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Complex patterns of actin molecular evolution in the red alga *Stylonema alsidii* (Stylonematophyceae, Rhodophyta)

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SUMMARY

The Stylonematophyceae is an early diverging red algal class that contains organisms in which sexual reproduction has not been reported. Previous studies have hypothesized a single copy of the actin gene in this class. Amplification of the actin gene in members of the Stylonematophyceae, including several isolates of *Stylonema alsidii*, reveals a more complex evolutionary history. The data support independent gene duplications in *Goniotrichopsis reniformis* and *Stylonema alsidii*. Three of the seven isolates of *S. alsidii* had three copies of actin. Analyses indicate that all copies are functional and not pseudogenes. Due to our lack of knowledge of the ploidy level in *Stylonema* and the asexual nature of these organisms, an ancient change in ploidy level, producing diploid or even triploid organisms, cannot be ruled out to explain two copies of actin within these organisms. Within *Stylonema alsidii* two of the actin copies (Groups 1 and 2) show similar levels of variation; p-distances and the number of synonymous and non-synonymous sites are roughly the same. Yet the changes are distributed differently. One group showed little shared variation among the samples, mutations therefore represent autoapomorphies, while variation in the other group is usually shared among samples (parsimony-informative). Variation in the other group is usually shared among samples (parsimony-informative). A third group of actin genes also observed in *S. alsidii* are highly divergent from the other two copies, yet they maintain all of the signatures of functional proteins. Our data reveal a complex evolutionary history of the actin gene in these species and highlight the lack of knowledge into the basic biology (ploidy level, life cycle characteristics) of this class.

Key words: actin, gene duplication, molecular evolution, Rhodophyta, Stylonema, Stylonematophyceae.

INTRODUCTION

The red algae, Rhodophyta, are a unique eukaryotic lineage lacking flagellated stages and containing a primary plastid with phycobiliproteins. The phylum consists of several early branching classes (e.g. Stylonematophyceae), and a class containing most of the described species, Florideophyceae (Yoon *et al*. 2006). Although species numbers are low in many of the early diverging groups their biochemical diversity and genetic divergence is large (Karsten *et al*. 1999, 2003; Zuccarello *et al*. 2008).

Actin is a ubiquitous cytoskeletal protein of eukaryotes. In association with actin-associated proteins (e.g. myosin), it is involved in multiple cellular processes from cell movement, cell shape change to membrane-protein localization (e.g. Staiger 2000; Drøbak *et al*. 2004). Among its various functions the role of actin has been specifically demonstrated in red algae in nuclear movement within egg cells (Wilson *et al*. 2002) and amoeboid motion in spores (Ackland *et al*. 2007). Actin has been used in phylogenetic analysis between and within eukaryotic kingdoms (e.g. Bhattacharya & Weber 1997; Baldauf *et al*. 2000; Nozaki 2005).

The actin gene has been characterized in a few red algae. Multiple copies of the actin gene are described from the morphologically complex red algae (Florideophyceae) (Hoef-Emden *et al*. 2005; Le Gall *et al*. 2005) and this has been attributed to the need for functional diversity in actins in these thalli (Hoef-Emden *et al*. 2005). Actin, and actin-homologs, have been observed in the genus *Porphyra* (Kitade *et al*. 2002; Le Gall *et al*. 2005) a member of the morphologically less complex Bangiophyceae (*sensu* Saunders & Hommersand 2004). In *Porphyra purpurea* three copies of the actin gene were observed, plus one pseudo-gene recognized by multiple stop codons and indels (Le Gall *et al*. 2005). Recently the actin gene has been characterized in a large subset of the early branching red algal lineages (Hoef-Emden *et al*. 2005). In many of these lineages (e.g. Stylonematophyceae,

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Compsopogonophyceae) a single copy of the actin gene was noted. In these algae, and other early diverging lineages, the lack of multiple copies was attributed to the fact that these algae would have less need for functionally diverse actin copies, as their morphologies were simple, so selection for duplication would be low.

The Stylonematophyceae, a class containing the genera *Chroodactylon*, *Goniotrichopsis* and *Stylonema* has recently received a molecular phylogenetic treatment using multiple plastid and nuclear markers and worldwide sampling (Zuccarello *et al*. 2008). For the most part, the genera are all well-supported by molecular data, although the relationships between genera were mostly unresolved. The most sampled species was *Stylonema alsidii*, which formed a well-supported clade. The sister taxa to this species were *Stylonema cornu-cervi* and *Goniotrichopsis reniformis*, this sister group relationship was not supported by bootstrap values. Within *Stylonema alsidii* several well-supported clades contain samples from disparate locations, while multiple samples from the same area did not group together (Zuccarello *et al*. 2008).

The Stylonematophyceae is one of the morphologically most diverse groups of red algae outside the Florideophyceae, consisting of unicellular (e.g. *Rhodosorus*), filamentous (e.g. *Stylonema*) and blade-like (e.g. *Goniotrichopsis*) representatives. Sexual reproduction and sexual structures have never been observed in this group. Convincing reports of sexual reproduction outside the Bangiophyceae (e.g. *Porphyra*) and Florideophyceae are lacking. The only class in which sexual stages have been reported is the Compsopogonophyceae. Sexual reproduction has also been reported in the Rhodochaetales (*Rhodochaete parvula*), which has a diplohaplontic life cycle (Magne 1960). Within the Erythropeltidales the genera *Chlidophyllon* and *Pyrophyllon* clearly have sexual structures similar to *Porphyra* (Nelson 1993; Nelson *et al*. 2003), suggesting that the upright life cycle phase is haploid. Within *Erythrotrichia,* reports of sexual reproduction, although unconvincing, suggested that the upright is diploid (Magne 1990).

In an attempt to study species in the presumed asexual Stylonematales we targeted the actin gene, which is nuclear encoded and hypothesized to be present as a single copy in this group.

MATERIALS AND METHODS

DNA was extracted from silica gel dried cultured material (Table 1) using a modified cetyl trimethylammonium bromide (CTAB) extraction procedure (Zuccarello & Lokhorst 2005). The polymerase chain reaction (PCR) primers used to amplify were forward: F97; CSGTSRTBGGTGTSCCBAAGCARAAGGG and reverse-R948; CTCYGGCGGSGCRATSACCTTGATCTTCA. These amplified a fragment 848 bp in length (approximately 75% of the complete gene from *Dixoniella grisea*, AY943972). The PCR reaction (30 μL) consisted of 1 μL DNA, 0.25 μM of each primer, 200 μM of each dNTPs, 2.5 mM $MgCl_2$, 0.1% bovine serum albumin (BSA), 1 unit of *Taq* polymerase (New England Biolabs, Ipswich, MA, USA), and 1× of the respective buffer provided by the manufacturer. The PCR protocol consisted of a touchdown-PCR: initial denaturing at 94°C for 5 min; nine cycles of 94°C for 1 min, initial annealing at 65°C for 1 min with a 1°C decrease in temperature each cycle and 72°C for 1 min, followed by 26 cycles of 94°C/30 s, 55°C/1 min, 72°C/1 min, with a final extension step at 72°C for 5 min. PCR products were confirmed on a 1% agarose gel stained with ethidium bromide.

Direct sequencing of the PCR products produced electropherograms with multiple peaks mostly concentrated at the 3rd codon positions. Cloning of the PCR products was therefore undertaken. PCR

Table 1. Location and culture numbers of samples (J. A. West culture collection)

Sample	Location, culture number	No. clones
Goniotrichopsis reniformis (Kajimura) Kikuchi	Ubara, Katsuura, Chiba, Japan, 4563	8
Stylonema cornu-cervi Reinsch	Ubara, Katsuura, Chiba, Japan 4564	5
Stylonema alsidii (Zanardini) K.M. Drew	Angeva, Madagascar, 4319	13
Stylonema alsidii	Hienghene, New Caledonia, 4390	8
Stylonema alsidii	Yoshio, Katsuura, Chiba, Japan, 4566	11
Stylonema alsidii	Penguin, Tasmania, Australia, 4423	11
Stylonema alsidii	Hienghene, New Caledonia, 4355	2
Stylonema alsidii	Williamstown, Victoria, Australia, 4279	9
Stylonema alsidii	Le Caro, Brittany, France, 4472	8
Downloaded sequences		
Chroodactylon ornatum (C. Agardh) Basson	AJ880400 (SAG103.79, Port Isabel, Texas, USA)	n.a
Chroodactylon ornatum	AJ880398 (CAM, Roscoff, France)	n.a
Stylonema alsidii	AJ880410 (UTEX samples 1424 = JAW 3610)	n.a

Number of clones sequenced.

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products were cleaned using a PCR purification kit (High Pure PCR Purification Kit, Roche Diagnostics GmbH, Basel, Switzerland) and cloned into competent *Escherichia coli* cells (JM109 High Efficiency Competent Cells, Promega, Madison, WI, USA) via the pGEM®-TEasy Vector (Promega) following the manufacturers protocols. Blue/white screening followed standard protocols (Sambrook & Russell 2001). Selected clones were grown overnight in Luria-Bertani (LB) broth containing ampicillin. Alkaline lysis mini-preparations were preformed to obtain plasmid DNA (Sambrook & Russell 2001), pellets were resuspended in 50 µL 0.1× TE (1 mM Tris, 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0) buffer. Plasmids were checked for inserts by electrophoresis in 1% agarose gel against vectors lacking inserts. Sequencing of plasmids using the vector primers M13F and M13R was done commercially (Macrogen Inc., Seoul, Korea). Sequences were assembled using the computer software VectorNTI (Invitrogen, Carlsbad, CA, USA). All sequences were compiled in Se-Al version2a11 (Rambaut 1996).

Analysis of actin data

Cloned actin sequences were screened for mis-incorporation artefacts as well as the formation of chimeric PCR products (Wang & Wang 1997). Singletons, base pair positions differing in one clone when compared with the other clones from that algal sample, were hypothesized to be mis-incorporation artefacts generated by the non-proof-reading ability of the *Taq* DNA polymerase enzyme, and removed. Additional evidence for these artefacts is derived from the fact that these point mutations occur randomly rather than being restricted to third codon positions. Chimeric sequences, a common PCR-artefact in mixed template samples, were detected using the Bellerophon server and removed from the dataset (Huber *et al*. 2004). Homology of the generated actin sequences to conventional actins was verified using the ARPAnno web server (http://bips.u-strasbg.fr/ARPAnno/ARPAnno.html; Muller *et al*. 2005).

A phylogenetic analysis of the 'total evidence' data presented in Zuccarello *et al*. (2008), containing only the specimens used in the actin analysis, was produced for comparison. This consisted of a concatenated data set for the genetic regions: partial nuclear encoded small sub-unit (SSU) rDNA, *psb*A, *tuf*A, and the RuBisCo spacer. The dataset consisted of 2842 characters (261 parsimony-informative positions, 241 parsimony uninformative positions, 2340 constant positions), be it that not all samples were sequenced with all the genes (see Zuccarello *et al*. 2008 for further details). Maximum-parsimony (MP) trees were constructed in PAUP*4.0b10 (Swofford 2002), using the heuristic search option, 500 random sequence additions, tree-bisection-reconnection (TBR) branch swapping, unordered and unweighted characters, and gaps treated as missing data. For bootstrap analysis (BP), 1000 bootstrap datasets were generated from resampled data (10 random sequence additions). Bayesian trees were inferred using Mr Bayes3.1.2 (Huelsenbeck & Ronquist 2001) of two parallel runs of each three heated chains and one cold one, and 5×10^6 generations with sampling every 1000 generations. The analyses were submitted to the Computational Biology Service Unit (CBSU, Cornell University, http://cbsuapps.tc.cornell.edu/mrbayes.aspx).

The actin dataset consisted of 22 sequences, belonging to 10 species, and 848 characters (GenBank accession numbers EU716338-EU716356) and was analyzed using three algorithms: a neighbor-joining procedure was implemented in PAUP* (Swofford 2002), using Tamura-Nei (Tamura & Nei 1993) distances bootstrap percentages were determined (1000 replicates); a MP analysis was implemented in PAUP*, with 500 random sequence additions, TBR branch swapping, unordered and unweighted characters. Bootstrap values were determined by 1000 replicates each with 10 random sequence additions; Bayesian trees were inferred using Mr Bayes 3.1.2 (Huelsenbeck & Ronquist 2001) of two parallel runs of each three heated chains and one cold one, and 3×10^6 generations with sampling every 1000 generations. The analyses were submitted to the Computational Biology Service Unit. Convergence between runs and stationarity of scores was checked using Tracer v1.4 (Rambaut & Drummond 2007). A burn-in of 1500 trees was removed from each run separately, well after stationarity, before constructing the consensus tree of 3000 trees. Shimodaira-Hasegawa tests (Shimodaira & Hasegawa 1999), as implemented in PAUP* (RELL optimization, 1000 bootstrap replicates), were used to test several alternative topologies, as discussed in results.

RESULTS

The 'total evidence' phylogeny was very similar to the data presented previously (Zuccarello *et al*. 2008). Maximum parsimony produced a single tree of 761 steps. A moderately supported relationship of *Goniotrichopsis reniformis* and *Stylonema cornu-cervi* was resolved as the sister clade of *Stylonema alsidii* (Fig. 1). The latter formed a well-supported clade with the UTEX samples 1424 (= JAW3610) being sister to the remaining samples. The groupings, even with this limited sample size show little biogeographic structure: samples from Australia and France (4279 and 4472) are nearly identical, while another sample from Australia is more distantly related (4423); two samples from

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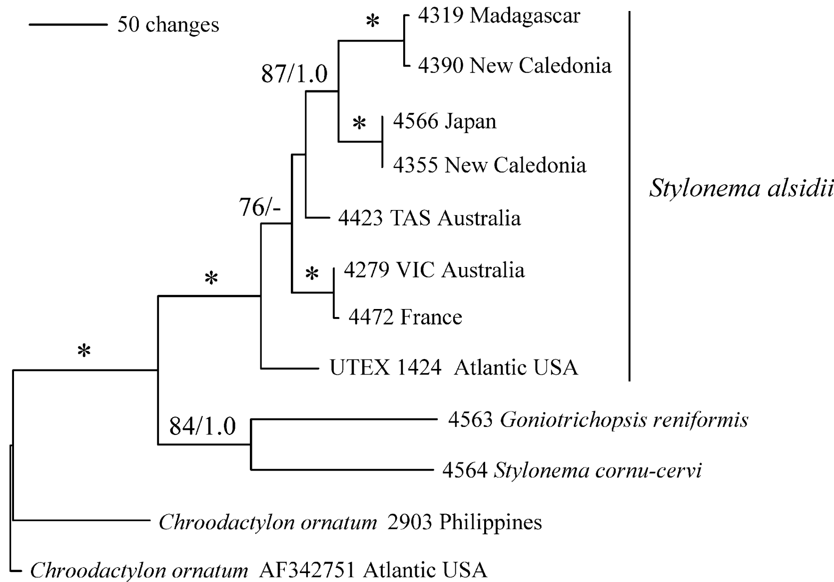

Fig. 1. Maximum-parsimony topology of 'total evidence' DNA dataset (2842 characters, 241 parsimony-informative characters) of select taxa in the Stylonematales. *Chroodactylon ornatum* chosen as outgroups. *≥95% MP-BP and 0.95 PP, otherwise BP/PP if over 70%. BP, bootstrap analysis; MP, maximum-parsimony; PP, posterior probabilities PP.

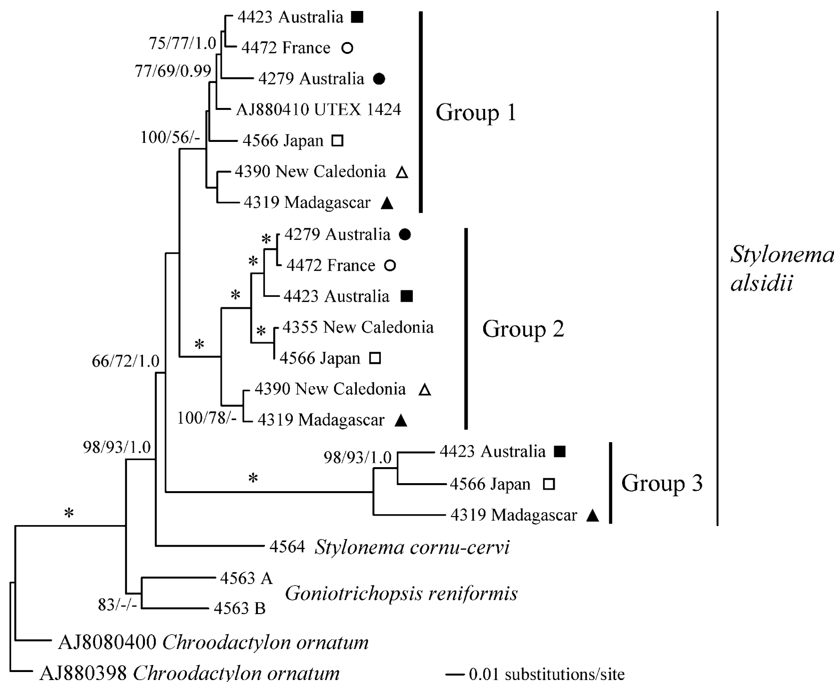

Fig. 2. Neighbor-joining topology of actin gene DNA sequences (848 characters, Tamura-Nei distances) of select taxa in the Stylonematales. *Chroodactylon ornatum* chosen as outgroups. *≥95% NJ-BP/MP-BP/0.95 PP, otherwise NJ.BP/MP.BP/PP if over 50% BP or 0.7PP. BP, bootstrap analysis; MP, maximum-parsimony; NJ, neighbour-joining; PP, posterior probabilities PP.

New Caledonia (4355 and 4390) are found in separate clades mixed with samples from distant locations, Japan and Madagascar, respectively.

All actin sequences have the conserved signature sequence LLTEAPLNPKANR (corresponding to position 104 in *Saccharomyces cerevisiae* actin, Swiss-Prot P60010) indicative of genuine actin. They also possessed a Prosite actin_1 signature (position 53 of the *Saccharomyces cerevisiae* actin) sequence of YV[1]GDAAQ[2]ARRG ([1] = V replaced by isoleucine (I) in Group 3 of *Stylonema alsidii*, and [2] = replaced by methionine (M) in *Chroodactylon*).

The actin phylogeny showed many of the relationships seen in the 'total evidence' tree. A single MP tree of 644 steps was produced (consistency index = 0.6118), the neighbor-joining (NJ) topology is illustrated in Figure 2. *Goniotrichopsis reniformis* and *Stylonema cornu-cervi* were earlier diverging taxa from the moderately supported *Stylonema alsidii* (66% for NJ BP, 72% for MP BP and 1.0 Bayesian posterior probabilities (PP)). The monophyly of *G. reniformis* and *S. cornu-cervi*, seen in the 'total evidence' tree, was not observed in this analysis, with *S. cornu-cervi* being the sister species to *Stylonema alsidii*.

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Within the single isolate of *Goniotrichopsis reniformis* two distinct copies of the actin gene were observed. These differed by 54 base pair (bp) changes with a majority of the changes in the third codon positions (49 3rd codon position changes and 5 1st to 2nd position changes; one amino acid change, methionine → leucine). Only one copy of actin was observed in *Stylonema cornu-cervi* although five clones were sequenced (five singleton base pair changes in the clones were removed, attributed to nucleotide mis-incorporation, see Materials and Methods), versus eight clones sequenced in *G. reniformis* (Table 1). For comparison the two *Chroodactylon ornatum* sequences downloaded from GenBank, one isolated from France the other from Texas USA, differed by 29 bp, all in the 3rd codon positions, and had identical amino acid sequences.

Within *Stylonema alsidii* the data presented several novel results (Fig. 2). Three groups of actins were seen within the seven samples analyzed. Two groups (Group 1 and 2) were observed to have different levels of support both of the groups and within groups.

Group 1 (Fig. 2) contained 67 variable sites (59 in 3rd codon positions, eight 1st to 2nd positions, and seven amino acid substitutions). Group 1 showed low phylogenetic resolution, and the group as a whole was only weakly supported (100% NJ BP, but other methods did not support this grouping well). Within Group 1 the actin sequences of the UTEX sample from GenBank was resolved.

Group 2 (Fig. 2) had levels of variation comparable to Group 1 (61 variable sites, 54 3rd codon changes, seven 1st to 2nd codon changes, and five amino acid substitutions). All relationships within Group 2 were well-supported except for the grouping of sample 4390 and 4319, which did not receive any support in the Bayesian analysis. The grouping of samples within Group 2, were similar to the 'total evidence' tree, with grouping of samples from Australia and France (4279 and 4472), New Caledonia and Japan (4355 and 4566) and the previous mentioned grouping of a Madagascar and New Caledonia sample (4319 and 4390), but the topology was reversed with the most derived grouping (actin sequences of 4279 and 4472) found in one of the earliest diverging groups in the 'total evidence' phylogeny. Although well-supported this topology was not significantly different (Shimodaira-Hasegawa test, $P > 0.05$) from the topology seen in the total evidence tree.

The difference in the level of phylogenetic resolution between these two actin groups (Group 1 and 2) is not surprising when the distribution of parsimony-informative (shared characters) characters is investigated. In Group 2 (61 variable characters), 49 characters are shared (parsimony-informative) between samples (80%), while in Group 1 (67 variable characters) only 16 are shared between samples (parsimony-informative) (24%).

Actin sequences in Group 3 were only observed in three isolates (4423, 4566, 4319) and showed more divergence among the isolates as well as compared with the other two groups. In these sequences the actin motifs were conserved and no stop codons were observed, indicating that these are possibly functional actin copies. Group 3 had 13 unique shared amino acids not found in the other two *S. alsidii* groups, and 10 amino acids were unique to all of the Stylomenatophyceae investigated. Within Group 3 there were 81 base pair changes, 69 in 3rd codon positions and 12 1st to 2nd codon position changes, many of the changes were unique to Group 3 samples. The grouping of 4423 and 4566, with 4319 and a sister sequence, reflects the phylogenetic relationships seen in the Group 1 relationships.

DISCUSSION

Our data reveals that the evolution of actin within the Stylonematophyceae is complex. There are multiple copies of actin within the Stylonematophyceae as opposed to the one gene hypothesis of Hoef-Emden *et al*. (2005). These actin copies cannot at present be attributed to pseudo-genes as: conserved motifs were maintained, most changes are in 3rd codon positions and synonymous, no indels or stop codons were discovered. Expression of these gene copies was not investigated, but if a particular copy is not expressed then the conditions mentioned above would possibly not hold.

Multiple copies, including pseudo-genes, of actin have been reported in the *Porphyra purpurea* (Le Gall *et al*. 2005) but this is the first study in which multiple samples within a species have been investigated in red algae. This study raises several questions: Are these multiple copies due to gene duplications and how many? Do our data shed light on the possibility of sexuality in this group? Why is the phylogeny and phylogenetic signal (parsimony informative sites), in the actin copies (especially Groups 1 and 2) so different?

The phylogeny of the actin data (all actin copies in all samples) supports multiple origin of the actin copies in these organisms. The phylogeny shows that *Goniotrichopsis reniformis* actins and *S. cornu-cervi* actins are sister copies to *S. alsidii* actins. If the duplication occurred on the branch leading to these taxa then we would predict, at least, two clades each containing at least one actin copy from *Goniotrichopsis reniformis* and one of the *S. alsidii* groups and this repeated for the other clade. So our data supports at least two independent duplication events, one in *Goniotrichopsis reniformis* and another in *Stylonema alsidii*.

Are possibilities other than gene duplication possible? To address this question we must be aware of the limited data we have on these species and recall that all of the genera within the class Stylonematophyceae are

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reportedly asexual. If true, meiotic recombination and segregation is unlikely and ploidy level is irrelevant, i.e. even if diploid, the homologous chromosomes and associated loci will evolve independently as has been seen in ancient asexual bdelloid rotifers (Welch & Meselson 2000). It is possible that two independent diploidization events have occurred in *Goniotrichopsis reniformis* and *Stylonema alsidii,* which may explain the two actin copies in *G. reniformis* and two of the copies in *S. alsidii*, though not the third actin group. So it is possible that this diploidization has occurred in the two genera followed by allelic divergence. The other possibility is that gene duplication has occurred twice. How could these two scenarios be compared? If we knew something about the karyology of this group we may be able to hypothesize a genome duplication from an ancestor in which the *n* chromosome value was known. To distinguish between duplication and diploidization would still be difficult as life cycle alternation of chromosomes numbers would not be observed in an asexual organisms. The only chromosome count known for this group is a value of '3–4' chromosomes in *Chroodactylon ornatum* (Brodie & Irvine 2003), and we were not able to count the chromosomes in *S. alsidii*, due to the diffuse nature of cell division and the small size of cells. Dominance of particular life history stage in related taxa is also uninformative as a sister class, Compsopogonophyceae (Yoon *et al*. 2006), in which sex has been reported (order Erythropeltidales and Rhodochaetales) have dominant diploids (e.g. *Erythrotrichia*, Magne 1990), dominant haploids (e.g. *Pyrophyllon*, Nelson 1993; Nelson *et al*. 2003) or isomorphic alternation of generations (*Rhodochaete*, Magne 1960). The development of other nuclear markers in *S. alsidii* would be an appropriate strategy to address this question, as in diploid organisms two copies of all loci should be detected, while if only actin, or a limited number of genes has been copied, this would not be the case.

Our data also indicate that if *Stylonema alsidii* became diploid through a sexual event, then this event has stopped as the two putative alleles (e.g. Group 1 actins versus Group 2) show for the most part congruent sample topology and all well sampled taxa have at least a Group 1 and a Group 2 copy, making their role as independently segregating alleles unlikely.

In *S. alsidii* there is also a third group of actin genes (Group 3) that shows high divergence from the other actin genes. There is no indication that these are pseudo-genes (see previous argument) and they are likely to be observed in all samples, as the samples in which the most clones have been sequenced (Table 1) are also the ones in which this third group has been noted. The phylogenetic relationships seen in Group 3 actins also reflects the relationships seen in the other actin groups (as far as this is deducible from three samples). It would appear that at least another actin gene duplication has occurred in *S. alsidii* (i.e. not due to possible diploidization), that this event was different from the event producing Group 1 and 2 actin groups, although whether this occurred before or after the divergence of the Group 1 and 2 copies is not resolved from our data (i.e. no branch support), and that this gene copy is possibly diverging under different selective regimes, hence the long branch.

We assume that the Group 1 and Group 2 actins were produced from a common event (a duplication of some sort, see discussion above). A second anomaly is the difference in parsimony informative sites in the two copies of actin in *S. alsidii*. Actin sequences of these samples from the Group 1 and the Group 2 actin have approximately equal levels of variation, mostly concentrated in 3rd codon positions and yet the number of shared variable positions (parsimony-informative variation) is greatly different with only 24% being informative in Group 1 and 80% of the variation being parsimony informative in Group 2. As this variation is mostly synonymous it is hard to explain this pattern of variation as selection for different functionality, and if we assume an origin before divergence of the samples, as the data clearly indicates, then differential age of the copies cannot be involved. This is a curious unresolved result of our analysis.

As nuclear markers continue to be developed for evolutionary studies in algae, for example to resolve difficult phylogenetic relationships and give insights into hybridization, the complexities of the nuclear genome and the history of genes and gene copies will continue to challenge our knowledge of these poorly known organisms.

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