in the type locality measured on 17 June 2008 were pH, 8.3; temperature, 15.9°C; conductivity, 839 µS · cm⁻¹; total dissolved phosphorus, 1.1 µg · L⁻¹; and total dissolved nitrogen, 1.4 µg · L⁻¹. *Z. californicum* was associated with *Nostoc verrucosum*,

TABLE 1. Comparison of vegetative and reproductive features of *Zygnema aplanosporum* and *Zygnema californicum* with related taxa. NR, not reported in the literature.

	Vegetative cells width (µm)	Zygospor e dimensions (µm)	Mesospor e color	Mesospor e ornamentation	Reproduction mode other than scalariform conjugation
<i>Z. aplanosporum</i>	24.1–33.2	26–42 × 39–53	Dark blue, bluish-green, dark brown	Densely granulate	Globose aplanospores; cylindrical akinetes
<i>Zygo. marocanum</i>	20–24	32–40 × 38–40 × 22–28	Blue	Finely scrobiculate	NR
<i>Zygo. sudanense</i>	15–17	28–30 × 26–28 × 20–22	Yellow-brown	Finely, deep scrobiculate	NR
<i>Zygo. seuratii</i>	21–28	30–40 × 42–30 × 30–35	Blue	Scrobiculate; pits 2.5–3 µm, 3–5 µm apart	NR
<i>Zygo. stephensiae</i>	20–29	30–43 × 42–54	Yellow-brown	Densely punctate	Conjugation-lateral and conjugation through end wall; cylindrical akinetes
<i>Z. californicum</i>	23–29	32–40 × 34–46	Dark blue to brown	Outer layer scrobiculate, pits 2–3 µm, 2–3 µm apart; inner layer finely denticulate	NR
<i>Z. coeruleum</i>	24–26	26–32 × 32–35	Blue	Scrobiculate, pits 1.5 µm, 3 µm apart	NR
<i>Z. gorakhporens e</i>	23–27	36–43 × 30–36	Blue	Scrobiculate, pits 4 µm, 1–3 µm apart	NR
<i>Z. synadelphum</i>	17–21	27–36 × 34–44	Blue	Irregularly punctuate; pits 1–2 µm	Cylindric-ovoid aplanospores
<i>Z. pawhuskae</i>	21–35.6	34–48 × 46–65	Seal-brown	Outer layer smooth; inner layer densely verrucose, reticulate-verrucose	NR
<i>Zygo. orientale</i>	18–21	30–48 × 30–45 × 30	Yellow-brown	Scrobiculate, pits 2–2.5 µm, 2.5–3 µm apart	NR
<i>Zygo. guineense</i>	20–22	41 × 45 × 35	Reddish-brown	Scrobiculate, pits 3–4 µm, 4–5 µm apart	NR
<i>Zygo. laetevirens</i>	27–32	51–60 × 42–45	Brown	Outer layer scrobiculate, pits 2–4 µm, 3–5 µm apart; inner layer minutely verrucose	NR

Anabaena pseudoscillatoria, as well as with other zygne matalean algae.

Related species. *Zygnema coeruleum* Czurda, *Z. gorakhporens e* R. N. Singh, and *Z. synadelphum* Skuja were similar to *Z. californicum* in zygospor e size, ornamentation, and color. However, formation of a sporangial wall around the zygospor es was not reported for these species. A similar pectic layer around the zygospor es is known for *Zygnema pawhuskae* Taft, which differs in zygospor e ornamentation. *Zygonium orientale* Wei, *Z. guineense* Gauth.-Lièvre, and *Z. laetevirens* (Klebs) Mig. had similar structure of sporangia wall and scrobiculated mesospor e, although blue-colored stages in mesospor e development were not described (Table 1).

Section *Collinsianum* (Czurda) H. Krieg.

Zygnema giganteum Randhawa (Figs. 1M and 3L).

(Transeau 1951, p. 27, plate III, figs. 9 and 10, Randhawa 1959, p. 225, fig. 161A–C, Kadłubowska 1984, p. 204, fig. 305A–D).

Vegetative cells 36–43 µm wide; filaments covered by a pectic sheath up to 7 µm in thickness (Fig. 3L). Conjugation scalariform; zygospor es formed in the conjugation tubes, extending into gametangia; zygospor es ovoid to globose, 35–50 × 53–69 µm (Fig. 1M). Mesospor e of two layers; yellow-brown; outer layer undulate pitted; inner layer granulate. Exospor e consists of two smooth colorless layers.

Notes: This species is known only from India, reproducing sexually by formation of zygospor es in the conjugation tubes or in the gametangia, and asexually by cylindrical aplanospores. In Californian

material, zygospores placed in the gametangia and aplanospores were not observed, and vegetative filaments were covered by pectic sheath, not described in the literature for *Z. giganteum*.

Strain: RS013: Ash Creek (41°19' N, 120°98' W), Modoc County, California, USA, August 3, 2010.

Water chemistry parameters in the locality measured on 03 August 2010 were pH, 8.5; temperature, 17°C; conductivity, 269 $\mu\text{S} \cdot \text{cm}^{-1}$.

Section *Leiospermum* (Czurda) H. Krieg.

Zygnema argillarii Kadłub. (Fig. 1R).

(Kadłubowska and Christensen 1979, p. 167, fig. 2, Kadłubowska 1984, p. 174, fig. 245, Rundina 1998, p. 70, fig. 27).

Vegetative cells 26–32 μm wide. Conjugation scalariform; zygospores formed in one of the gametangia. Zygospores lenticular, in frontal view spherical or broadly elliptical 31–39 \times 39–42 μm . Mesospore yellow-brown, densely punctate. Exospore smooth, thin, colorless, expanded, and detached from zygospore; it remained in the filaments when zygospores were released.

Notes: It is a rare and little-known species from Europe (Rundina 1998). In the original description, the formation of separate wall surrounding the zygospores has been noted of taxonomic importance and related to the gametangial walls in *Zygogonium* (Kadłubowska and Christensen 1979). In contrast, Rundina (1998) considered that expanded thin layer surrounding the zygospores as being an exospore. Calcofluor staining showed that expanded colorless exospore is the only cellulose layer above the mesospore, and rejected the presence of additional separate wall in Californian material.

Strains: RS005: Indian Creek (32°90' N, 116°49' W), Tijuana River watershed, San Diego County, June 2, 2010; RS008: Cottonwood Creek (32°19' N, 116°49' W), Tijuana River watershed, San Diego County, California, USA, May 30, 2010.

Water chemistry parameters in Indian Creek measured on 02 June 2010 were pH, 7.3; temperature, 8.2°C; conductivity, 346.8 $\mu\text{S} \cdot \text{cm}^{-1}$.

Zygnema carinthiacum Beck-Mannagetta (Fig. 1, I–L)

(Transeau 1951, p. 39, plate VI, figs. 16, Kadłubowska 1984, p. 195, fig. 284, Rundina 1998, p. 89, fig. 36, 48, Johnson 2002, p. 506, fig. 127A)

Vegetative cells 26–28 μm wide. Conjugation scalariform; zygospores formed in one of the gametangia. Zygospores globose to ovoid, 30–38 \times 30–49 μm . Exospore colorless. Mesospore of two layers, dark blue (Fig. 1J) to brown in fully matured zygospores (Fig. 1, K and L). Outer mesospore layer scrobiculate, with pits 3–5 μm in diameter, spaced 2–5 μm apart, inner layer finely denticulate, distinguishable only in brown zygospores (Fig. 1, K and L).

Notes: Cosmopolitan, but rare and little-known species (Rundina 1998, Johnson 2002). According to Transeau (1951), Kadłubowska (1984), and Johnson (2002) the mesospore is composed of single thick, blue, scrobiculate layer. Rundina (1998)

described a shift in the mesospore color from blue to blue-green in fully matured zygospores and two obvious mesospore layers in some populations. The inner finely denticulate mesospore layer is easily dissolved in nitric acid and lactic acid, and thus probably overlooked (Rundina 1998). Californian material resembles closely the description of Rundina (1998), except for the brown color in mature zygospores.

Strain: RS011: Elizabeth Lake Canyon (34°64' N, 118°51' W), Los Angeles County, California, USA, June 2, 2010.

Water chemistry parameters in the locality measured on 02 June 2010 were pH, 8.5; temperature, 24.5°C; conductivity, 645 $\mu\text{S} \cdot \text{cm}^{-1}$.

Section *Cylindricum* H. Krieg.

Zygnema irregulare H. Krieg. (Fig. 1O).

(Transeau 1951, p. 43, plate VII, fig. 8, Randhawa 1959, p. 255, fig. 227, Kadłubowska 1984, p. 214, fig. 323, Rundina 1998, p. 91, fig. 38, 3).

Vegetative cells 36–40 μm wide; conjugation unknown; reproduction by akinetes. Akinetes cylindrical, completely filling the cell, 33–40 \times 20–54 μm . Mesospore brown, thick, with irregularly spaced small granules and pits 3–7 μm in diameter; suture oblique.

Strain: RS012: Indian Creek (32°90' N, 116°49' W), Tijuana River watershed, San Diego County, California, USA, June 2, 2010.

Notes: A rare species known only from Europe. Some Californian specimens have narrower vegetative filament (<40 μm).

Water chemistry parameters in the locality measured on 02 June 2010 were pH, 7.3; temperature, 8.2°C; conductivity, 346.8 $\mu\text{S} \cdot \text{cm}^{-1}$.

Zygnema sterile Transeau (Figs. 1, H and N; and 3M)

(Transeau 1951, p. 41, plate VII, Fig. 11, Randhawa 1959, p. 255, fig. 228, Kadłubowska 1984, p. 208, fig. 306, Rundina 1998, p. 92, fig. 38, 6).

Vegetative cells 45–55 μm wide, filaments covered by a pectic sheath up to 10 μm in thickness, often deeply constricted at the plane of the cross walls (Fig. 3M). Conjugation unknown; reproduction by akinetes. Akinetes heavy-walled, completely filling the cells, 47–55 \times 30–70 μm (Fig. 1N); mesospore colorless, smooth (Fig. 1H). The brown color and granulation of the akinetes apparently resulted from chemical changes in the protoplasts and chloroplasts.

Strain: RS002: Santa Ysabel Creek (33°12' N, 116°67' W), San Dieguito watershed, San Diego County, California, USA, June 04, 2008.

Notes: A common species known from Europe, North America, Asia (Transeau 1951, Kadłubowska 1984, Rundina 1998). Californian specimens fulfill the Transeau (1951) description, including multilayered appearance of akinete walls.

Water chemistry parameters in the locality measured on 15 May 2008 were pH, 8.3; temperature, 18°C; conductivity, 402 $\mu\text{S} \cdot \text{cm}^{-1}$.

Zygnema subcylindricum H. Krieg. (Fig. 1, P and Q).

(Transeau 1951, p. 43, plate VII, fig. 13, Randhawa 1959, p. 254, fig. 225A and B, Kadłubowska 1984, p. 214, fig. 325, Rundina 1998, p. 91, fig. 38,4 and 5)

Vegetative cells 26–30 μm wide. Reproduction mostly by means of akinetes; conjugation was known only from culture. Akinetes cylindrical completely filled the cell, 27–30 \times 33–80 μm (Fig. 1P). Mesospore yellow-brown, thick, regularly granulated; granules fine, about 1 μm apart. Suture irregular, oblique or envelope-like, one or two per akinete (Fig. 1P). Conjugation scalariform; zygospores formed in one of the gametangia (Fig. 1Q). Zygospores subglobose 33–35 \times 40–42 μm ; mesospore, yellow-brown, granulated.

Strain: RS003: Arrowbear Lake (34°12' N, 117°04' W), San Bernardino County, April 03, 2008, California, USA.

Notes: Rare and little-known species from Europe and Asia (Rundina 1998). Californian specimens do not reach maximum filament width of 35 μm . The sexual reproduction was observed for the first time in this work.

Water chemistry parameters in the locality measured on 03 April 2008 were pH, 8.3; temperature, 5°C; conductivity, 19.7 $\mu\text{S} \cdot \text{cm}^{-1}$.

Phylogenetic analyses. Sequence alignments for *cox3* and *rbcL* were assembled and analyzed separately. The *cox3* alignment included 577 sites of which 237 were variable and 204 were parsimony-informative. Within *Zygnema*, 66 sites were variable and 51 sites were parsimony informative. The *rbcL* alignment included 1,317 sites, of which 475 were variable and 406 were parsimony informative. Within *Zygnema*, 143 sites in the *rbcL* alignment were variable and 107 sites were parsimony informative. Combining the *cox3* and *rbcL* data into a single data set did not significantly increase support for most relationships within *Zygnema* when compared to the *rbcL* data alone (not shown).

Strains of *Zygnema* were positioned in a single monophyletic clade sister to *Zygonium tunetanum* (Figs. 5 and 6). Strains from California were intermixed with strains from other parts of the world. In both the *rbcL* and *cox3* phylogenies, strains of *Zygnema* formed two major clades. These clades were moderately to strongly supported in *rbcL* analyses (Fig. 5), although one of the clades (minus *Z. circumcarinatum* Czurda for which *cox3* data were not available) received little support in the *cox3* likelihood and Bayesian analyses (Fig. 6). The first clade contained *Z. californicum* and *Z. aplanosporum* (section *Pectinatum*), *Z. carinthiacum* and *Z. peliosporum* Witt. (section *Leiospermum*), and *Z. sterile* (section *Cylindricum*) and several unidentified strains (Figs. 5 and 6). All these species have a blue stage during the development of the mesospore, although the mesospore color of the mature zygospore may be brown, or colorless for akinetes. The second clade

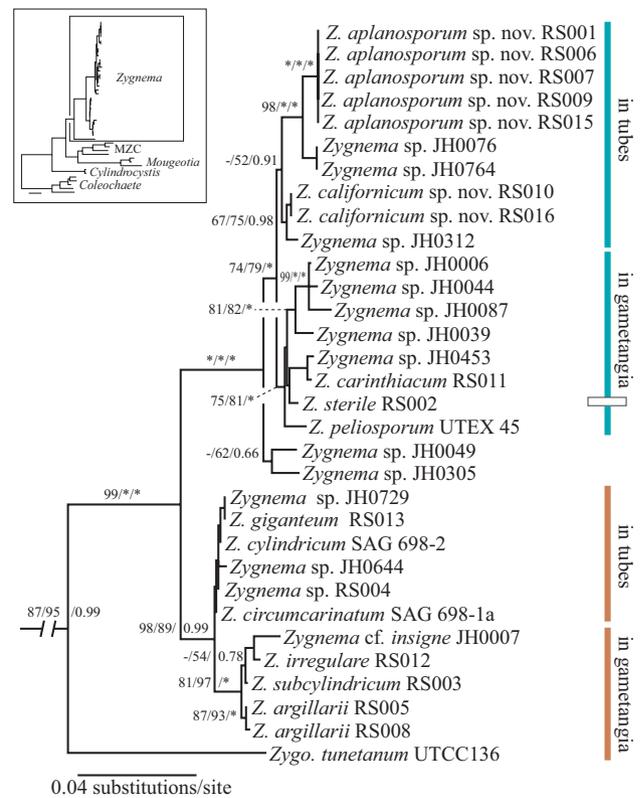


FIG. 5. Phylogeny of *Zygnema* and *Zygonium* based on *rbcL* data. Maximum-likelihood tree found using the GTR + I + Γ model. Numbers above the branches are bootstrap values from Parsimony analysis, RAxML, and posterior probabilities from a Bayesian analysis, respectively. An asterisk indicates bootstrap support of 100 or a posterior probability of 1.0. A dash indicates bootstrap support of <50 or a posterior probability <0.5. Mesospore color is indicated by the color of the bar on the right.

contained *Z. circumcarinatum* and *Zygnema* sp. JH0644 (section *Pectinatum*), *Z. giganteum* (section *Collinsianum*), *Z. argillarii* and *Z. cf. insigne* (Hassall) Kütz. (section *Leiospermum*), *Z. cylindricum*, *Z. subcylindricum* and *Z. irregulare* (section *Cylindricum*), and two other strains of *Zygnema*. All these species have yellow or brown mesospores, but never blue. These two major clades each contain subclades of taxa with zygospores produced either in conjugation tubes or the gametangia (Fig. 5). Reproductive characteristics of many strains remain unknown because only sterile filaments were encountered. *Zygnema sterile*—although phylogenetically related to species with blue spores formed in gametangia—is not known to reproduce sexually and forms akinetes with colorless walls. Species with many characteristics typical of *Zygonium* (i.e., *Z. aplanosporum* and *Z. californicum*) were deeply embedded in a clade of many strains of *Zygnema* in both *rbcL* and *cox3* analyses (Figs. 5 and 6). In the *rbcL* phylogeny, these species were positioned together in a clade with three unidentified species of *Zygnema* (strains JH0076, JH0312, and JH0764) with low statistical support (Fig. 5). Strain JH0076 was collected from

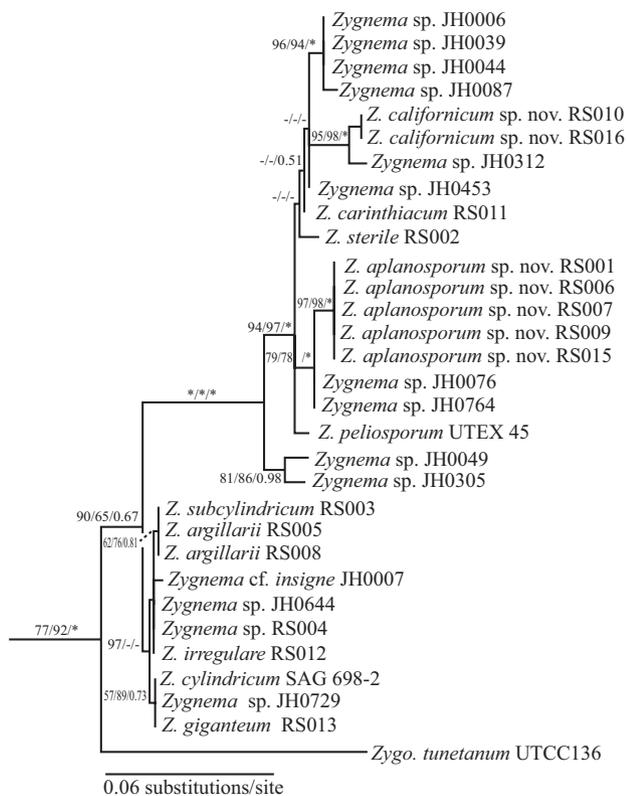


FIG. 6. Phylogeny of *Zygnema* and *Zygo. tunetatum* based on *cox3* data. Maximum-likelihood tree found using the GTR + I + Γ model. Numbers above the branches are bootstrap values from Parsimony analysis, RAxML, and posterior probabilities from a Bayesian analysis, respectively. An asterisk indicates bootstrap support of 100 or a posterior probability of 1.0. A dash indicates bootstrap support of <50 or a posterior probability <0.5.

damp soil, and strain JH0764 was collected from an acid stream (Table S1). Although these are habitats considered typical for *Zygo. tunetatum*, identity of neither strain JH0076 nor JH0764 could be confirmed because of a complete absence of reproductive spores.

DISCUSSION

Synapomorphies in clades of Zygnema. The existing infrageneric classification of *Zygnema* is not consistent with our molecular phylogeny. The previous classification used the reproductive mode to divide *Zygnema* species in three or four groups (sections) according to the presence/absence of sexual reproduction, and the position of zygospores in the conjugation tube, in gametangia, or in tubes but extended into the gametangia (Gauthier-Lièvre 1965, Kadłubowska 1984). Although these characteristics may be useful in distinguishing species, they do not correspond to natural groups. We propose that mesospore color may be a useful characteristic for the infrageneric classification of *Zygnema* species. However, we could not confirm mesospore color for many of the strains included in this study (because

the strains were derived from sterile filaments) and many species of *Zygnema* remain untested. Some recent studies (Butt 2003, Ghazala et al. 2004) suggested chemotaxonomic properties could be important in *Zygnema* taxonomy, similar to other algal groups (Kamenarska et al. 2006). Phycochemical study of three species of *Zygnema* showed that *Z. czurdae* Randhawa, which has blue-colored mesospore, differed from two *Zygnema* species with brown-colored mesospore in composition of fatty acids, sterols, and terpenes (Butt 2003, Ghazala et al. 2004).

Based on our phylogenetic analyses, it appears that mesospore coloration is a principal character in the classification of *Zygnema*. One lineage of *Zygnema* contained those strains with blue mesospore coloration in their development and the other lineages contained species without a blue mesospore stage (Figs. 5 and 6). This first lineage included species in which zygospores are formed either in the conjugation tube or in the gametangia (*Zygnema* sections *Pectinatatum* and *Leiospermum*) as well as one strain reproducing only by akinetes (section *Cylindricum*). The structure of *Z. sterile*'s akinetes has not been described in detail (Rundina 1998). This study showed that akinetes appeared brown in color, but when their cytoplasmic contents had been discharged, the wall proved to be colorless and lamellate. This finding confirmed Transeau's (1951) statement that the brown color and granulation of *Z. sterile* akinetes resulted from chemical changes in the protoplasts and chloroplasts.

The second main *Zygnema* lineage included species with zygospores placed in the conjugation tubes or in the gametangia or both (*Zygnema* sections *Pectinatatum*, *Leiospermum*, and *Collinsianum*) as well as those reproducing primarily by akinetes (*Zygnema* section *Cylindricum*), with mesospore coloration that turned directly to yellow or brown. Furthermore, species known to reproduce only by means of akinetes were positioned within three different clades. However, it is likely that many of the *Zygnema* species not known to conjugate are capable of sexual reproduction. This assumption was supported by observation of sexual reproduction in *Z. subcylindricum*, which was similar to the other members of that clade (i.e., *Z. argillarii* and *Z. cf. insigne*). Sexual reproduction was not previously known in *Z. subcylindricum* (Kadłubowska 1984). Based on *Z. subcylindricum*'s reproductive characteristics, it should be classified in section *Leiospermum* in the traditional classification.

Zygnema and Zygo. tunetatum. *Zygo. tunetatum*, a strain isolated from a Canadian lake, was sister to the *Zygnema* clade (Figs. 5 and 6). Morphological features of *Zygo. tunetatum* from Canada (Wei et al. 1989) differed from the original description of that species (Gauthier-Lièvre 1965). However, formation of cytoplasmic residue in gametangia after conjugation—a characteristic some-

times considered typical of *Zygonium*—was not mentioned for *Zygo. tunetanum* (Gauthier-Lièvre 1965, Wei et al. 1989), so the identity of this strain remains uncertain. A previous study (Hall et al. 2008) reported that a strain of *Z. circumcarinatum* was closely related to *Zygo. tunetanum*. The chloroplast genome of a different strain of *Z. circumcarinatum* has been sequenced (Turmel et al. 2005) and the *rbcL* sequence from that strain indicates that *Z. circumcarinatum* is closely related to other strains of *Zygnema* (Fig. 5) (see “Publication Note”).

The phylogenetic position of *Zygonium* remains unclear due to the lack of strains that could be confidently identified (Hall et al. 2008, this study). In most recent studies, (e.g., Kleeberg et al. 2006, Holzinger et al. 2010) the identification of *Zygonium ericetorum* Kütz., the type species of *Zygonium*, was based only on vegetative filament features, which are not sufficient to reliably distinguish *Zygonium* from *Zygnema*. Two species attributed here to *Zygnema*, *Z. aplanosporum* and *Z. californicum*, were characterized by zygospores formed in the conjugation tubes and separated from gametangia by a cellulose sporangial wall, features typical of *Zygonium*. In addition, *Z. aplanosporum* possessed a combination of vegetative and reproductive features noted as characteristic of *Zygonium*, such as presence of short branches, single-celled or filamentous rhizoidal outgrowths, thickened vegetative cell walls, purple colored cell content and transverse walls, small compressed-globular chloroplasts, as well as predominant asexual reproduction (Transeau 1951, Gauthier-Lièvre 1965, Kadłubowska 1984, Rundina 1998). Another important and unique feature of *Zygonium* is the cytoplasmic residue that remained in the sporangium after both aplanospore and zygospore formation. However, in some *Zygonium* species, cytoplasmic residue was not illustrated or described (Transeau 1951, Gauthier-Lièvre 1965). Therefore, this characteristic is probably not universal in *Zygonium* and was not observed in *Z. aplanosporum* or *Z. californicum*.

In gross morphology and reproductive mode, *Z. aplanosporum* resembled *Z. terrestre* Randhawa (Randhawa 1959). Despite its terrestrial distribution, *Z. terrestre* has not been assigned to the genus *Zygonium* because of the lack of sporangial wall (Randhawa 1959). However, Randhawa (1959) considered *Z. terrestre* to have characteristics linking *Zygnema* and *Zygonium* and speculated that adoption of the terrestrial habit, partial utilization of the protoplast of the gametangia, and isolation of the zygospores by walls from the gametangia can be taken as advanced features of *Zygonium*. Our data indicate that *Z. aplanosporum* and *Z. californicum*, which possess all the features characteristic of *Zygonium*, are embedded in a larger clade of *Zygnema*. Based on our observations, there are no features or combinations of features that separate *Zygnema* and *Zygonium*. Although it will be necessary to study the type species of *Zygonium* (*Zygo. ericetorum*) before we

can be absolutely certain, we conclude that *Zygonium* is probably a synonym of *Zygnema*.

Additional criteria for Zygnema systematics. The mesospore color could be obscure, particularly in the representatives from the first lineage from phylogenetic analysis. Among these species, the mesospore was blue colored with detectable ornamentation during early developmental stages. Only in some of the oldest zygospores did the mesospore turn brown, and then ornamentation was more pronounced and the suture became prominent. Two years of continuous observation of aplanospore development in *Z. aplanosporum* from cultures and numerous natural populations revealed that most of the aplanospores in one filament had blue, bluish-green to dark-blue mesospores, and only in a few (the oldest ones) did the mesospore become dark brown with more prominent granulations and suture. The brown-colored mesospore was thicker compared to blue colored, and only aplanospores with a brown mesospore germinated (Fig. 20). Similar changes in mesospore color, thickness, and ornamentation were observed in *Z. californicum* and *Z. carinthiacum*. In the zygospores with blue-colored mesospore, four chloroplasts were still detectable, and the mesospore ornamentation was less developed (Fig. 1, B and J) compared to brown-colored zygospores (Fig. 1, D and L). Thus, the aplanospores and zygospores with dark-brown mesospore were considered to be fully mature. In contrast, Transeau (1951, p. 28) described the zygospore mesospore in *Z. terrestre* as “blue when mature, brownish when immature.” Confusion might arise during the identification of *Zygnema* species which have blue-colored stages during the development of mesospore because field samples or cultures contain a mixture of zygospores with blue and brown mesospore (this study, Poulíčková et al. 2007). Therefore, fully mature zygospores should be considered to support correct species identification of *Zygnema* species. Our data showed that the most reliable character for identification of *Z. aplanosporum* was the structure of the aplanospore and its sporangial wall. Vegetative filament morphology and akinete formation were variable among the populations, and other authors have suggested that these features may be dependent on environmental conditions (Randhawa 1959, Wei et al. 1989).

Local distribution patterns in California. This study has increased our knowledge of the diversity of *Zygnema* in California. Previously, only four *Zygnema* species were known from the studied area: *Z. chalybeospermum* Hansg., *Z. insigne*, *Z. peliosporum*, and *Z. stellinum* (Vaucher) C. Agardh (Collins 1909, 1918, Transeau 1951). This work added eight *Zygnema* species to the algal flora of California, and none of the previously identified species were encountered. Similar studies of lotic ecosystems in New Zealand doubled the number of species belonging to *Spirogyra* and *Zygnema* known

to occur in that region (Novis 2004), so it is not unprecedented to find unreported species in poorly studied regions.

Little is known about how environmental conditions affect distribution and reproduction of zygne-mataleans especially in arid areas such as southern California. Smith (1950) stated that *Zygnema* is more abundant than *Spirogyra* in southern California, whereas in the other parts of the United States, it is considerably rarer. We recorded *Spirogyra* more often (24% of sites) than *Zygnema* (15% of sites) in southern California streams (a total of 175 stream sites from this region). However, our study was focused on running, neutral to alkaline waters, although the Zygnematophyceae in general occur in neutral to slightly acidic lentic habitats (ditches, ponds, and lakes) (Gerrath 2003), which could explain the lack of agreement with existing knowledge about the local *Zygnema* biodiversity.

Species-level identification of *Zygnema* may have potential for water-quality monitoring. For instance, *Z. applanosporum* has been collected only in habitats dominated by other algae regarded as indicative of good water quality, such as the red algae *Paralemana catenata* and *Batrachospermum boryanum*, and the nitrogen-fixing cyanobacterium *Nostoc verrucosum* (Komárek et al. 2002, Sheath 2002). Thus, studies that aim to link the distribution and diversity of filamentous Zygnematophyceae to factors that regulate lotic communities are needed to improve their application in regional stream bioassessment.

PUBLICATION NOTE

After this manuscript was reviewed for publication, additional information about the phylogenetic position of *Zygnema circumcarinatum* became available. Two strains of *Z. circumcarinatum* were received from the Microalgae and Zygnemophyceae Collection of Hamburg (MZCH, formerly SVCK). The *rbcL* sequences from these strains were almost identical to the sequence from *Z. circumcarinatum* strain UTEX 42 reported by Hall et al. (2008). The sequences from the MZCH and UTEX strains are markedly different from the *rbcL* sequence derived from the whole chloroplast genome of *Z. circumcarinatum* strain SAG 698-1a (Turmel et al. 2005) (compare the phylogenetic position of *Z. circumcarinatum* SAG 698-1a in this study to that of UTEX 42 in Hall et al. 2008). This finding suggests that the published chloroplast genome of strain SAG 698-1a is not that of *Z. circumcarinatum*, but rather some other species of *Zygnema*.

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Supplementary Material

The following supplementary material is available for this article:

Table S1. Information of samples used in the present study.

This material is available as part of the online article.

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