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18S and ITS2 rRNA gene sequence-structure phylogeny of the Phaeophyceae (SAR, Stramenopiles) with special reference to Laminariales

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ABSTRACT

The phylogeny of brown algae (Phaeophyceae) has undergone extensive changes in the recent past due to regular new scientific insights. We used nuclear 18S rDNA with an extensive dataset, aiming to increase the accuracy and robustness of the reconstructed phylogenetic trees using a simultaneous sequence-structure approach. Individual secondary structures were generated for all 18S rDNA sequences. The sequence-structure information was encoded and used for an automated simultaneous sequence-structure alignment. Neighbor-joining and profile neighbor-joining trees were calculated based on 186 phaeophycean sequence-structure pairs. Additionally, sequence-structure neighbor-joining, maximum parsimony and maximum likelihood trees were reconstructed on a representative subset. Using a similar approach, ITS2 rDNA sequence-structure information was used to reconstruct a neighbor-joining tree including 604 sequence-structure pairs of the Laminariales. Our study results are in significant agreement with previous single marker 18S and ITS2 rDNA analyses. Moreover, the 18S results are in wide agreement with recent multi-marker analyses. The bootstrap support was significantly higher for our sequence-structure analysis in comparison to sequence-only analyses in this study and the available literature. This study supports the simultaneous inclusion of sequence-structure data at least for 18S to obtain more accurate and robust phylogenetic trees compared to sequence-only analyses.

1. Introduction

Phaeophyceae are multicellular photosynthetic organisms that vary in size from microscopic, branched filaments less than a millimeter in diameter to large macroscopic fleshy thalli, which may attain heights in excess of 50 m. The Phaeophyceae received their fitting name “brown algae” due to their characteristic color, which is caused by the large amounts of the carotenoid fucoxanthin (Lee, 2018). The phylogeny of this morphologically extremely diverse group, which comprises approximately 2000 described species, has been repeatedly revised in the past in seeking a general consensus (Akita et al., 2022; Bringloe et al., 2020; Draisma et al., 2001; Kawai et al., 2015; Phillips et al., 2008; Silberfeld et al., 2011, 2014). At the very beginning of phylogenetic

molecular analyses, Tan and Druehl (1993) performed phylogenetic reconstructions based on cytosolic 18S rDNA in order to evaluate the previously established systematics of the brown algae. Later on, multi-marker analyses revealed a steady series of additional insights (Akita et al., 2022; Bittner et al., 2008; Bringloe et al., 2020; Cho et al., 2004; Draisma et al., 2001, 2010; Jackson et al., 2017; Kawai, 2014; Kawai and Hanyuda, 2021; Kawai et al., 2015, 2017; McCauley and Wehr, 2007; Silberfeld et al., 2011, 2014; Starko et al., 2019). Recently, Akita et al. (2022) inferred an almost fully supported phylogeny of only eight of the 19 phaeophycean orders. They concluded that “much effort is still needed to provide a comprehensive phylogeny robustly resolving inter-ordinal relationships in brown algae”.

In this study, we revisit the nuclear 18S rDNA as a marker for the

Abbreviations: GTR, general time-reversible; ITS1, internal transcribed spacer 1; ITS2, internal transcribed spacer 2; JC, Jukes–Cantor; ML, maximum likelihood; MP, maximum parsimony; NCBI, National Center for Biotechnology Information; NJ, neighbor-joining; NNI, nearest neighbor interchange; PNJ, profile neighbor-joining; rDNA, ribosomal DNA; SSU, small subunit; TBR, tree bisection and reconnection.

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Phaeophyceae with a much more comprehensive dataset compared to previous 18S rDNA analyses (Boo et al., 1999; Kawai and Sasaki, 2000; Kawai et al., 2007; Lee and Bae, 2002; Lee et al., 2003; Tan and Druhl, 1993, 1994). A pivotal group within the Phaeophyceae are the Laminiarales, also known as kelp, which have long been recognized for their

ecological significance in marine ecosystems and their economic importance in various industries (Chapman, 1970; Ohno and Critchley, 1998). Molecular studies have significantly impacted the taxonomic landscape of this order, leading to revisions at various taxonomic levels (Sasaki and Kawai, 2007). For 18S rDNA sequences within the

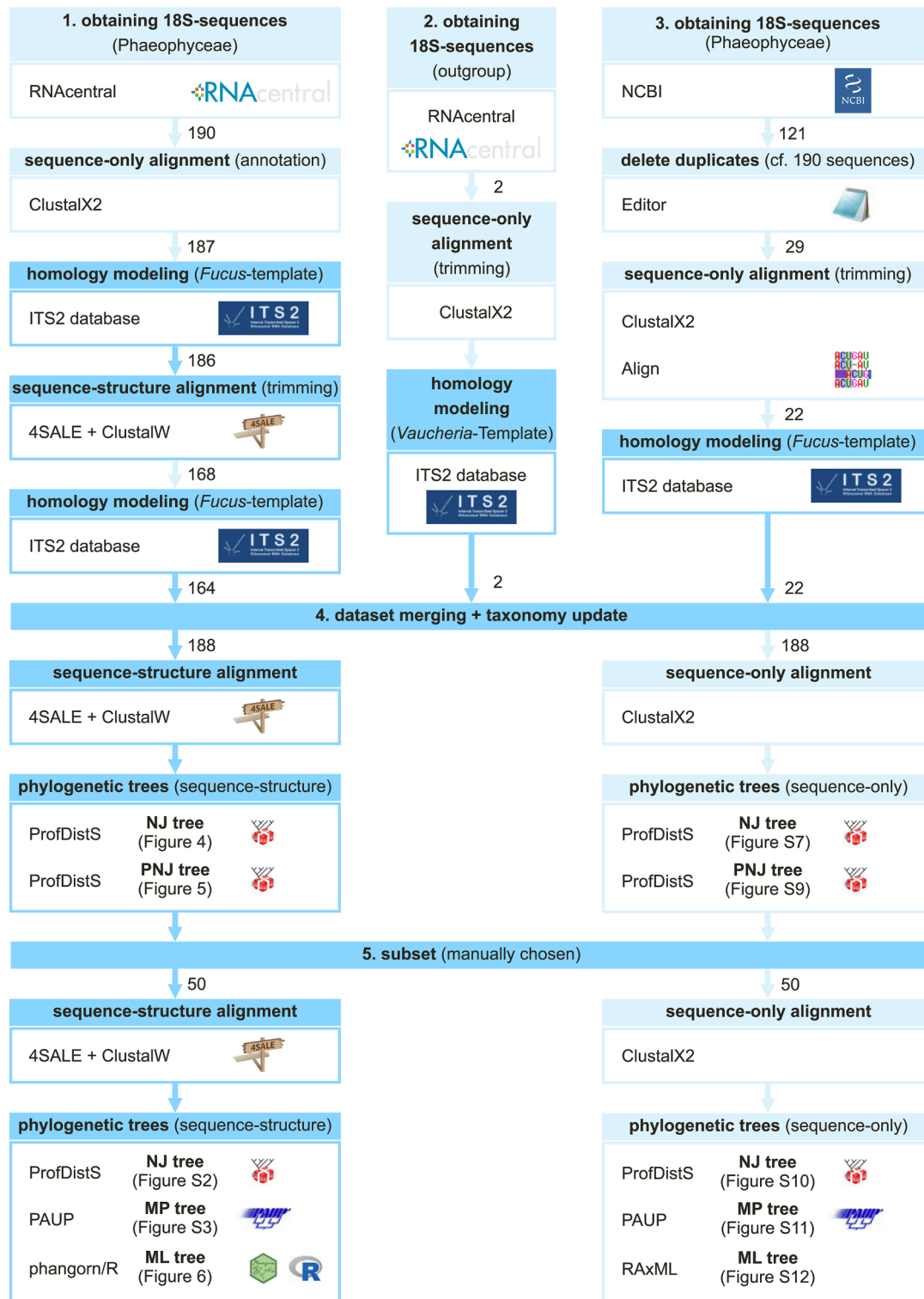


Fig. 1. Flowchart of all materials and methods for 18S rDNA analyses. 18S rDNA sequences were consecutively obtained from RNAcentral (The RNAcentral Consortium, 2019) and supplemented with sequences from the GenBank (Benson et al., 2013) database from the National Center of Biotechnology Information (NCBI) (Sayers et al., 2022). Organisms classified as “sp.” were discarded, as well as sequences that could not be properly aligned. For each step, the used programs are given inside a frame. Based on the Neighbor-Joining (NJ) (Saitou and Nei, 1987) overview tree generated with ProfDistS (Friedrich et al., 2005; Wolf et al., 2008), a subset was manually chosen for further phylogenetic analyses. Associated figure numbers are given inside the frames.

Laminariales, a resolution limit has been reached (Boo et al., 1999). Here, we use the internal transcribed spacer 2 (ITS2) or 18S rDNA to reconstruct phylogenetic relationships. Unlike previous sequence-only analyses, this study uses an approach that simultaneously includes the individual secondary structure for each sequence when performing the alignment. This approach has been shown to increase the accuracy and robustness of inferred ITS2 rDNA neighbor-joining (NJ) trees, as demonstrated by Keller et al. (2010). The resulting recommendation by Keller et al. (2010) to apply such a sequence-structure approach to other variable sequences with a conserved structure, such as 18S rDNA, and other methods of phylogeny, such as maximum parsimony (MP) and maximum likelihood (ML), has already been fruitfully applied in numerous studies (Borges et al., 2021; Buchheim et al., 2017; Czech and Wolf, 2020; Heeg and Wolf, 2015; Lim et al., 2016; Markert et al., 2012; Plieger and Wolf, 2022; Rackevei et al., 2023; Weimer et al., 2023).

To our knowledge, this study is the first with such a comprehensive nuclear 18S rDNA sequence dataset and also the first to analyze the phylogeny of the Phaeophyceae and Laminariales based on a simultaneous RNA sequence-structure approach.

2. Material and methods

2.1. Dataset generation

Aiming to compile an 18S rDNA dataset for the Phaeophyceae (SAR, Stramenopiles) that is as comprehensive and consequently representative as possible, the two databases RNAcentral (The RNAcentral Consortium, 2019) and GenBank (Benson et al., 2013) were utilized (Fig. 1).

The latter is a service of the National Center of Biotechnology Information (NCBI) (Sayers et al., 2022). The search covered full-length nuclear 18S rDNA (1500 to 3000 nucleotides). Only organisms classified down to the species level were further processed.

18S rDNA sequences for phaeophycean organisms were first obtained from RNAcentral (retrieved on 07/11/2023). In a ClustalX (Larkin et al., 2007) sequence-only alignment, sequences that could not be properly aligned were removed. Homology modeling (Selig et al., 2008; Wolf et al., 2005) as implemented in the ITS2 database (Ankenbrand et al., 2015), was used to include the individual secondary structure when trimming all sequences equally. The secondary structure of *Fucus ceranoides* (URS0001A45864) from RNAcentral was used as a template for all phaeophycean sequences (Fig. 2).

In order to represent the entire 18S rDNA structure as one helix with side arms, a binding nucleotide pair was manually inserted at both ends of the template and subsequently removed. In this way, even the modeled template only achieved a structure transfer value of 99.749 %. Sequences with a structure transfer value of less than 75 % were discarded.

The trimming of both ends of the helix was performed in 4SALE (Seibel et al., 2006, 2008) by using the implemented ClustalW (Larkin et al., 2007) with its 12×12 scoring matrix (Wolf et al., 2014). Sequences that were too short were discarded. In order to obtain the appropriate structure transfer values [%], homology modeling was performed again (the values obtained are listed in Suppl. Table S1). Sequences with a structure transfer value of less than 94 % were discarded (Suppl. Table S2).

Considering the Phaeophyceae as the sister group of the

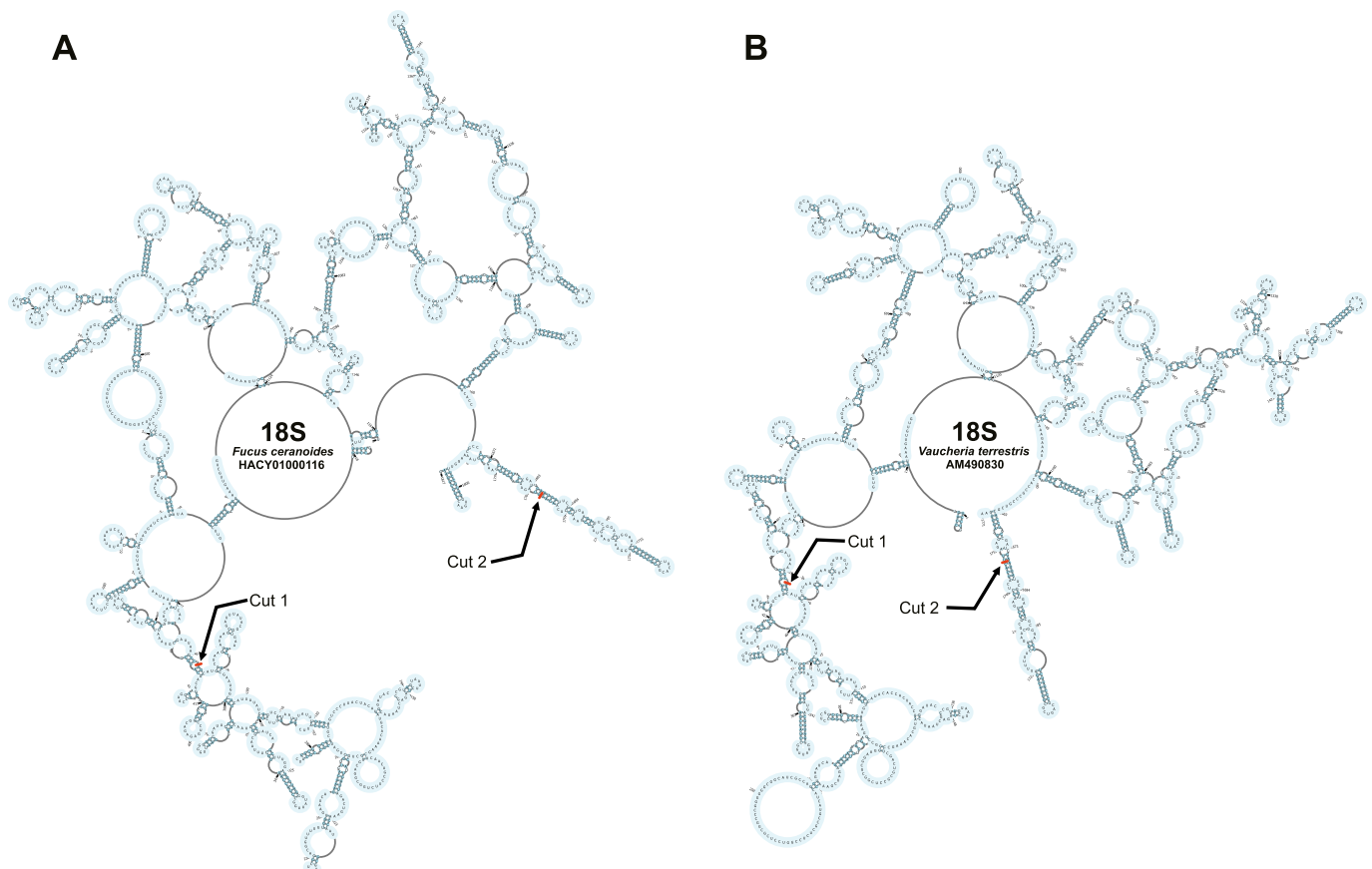


Fig. 2. The two structural 18S rDNA templates for homology modeling visualized with the PseudoViewer web application (Byun and Han, 2006). The sequence-structure data was obtained from RNAcentral, and then equally trimmed at the indicated positions in 4SALE. Paired nucleotides are indicated in dark blue, unpaired nucleotides in light blue. *Fucus ceranoides* (A) was used as the template for all phaeophycean sequences. *Vaucheria terrestris* (B) was used as the template for the outgroup taxa. This allowed only sequences with structural homology greater than 94 % to be used for the analyses. Below the taxon name is the GenBank accession number. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Xanthophyceae (Derelle et al., 2016), *Vaucheria terrestris* and *Vaucheria bursata* 18S rDNA sequences were obtained as outgroup data from RNACentral (retrieved on 07/11/2023). They were trimmed using the method described above based on a sequence-only alignment performed in ClustalX. To maintain a structure transfer value greater than 94 %, the secondary structure of the *Vaucheria terrestris* 18S rDNA from RNACentral was used as a template (Fig. 2) in the same way as the template of *Fucus ceranoides* (with the secondary structure of *Fucus ceranoides* as a template, only values between 88 % and 92 % would have been achieved).

The GenBank database provided additional sequences for phaeophycean organisms (retrieved on 07/31/2023) after removing already collected records (Fig. 1). Using only GenBank with the defined query excluding RNACentral, some transcriptome sequences would not be included in the dataset.

The merged dataset was updated taxonomically according to AlgaeBase (Guiry and Guiry, 2024) and recent literature, the latter was used to include the Arthrothamnaceae (Jackson et al., 2017; Starko et al., 2019).

2.2. Reconstruction of the sequence-structure trees

An automatic sequence-structure alignment (Wolf et al., 2014) was generated in 4SALE using the implemented ClustalW along with its 12×12 scoring matrix (Wolf et al., 2014). The 12-letter encoding is explained in detail in Figure 3. ProfDistS (Friedrich et al., 2005; Wolf et al., 2008) was used to calculate the overview sequence-structure NJ (Saitou and Nei, 1987) tree (Fig. 4) using a sequence-structure specific Jukes–Cantor (JC) correction (Jukes and Cantor, 1969). To determine the basal branching patterns of the monophyletic subclades with higher accuracy and robustness, an additional profile neighbor-joining (PNJ) (Müller et al., 2004) tree was calculated with ProfDistS (Fig. 5). The necessary profiles were defined manually based on the overview sequence-structure NJ tree. Profiles are alternately highlighted in light and dark blue. Organisms that could not be clearly assigned to a monophyletic subclade were not included in any manually predefined profile.

Based on the overview sequence-structure NJ tree, a subset of 50 organisms was selected, choosing two representatives from each established family whenever possible (Fig. 1). For better comparability, the taxa selection from other works was considered (Bittner et al., 2008; Lee and Bae, 2002; Starko et al., 2019). As resolving the phylogeny of Laminariales was particularly challenging in the past (Jackson et al., 2017), four representatives of the Arthrothamnaceae were chosen instead of only two. For further analyses, a sequence-structure alignment was generated using 4SALE (Fig. 1). The subset sequence-structure tree was reconstructed for NJ, MP (Camin and Sokal, 1965) and ML (Felsenstein, 1981) respectively using ProfDistS, PAUP* (Swofford, 2002) and R (R Core Team, 2022) (Fig. 6 and Suppl. Fig. S2 and S3). For all calculations performed in PAUP*, the maxtree limit was set to 10,000 and the tree rearrangement algorithm to “tree bisection and reconnection” (TBR) (Allen and Steel, 2001). In R, the phangorn (Schliep, 2011) package was used, which only handles “nearest neighbor interchange” (NNI) (Li et al., 1996) as its tree rearrangement algorithm. Thereby the Generalised time reversible (GTR) (Lanave et al., 1984) + Proportion of invariant sites (I) + Among-site rate heterogeneity Gamma (G) model was used as the nucleotide substitution model. Due to the complexity of the sequence-structure approach with its 12×12 matrix (Wolf et al., 2014), the bootstrap support (Felsenstein, 1985) was estimated based on only 100 pseudo-replicates for all trees.

Sequence-structure analyses of the Laminariales using ITS2 rDNA as a marker were conducted as described for 18S rDNA. A workflow is shown in Supplementary Figure S1. The Chordaceae were excluded from this analysis because, based on current literature, they should be classified under Chordales rather than Laminariales (Starko et al., 2019). The secondary structures of *Lessonia flavicans* (AF319032) and *Pelagophycus porra* (AF319039) were predicted and used as templates (Fig. 7).

The ITS2 sequence-structure pairs were used to reconstruct an overall sequence-structure NJ tree (Fig. 8). From a manually chosen subset, sequence-structure NJ, MP and ML trees were generated (Suppl. Fig. S4–S6).

2.3. Reconstruction of the sequence-only trees

A sequence-only alignment was generated in ClustalX. An 18S rDNA and an ITS2 rDNA overview sequence-only NJ tree (Suppl. Fig. S7 and S8) was calculated in ProfDistS. Additionally, a sequence-only PNJ tree was reconstructed for 18S rDNA using ProfDistS (Suppl. Fig. S9). The same organisms as in the sequence-structure subset were selected for the sequence-only subset. In ClustalX, a sequence-only subset alignment was generated for 18S rDNA and ITS2. Based on the subsets, sequence-only NJ, MP and ML trees were reconstructed for 18S rDNA (Suppl. Fig. S10–S12) and ITS2 rDNA (Suppl. Fig. S13–S15) using ProfDistS, PAUP* and RAXML, respectively (Kozlov et al., 2019; Stamatakis, 2014). RAXML uses NNI as its tree rearrangement algorithm and GTR + I + G as its nucleotide substitution model, as does phangorn. For all trees, the bootstrap support was estimated based on only 100 pseudo-replicates.

3. Results

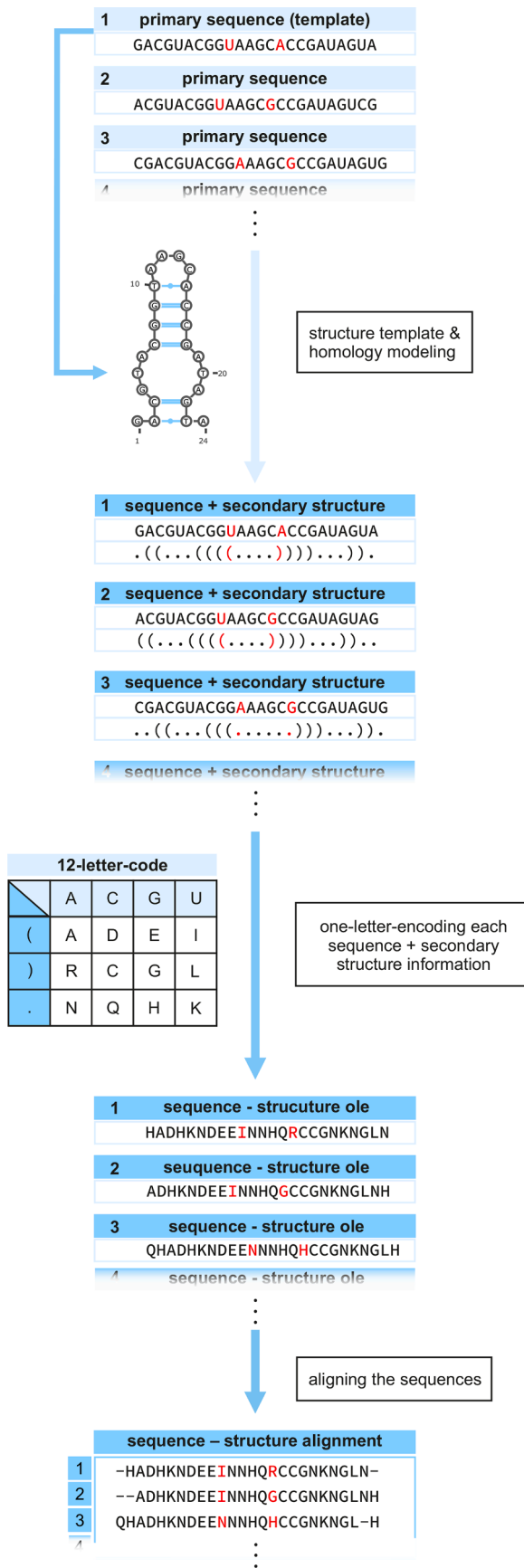
3.1. 18S dataset generation

Results concerning the 18S dataset generation are provided in Supplementary text file (Text S1).

3.2. 18S sequence-structure NJ overview tree

A global multiple sequence-structure alignment of 188 organisms was generated with 4SALE. Based on this alignment, a sequence-structure NJ overview tree (Fig. 4) was reconstructed using ProfDistS. Most orders and even most families were recovered as monophyletic. In total, eight of the 15 included phaeophycean orders were monophyletic (Asterocladales, Desmarestiales, Dictyotales, Discosporangiales, Ectocarpales, Fucales, Laminariales, Sphacelariales) and three polyphyletic (Chordales, Stschapoviales, Tilopteridales). Four orders were represented by only one taxon each (Ascoseirales, Ishigeales, Phaeosiphoniellales, Sporochnales). Nine of the 30 included phaeophycean families were shown to be monophyletic (Asterocladaceae, Chordaceae, Chordariaceae, Dictyotaceae, Ectocarpaceae, Fucaceae, Lessoniaceae, Pseudochordaceae, Sargassaceae) and seven polyphyletic (Agaraceae, Alariaceae, Arthrothamnaceae, Desmarestiaceae, Lithodermataceae, Scytosiphonaceae, Tilopteridaceae). Fourteen families were represented with only one taxon each (Acinetosporaceae, Akkesiphycaceae, Arthrocladiaceae, Ascoseiraceae, Choristocarpaceae, Discosporangiaceae, Halosiphonaceae, Ishigeaceae, Laminariaceae, Phaeosiphoniellaceae, Phaeostrophaceae, Phyllariaceae, Sporochnaceae, Stschapoviaceae). Due to the placement of *Ishige sinicola* (Ishigeophycidae, Ishigeales, Ishigeaceae) as a sister taxon to the Ectocarpales (= Acinetosporaceae, Chordariaceae, Ectocarpaceae, Scytosiphonaceae), the Fucophycidae are depicted as polyphyletic. According to GenBank, the sample of *Scytosiphon lomentaria* (GFKH01045785) (“Scytosiphonaceae 2”) is the same biosample as *Scytosiphon lomentaria* (GFKH01021693) (“Scytosiphonaceae 1”). Both entries have been removed from NCBI and can only be found in RNACentral (04.10.2023). The two otherwise identical sequences differed significantly in the region from nucleotide 1684 to nucleotide 1760. The three families of the Chordales were represented as polyphyletic groups. The two organisms assigned to the Stschapoviales, *Stschapovia flagellaris* and *Halosiphon tomentosus*, were shown to be polyphyletic. *Phaeosiphoniella cryophila* has been placed within the Tilopteridaceae, causing them to form a polyphyletic clade. The backbone bootstrap support values of the monophyletic clades were detailed using a PNJ tree.

When compared with the sequence-only NJ overview tree (Suppl.



(caption on next column)

Fig. 3. Generation of one-letter encoded sequence-structure alignments starting from primary sequences. An available secondary structure template is applied to the entire 18S rDNA dataset by homology modeling (Selig et al., 2008; Wolf et al., 2005) as implemented in the ITS2 database (Ankenbrand et al., 2015). Using a 12-letter translation table, each nucleotide is translated into one letter along with its structural pairing state (Wolf et al., 2014). When generating an alignment, 4SALE (Seibel et al., 2006, 2008) treats this one-letter encoded information as pseudo-protein sequences. This involves the use of scoring matrices with re-estimated substitution and gap costs (Wolf et al., 2014). For the maximum parsimony (MP) (Camin and Sokal, 1965) and maximum likelihood (ML) (Felsenstein, 1981) analysis, PAUP* (Swofford, 2002) and R (R Core Team, 2022) are directly using one-letter encoded.fasta files. ProfDistS NJ analyses use encoded x.fasta files, converting them internally to one-letter encoded files.

(Fig. S7), the same monophyletic and polyphyletic orders could be identified. Furthermore, *Ishige sinicola* was also presented as a sister taxon to the Ectocarpales, representing the Fucophycidae as polyphyletic. Similarly, the Chordales were presented as a polyphylum, but the Pseudochordaceae sistered with the one taxon of the Akkesiphycaceae. The Pseudochordaceae/Akkesiphycaceae clade in turn sistered with a clade consisting of the Chordaceae and the Laminariales. In the sequence-structure tree, the Pseudochordaceae sistered with the Tilopteridaceae/Phaesiphoniellaceae clade, the Akkesiphycaceae appeared as a sister clade to the Asterocladales/Ectocarpales/Fucales/Ishigeales clade, and the Chordaceae sistered with the Laminariales. Similarly, the 30 families were mono- and polyphyletic in the same way, with the exception of the Desmarestiaceae. In the sequence-structure tree, the Arthrocladiaceae were intercalated into the Desmarestiaceae resulting in the latter being polyphyletic. In the sequence-only tree, the single taxon of Arthrocladiaceae clustered with the Desmarestiaceae. *Phaeosiphoniella cryophila* likewise clustered with *Haplospora globosa*. *Tilopteris mertensii* was a sister taxon to this *Phaeosiphoniella/Haplospora* clade and consequently, the Tilopteridaceae were polyphyletic.

3.3. 18S sequence-structure PNJ tree

Thirteen monophyletic clades, forming the manually predefined profiles, were derived from the sequence-structure overview NJ tree (Fig. 4) at the lowest possible taxonomic level. (Highlighted in light and dark blue throughout the entire paper.) Twelve phaeophycean organisms could not be assigned to any predefined profile. A three times iterated sequence-structure PNJ tree (Fig. 5A) was reconstructed using ProfDistS. The phaeophycean clade was fully supported (bootstrap support = 100). The Discosporangiales were located at the base of the tree, forming a well-supported (98) sister clade to all the remaining phaeophycean organisms. The Dictyotaceae and the Sphacelariales successively split off from the remaining Phaeophyceae, presenting the Dictyotophycidae subclass as a polyphylum. The family Dictyotaceae was represented by 61 taxa and the order Sphacelariales by four taxa. The remaining well-supported (98) phaeophycean organisms split into two clades.

One clade with low support (49) included the moderately supported (76) Fucales at its base. The family of the Fucaceae was represented by six taxa and the family of the Sargassaceae by 28 taxa. Both clades were fully supported (100). The moderately supported (75) sister clade included the Asterocladaceae at its base. This fully supported (100) family was represented by two taxa. *Ishige sinicola* (AY232600) formed a moderate support (76) clade with the fully supported (100) Ectocarpales. Since the associated family of the Ishigeaceae is assigned to the Ishigeophycidae subclass, the subclass of the Fucophycidae was presented as polyphyletic. The Ectocarpales had *Scytosiphon lomentaria* (GFKH01045785) (“Scytosiphonaceae 2”) at its base, presenting the Scytosiphonaceae as polyphyletic. The “Scytosiphonaceae 1” clade was located within the bifurcation of the remaining Ectocarpales, sistering with the Ectocarpaceae, and diverged after *Scytosiphon lomentaria*

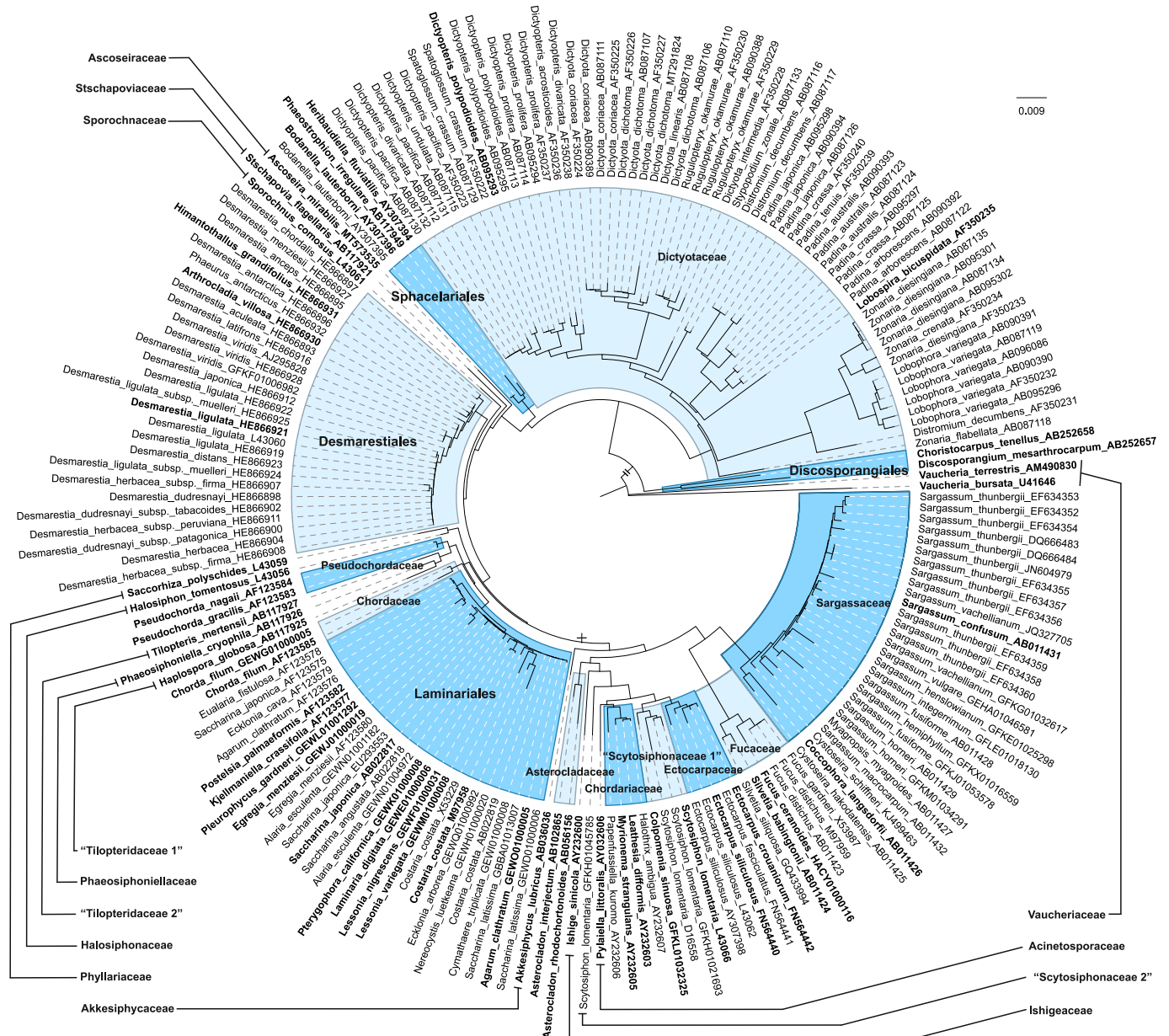


Fig. 4. 18S rDNA sequence-structure neighbor-joining overview tree obtained from ProfDistS. The tree was based on a global multiple sequence-structure alignment of 188 organisms generated with 4SALE. It includes 186 organisms classified as Phaeophyceae, whose sister group are the Xanthophyceae (Derelle et al., 2016). Therefore, the associated *Vaucheria terrestris* and *Vaucheria bursata* were used as outgroup taxa. The branch length to the outgroup was cut to 6.4 %. Each taxon name is accompanied by the GenBank accession name. Manually defined profiles for ProfDistS are alternately highlighted in light and dark blue. Within monophyletic orders, the families may be non-monophyletic. Quotation marks indicate clades that are not monophyletic. Taxonomic designations are shown for colored clades or with dashes if the organism has not been assigned to a profile. Taxa that have been manually selected for the subset are marked in bold. The scale bar indicates evolutionary distances. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(GFKH01045785). The Ectocarpaceae family was represented by five taxa and the “Scytosiphonaceae 1” clade by four. Both formed a moderately supported (86) sister clade to *Pylaiella littoralis* (AY032606) and the Chordariaceae.

The other low supported (66) clade had *Akkesiphycus lubricus* (AB036036) at its base, representing the Chordales as a polyphyletic group. The sister clade to the Akkesiphycaceae included the remaining phaeophycean organisms and was not supported (35). The splitting revealed two unsupported (33/48) clades. At the base of the least supported (33) one, there was a well-supported (99) clade consisting of *Halosiphon tomentosus* (L43056) (Halosiphonaceae) and *Saccorhiza polyschides* (L43059) (Phyllariaceae). Since the Halosiphonaceae belong to the Stschapoviales and the Phyllariaceae belong taxonomically to the

Tilopteridales, both groups were polyphyletic. The *Halosiphon/Saccorhiza* clade clustered as a well-supported clade (94). At its base were the Laminariales, which were represented by 27 taxa. Two more families of the polyphyletically represented Chordales, the Chordaceae and the Pseudochordaceae branched off successively. *Tilopteris mertensii* (AB117927) (Tilopteridaceae) formed a well-supported (99) clade together with the sister taxa *Haplospora globosa* (AB117925) (Tilopteridaceae) and *Phaeosiphoniella cryophila* (AB117926) (Phaeosiphoniellaceae). The *Haplospora/Phaeosiphoniella* clade was well supported (91). The Tilopteridaceae family was shown to be polyphyletic based on the taxonomic classification of *Phaeosiphoniella cryophila* (AB117926). *Sporochnus comosus* (L43061) diverged from a poorly supported (43) clade. At its base was *Stschapovia flagellaris* (AB117921), a

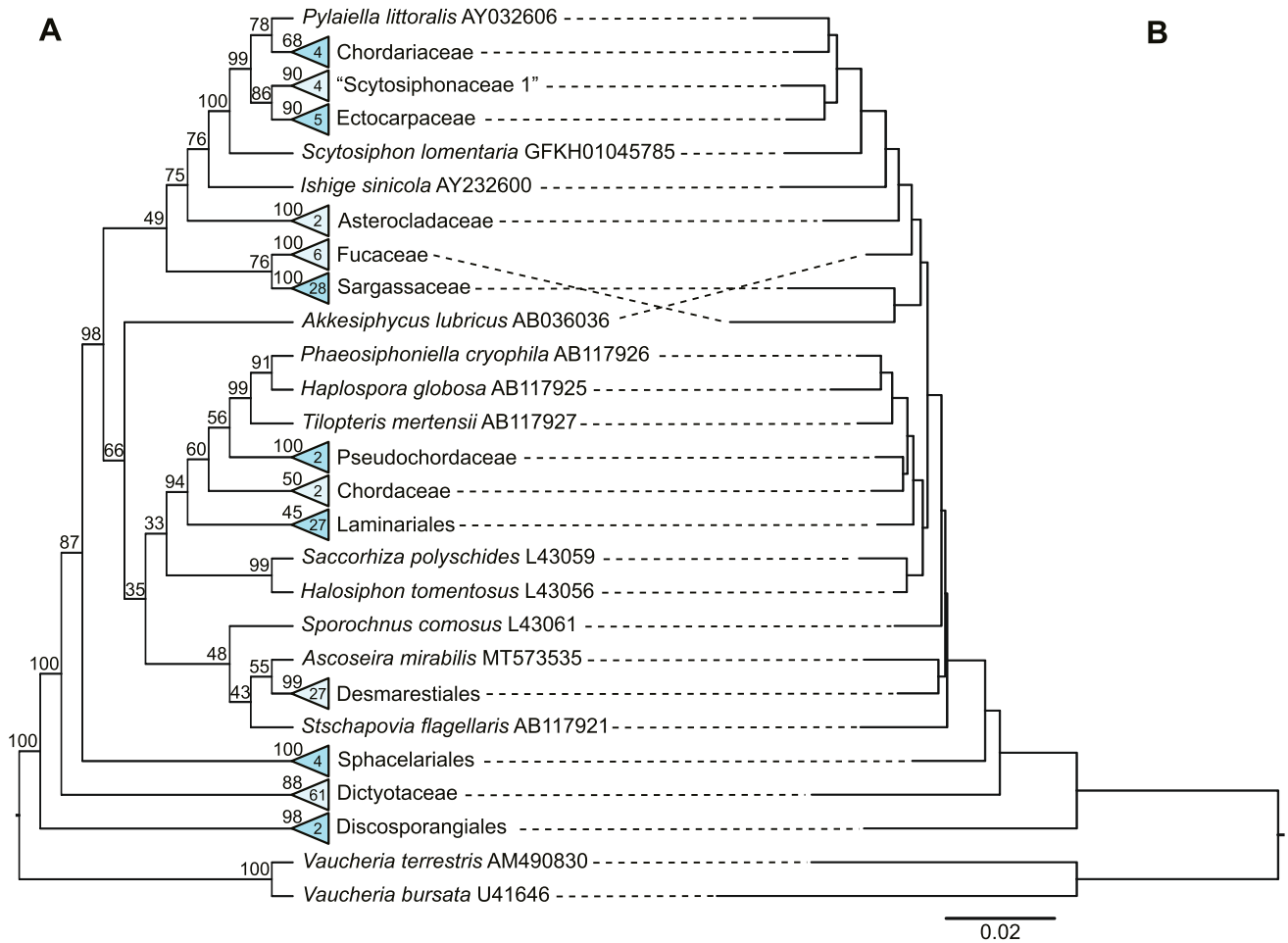


Fig. 5. 18S rDNA sequence-structure profile neighbor-joining (PNJ) (Müller et al., 2004) tree. The tree was generated with ProfDistS based on 188 organisms. Taxa names are accompanied by the GenBank accession name. The global multiple sequence-structure alignment was performed in 4SALE. The cladogram on the left (A) shows a three-times iterated 18S rDNA sequence-structure PNJ tree. The bootstrap values of 100 pseudo-replicates are shown at the internal nodes. In each iteration, super-profiles were generated based on the existing profiles and bootstrap values (>75). The numbers in the alternating light and dark blue triangles denote the number of sequences in the manually defined neighbor-joining profiles. The profile bootstrap values were derived and transferred using 100 pseudo-replicates of a sequence-only neighbor-joining overview tree generated in ProfDistS. The phylogram on the right (B) shows the original tree of profiles without further iterations. Both trees are rooted with *Vaucheria terrestris* and *Vaucheria bursata*. The scale bar indicates evolutionary distances. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

representative of the polyphyletic *Stschapoviales* clustered with the poorly supported (55) *Desmarestiales* and *Ascoseira mirabilis* (MT573535) clade. The well supported *Desmarestiales* (99) were represented by 27 taxa.

In comparison to the NJ tree, the PNJ tree featured several differences. In the sequence-structure NJ overview tree, two distinct clades were not seen after the divergence of the *Sphacelariales*. Instead, the *Ascoseira mirabilis* (MT573535), *Stschapovia flagellaris* (AB117921), *Sporochnus comosus* (L43061), the *Desmarestiales* and the *Halosiphon/Saccorhiza* clades each branched off successively, as successive sister clades to the remaining *Phaeophyceae*. The remaining *Phaeophyceae* split into two clades, some of which are also found in the PNJ tree. The well-supported (94) clade consisting of the *Laminariales*, *Chordaceae*, *Pseudochordaceae*, and the *Tilopteris/Haplospora/Phaeosiphoniella* clade present in the PNJ tree can also be found in the NJ tree. The *Laminariales* and the *Chordaceae* family co-clustered with the *Pseudochordaceae* and the *Tilopteris/Haplospora/Phaeosiphoniella* clade. The *Fucales/Asterocladales/Ishigeales/Ectocarpales* clade is also found in the NJ tree with an identical topology except for the sister taxon *Akkesiphycus lubricus* (AB036036).

The original PNJ tree (Fig. 5B) featured a slightly different topology compared to the three-times iterated PNJ tree (Fig. 5A). After the

Sphacelariales, *Stschapovia flagellaris* (AB117921), the *Desmarestiales/Ascoseirales* clade and *Sporochnus comosus* (L43061) branched off sequentially before the rest of the *Phaeophyceae* and did not form a clade of their own. The *Chordaceae* clustered with the *Pseudochordaceae*. *Akkesiphycus lubricus* (AB036036) was located at the base of the *Asterocladales/Ishigeales/Ectocarpales* clade.

Comparison of the sequence-structure PNJ tree with the sequence-only variant revealed a partially different topology, which is available in its entirety in [Supplementary Figure S9](#). If the same large clades were considered, the sequence-only variant usually yielded lower bootstrap values. Overall, the backbone of the sequence-only PNJ tree was poorly supported. The clade of all remaining *phaeophycean* organisms, which only excludes the *Discosporangiales* and the *Dictyotaceae*, was poorly supported in the sequence-only variant (61), whereas it was moderately supported in the sequence-structure variant (87). The *Tilopteris/Haplospora/Phaeosiphoniella* clade was poorly supported (51) in the sequence-only PNJ tree, whereas the inclusion of structure resulted in an almost fully supported (99) clade. The less strongly supported *Ectocarpales* (99) again clustered with *Ishige sinicola*. The resulting *Ectocarpales/Ishigeales* clade was even better supported in the sequence-only variant (86) than in the sequence-structure variant (76).

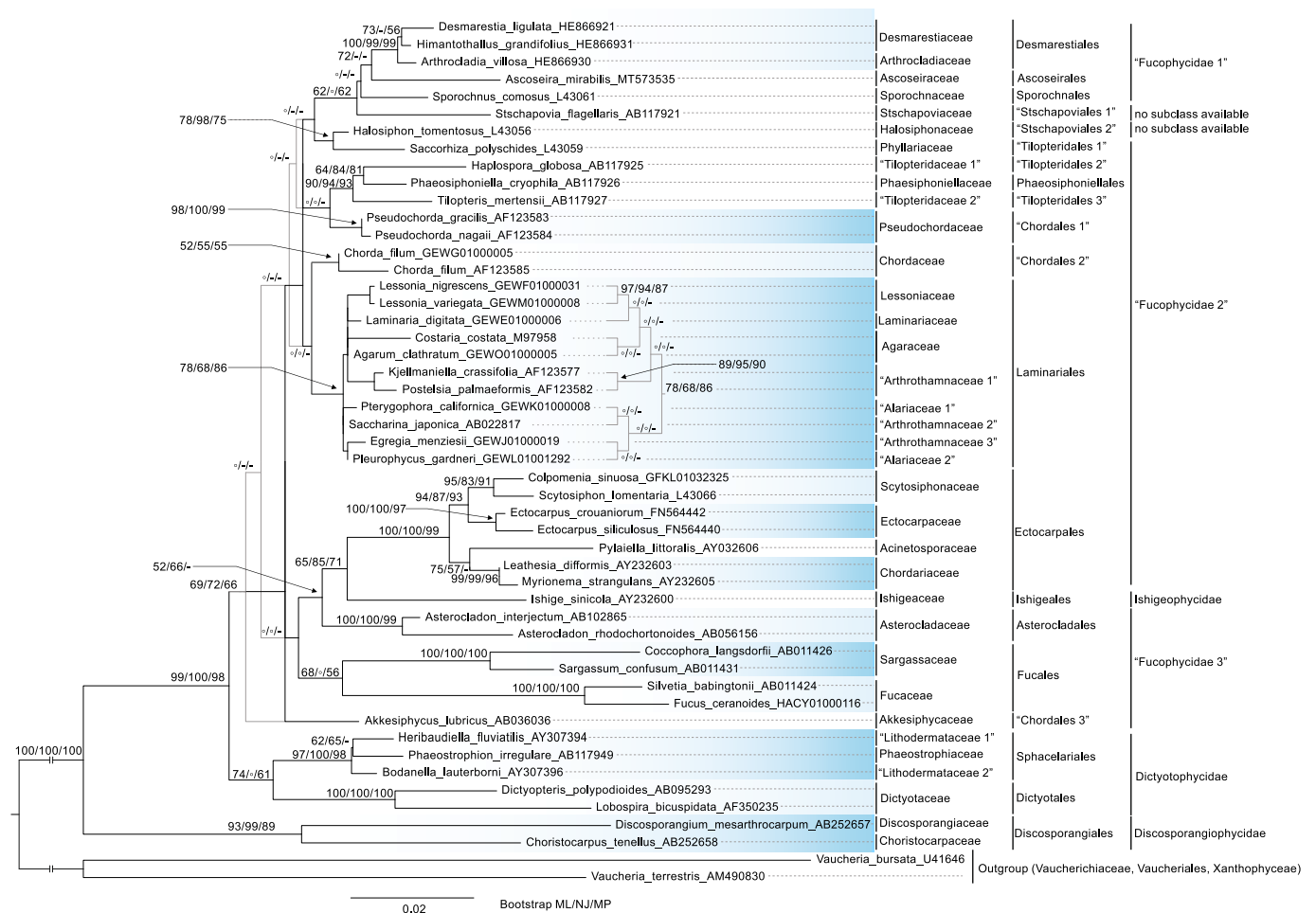


Fig. 6. 18S rDNA sequence-structure ML subset tree. The tree was generated by using R with phangorn (Schliep, 2011). The manually selected representative subset consists of 50 organisms. Manually defined neighbor-joining profiles used in ProfDistS are alternately highlighted in light and dark blue. Within monophyletic orders, the families may be non-monophyletic. Quotation marks indicate clades that are non-monophyletic. Taxa names are accompanied by the GenBank accession name. Bootstrap values mapped at internal nodes are from ML / NJ (Suppl. Fig. S2) / MP (Suppl. Fig. S3) analyses. Light gray lines in the tree serve as an aid for poorly resolved areas. Bootstrap values below 50 are indicated with “o”. Differing tree topologies are marked with “-”. The NJ tree was generated by using ProfDistS. The MP tree was generated by using PAUP*. The trees were rooted with *Vaucheria terrestris* and *Vaucheria bursata*. The branch length to the outgroup was cut to 10 %. The scale bar indicates evolutionary distances. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. 18S sequence-structure ML subset tree

The manually selected representative subset from the sequence-structure NJ overview tree (Fig. 4) contained 50 organisms. For all trees, a sequence-structure alignment generated in 4SALE was used. Figure 6 shows the ML tree with the bootstrap values of the ML, NJ and MP analyses at the internal nodes.

The subclass of the Fucophycidae was shown to be polyphyletic due to the lack of taxonomic assignment of the Stschapoviales and the placement of the Ishigeales. Most orders were represented to be monophyletic, except for Stschapoviales, Tilopteridales and Chordales. Likewise, most families were shown to be monophyletic, except for Alariaceae, Arthrothamnaceae, Lithodermataceae and Tilopteridaceae.

The Discosporangiales formed a well-supported (93/99/89 = bootstrap support from ML/NJ/MP) basal clade of the Phaeophyceae. At the base of the sister clade was the poorly supported (74/-/61) subclass of the Dictyotophycidae. Therein the fully supported (100/100/100) Dictyotales clustered with the well supported (97/100/98) Sphacelariales. The latter showed the Lithodermataceae family to be polyphyletic due to the intercalated Phaeostrophaceae. Based on extremely short evolutionary distances, the poorly supported (69/85/71) clade of the remaining Phaeophyceae was initially not very well resolved. The resulting backbone of the orders was unsupported in many places.

Therefore, it is not expedient to discuss this more explicitly. *Akkesiphycus lubricus* was located at the base of the Fucophyciae/Ishigeophycidae clade, causing the Chordales to appear polyphyletic. The remaining phaeophycian organisms split into two clades.

In the first, unsupported (o/o/-) clade, the Fucales clustered with the Asterocladales/Ishigeales/Ectocarpales clade. The poorly supported (68/o/56) Fucales clade included the fully supported families of the Fucaeae and the Sargassaceae. From the poorly supported (52/66/-) Asterocladales/Ishigeales/Ectocarpales clade, the Asterocladales branched off at the base of the poorly supported (65/85/71) Ishigeales/Ectocarpales clade. As the Ishigeales belong to the Ishigeophycidae, the Fucophycidae are presented as polyphyletic. The well supported (100/100/99) Ectocarpales clade split into the well supported (94/87/93) Ectocarpaceae/Scytosiphonaceae clade and the moderately supported (75/57/-) Acinetosporaceae/Chordariaceae clade.

The second, unsupported clade (o/-/-) included the unsupported (o/o/-) Laminariales/Chordaceae clade at its base. The moderately supported (78/68/86) clade of the Laminariales showed internal evolutionary distances between organisms that were occasionally extremely short. In the basal region of the Laminariales, the Alariaceae together with the Arthrothamnaceae were each shown to be polyphyletic. In the unsupported (o/o/-) Agaraceae/Laminariaceae/Lessoniaceae clade, the unsupported clade of the Agaraceae branched off first.

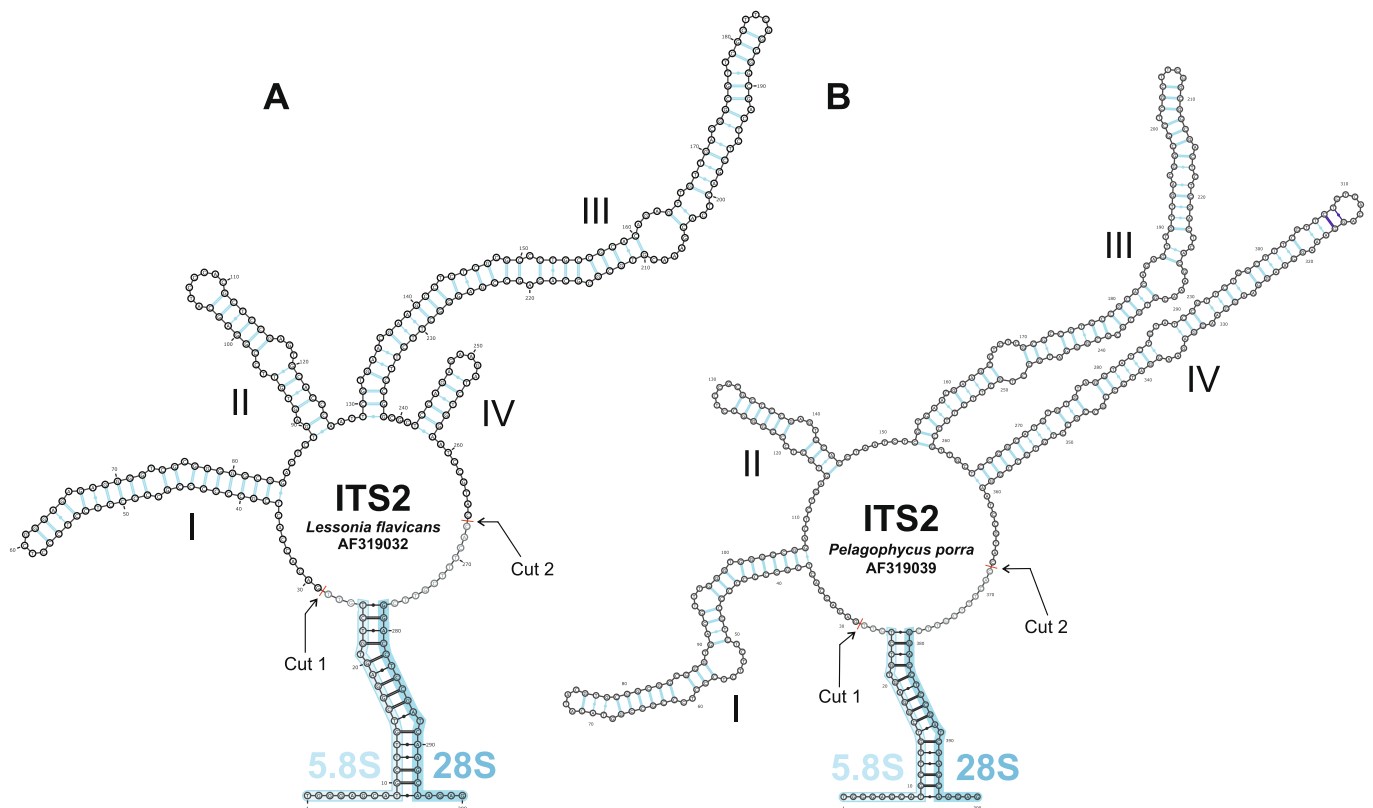


Fig. 7. ITS2 structure templates for homology modeling. The predicted ITS2 secondary structure of (A) *Lessonia flavicans* (AF319032) and (B) *Pelagophycus porra* (AF319039) including the proximal stem. The last/first 25/21 nucleotides of the 5.8S/28S stem are indicated in light/dark blue. ITS2 helices are numbered from I to IV. For homology modeling, the whole ITS2 including the proximal stem was used. The taxon sampling included many partial ITS2 sequences missing the first few and the last few nucleotides of the ITS2 as well as the proximal stem. Therefore, the final alignment used for further phylogenetic analyses starts with Cut 1 and ends with Cut 2 (indicated by arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Laminaria digitata was the sister taxon to the well supported (97/94/84) clade of the Lessoniaceae. The remaining Phaeophyceae again split into two unsupported clades. The Pseudochordaceae/ Phaeosiphoniellales/ “Tilopteridales 2 + 3” clade (o/o/-) consisted of the well-supported Pseudochordaceae family as a sister group to the Phaeosiphoniellales/ Tilopteridales clade. Within the well supported (90/94/93) Phaeosiphoniellales/Tilopteridales clade, the Tilopteridaceae are represented as polyphyletic since *Haplospora globosa* together with *Phaeosiphoniella cryophila* form a low supported (64/84/81) clade. The second clade included the poorly supported (62/o/62) Ascoseirales/Desmarestiales/ Sporochnales/ “Stschapoviales 1” clade clustering with the moderate supported (78/98/75) Halosiphonaceae/Stschapoviaceae clade (“Stschapoviales 1”/ “Tilopteridales 1”). In this way, the Stschapoviales were shown to be polyphyletic. *Stschapovia flagellaris* (“Stschapoviales 1”), *Sporochnus cosmosus* (Sporochnales) and *Ascoseira mirabilis* (Ascoseirales) all branched off sequentially ahead of the well supported (100/99/99) Desmarestiales clade. Therein the Arthrocladiaceae represented the sister group to the poorly supported (73/-/56) Desmarestiaceae.

Comparison of the sequence-structure ML subset tree with the sequence-only variant revealed a partially different topology, which is available in its entirety in [Supplementary Figure S12](#). On average, the sequence-only variant yielded lower bootstrap support values. The *Tilopteris/Haplospora/Phaeosiphoniella* clade was not supported (41/46/54) in the sequence-only ML subset tree, whereas the inclusion of structure produced a well supported (90/94/93) clade. The unsupported (34/-/-) *Kjellmaniella/Postelsia* clade and the well supported (87/93/86) Lessoniaceae clade both had significantly better bootstrap support in the sequence-structure variant (89/95/90 and 97/94/87, respectively). The Desmarestiaceae were also represented as an unsupported (39/50/55) clade in the sequence-only variant, whereas the inclusion of structure

produced a moderately supported (73/-/56) clade.

3.5. ITS2 sequence-structure NJ overview tree

A total of 680 ITS2 sequences from the Laminariales and the two outgroup taxa, *Chorda filum* (AB197760) and *Chorda borealis* (LC430541), were obtained from NCBI. Homology modeling using the two template structures yielded structural homology for at least 75 % of the taxa but not for the two outgroup taxa. The dataset was generated as described for the 18S rDNA data ([Suppl. Fig. S1](#)). The final dataset was composed of 604 ITS2 sequences from the Laminariales and the two outgroup taxa. Information about all gathered sequences can be found in [Supplementary Tables S3 and S4](#). Based on the alignment of 604 ITS2 sequence-structure pairs, an overall sequence-structure NJ tree of the Laminariales was reconstructed ([Fig. 8](#)). The resulting tree consisted of clades representing the genera of the Laminariales. However, not all genera were found to be monophyletic. Genera that were recovered as monophyletic were: *Agarum*, *Aureophycus*, *Costaria*, *Dictyoneurum*, *Ecklonia*, *Egregia*, *Eualaria*, *Kjellmaniella*, *Lessonia*, *Lessoniopsis*, *Macrocyctis*, *Nereocystis*, *Pelagophycus*, *Postelsia*, *Pterygophora*, *Saccharina*, *Tauya*, *Thalassiophyllum* and *Undaria*. A sister group to all other genera is the monophyletic *Egregia*. *Alaria* was polyphyletic. While most members were grouped into one clade (“*Alaria* II”), two organisms *A. crassifolia* (AF319001) and *A. praelonga* (AB022814) (“*Alaria* I” and “*Alaria* III”) branched outside this clade. “*Alaria* I” was a sister to the clade in which *Lessoniopsis* and “*Alaria* III” formed a sister to “*Alaria* II”. Other non-monophyletic genera were *Arthrothamnus*, *Cymathere*, *Hedophyllum*, *Laminaria*, *Neoagarum* and *Pseudolessonia*. The genus *Pseudolessonia* was represented by only one organism, *P. laminarioides* (DQ473541), which was a sister to the monophyletic *Postelsia*. Another singleton and the

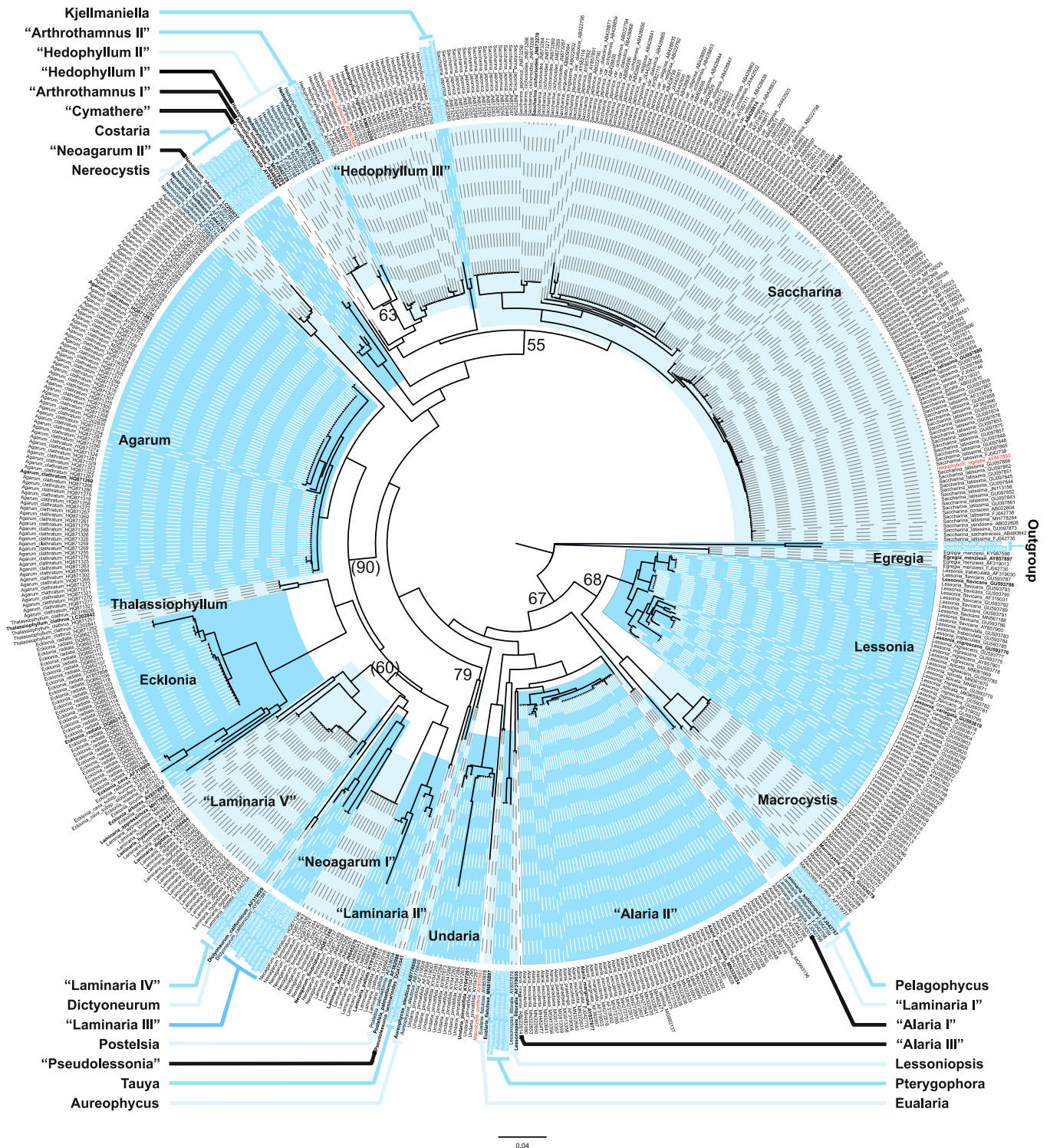


Fig. 8. ITS2 overall sequence-structure neighbor-joining (NJ) tree. The tree was based on 604 sequence-structure pairs and reconstructed using ProfDistS (Friedrich et al., 2005; Wolf et al., 2008). 4SALE (Seibel et al., 2006, 2008) was used for the global multiple sequence-structure alignment. *Chorda filum* (AB197760) and *Chorda borealis* (LC430541) were used as outgroup taxa. Each taxon name is accompanied by its GenBank accession number. Clades are highlighted alternating in a light and dark blue. Singletons are not highlighted with a specific color. All colored clades have bootstrap values > 50 except for “Alaria II” (bootstrap value = 45) and “Hedophyllum III” (28). Non-monophyletic clades and singletons are indicated by quotation marks. Taxa marked in red could not be assigned to a specific clade. Taxa manually chosen for the subset for further phylogenetic analyses are in bold. Bootstrap values for nodes outside of colored clades are indicated when > 50. Bootstrap values are mapped onto the original distance tree. Bootstrap values in parentheses are valid only when “Laminaria I” is included in the indicated clades as obtained by the consensus tree derived from the bootstrap analysis. The scale bar indicates evolutionary distances. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

only organism representing *Cymathere* is *Cymathere triplicata* (AY857884), which was a sister to the clade that includes *Hedophyllum*, *Arthrothamnus* and *Saccharina*. There were two different *Neoagarum* species included in the sampling. *Neoagarum fimbriatum* was sister to some of the *Laminaria* species, while *N. ohaerense* (LC202837) was a sister group to *Arthrothamnus*, *Costaria*, *Cymathere*, *Hedophyllum* and *Saccharina*. *Laminaria* was divided into five different clades. Represented as the “Laminaria I” clade, the *Laminaria solidungula* organisms formed a sister group to all other groups and genera except for *Egregia*, *Lessonia*, *Macrocystis* and *Pelagophycus*. The other *Laminaria*, represented as the clades “Laminaria II to V”, were polyphyletically separated from each other by intercalating sister groups. A few taxa (marked red in Fig. 8) seemed to be outliers that taxonomically do not belong in the clades in which they were located. Utilizing BLAST (Altschul et al., 1990) as implemented in NCBI, suggests that these taxa were most likely named incorrectly in GenBank. Nodes with bootstrap values above 50 are indicated in Figure 8. However, most nodes in the backbone of the tree are not well supported. This was also seen in the sequence-structure NJ, MP and ML subset trees as well as in all sequence-only trees (Suppl. Fig. S4–S6).

4. Discussion

4.1. Backgrounds of secondary structure inclusion

Several studies investigating the phylogeny of the Phaeophyceae have already considered secondary structures when generating their alignments (Cho et al., 2012; de Clerck et al., 2006; Harvey and Goff, 2006; Peters et al., 1997; Rousseau et al., 2001; Stiger et al., 2000, 2003). This inclusion of secondary structure models for the alignment of rDNA sequences was based on the fact that secondary structures are more likely to be conserved compared to nucleotides and should therefore be given special consideration (Kjer, 1995). However, this often involved a time-consuming manual alignment process in which the secondary structure was only used to guide the alignment (Cho et al., 2012; Peters et al., 1997; Rousseau et al., 2001; Stiger et al., 2000, 2003). In contrast, in this study, we included the sequence and the individual secondary structure information for each sequence simultaneously when generating the alignment by using a 12×12 scoring matrix (Wolf et al., 2014). It has already been shown for such a simultaneous inclusion of sequences and structural information by Keller et al. (2010) that the accuracy and robustness of the reconstructed NJ trees are improved. As one result of their ITS2 study, Keller et al. (2010) recommended that other ribosomal genes and analysis methods like MP and ML could equally benefit from this structural inclusion. Gene segments with a conserved structure and variable sequence could probably benefit the most. Consequently, 18S and ITS2 sequence-structure analyses have already been successfully applied for NJ, MP and ML analysis in several studies (Borges et al., 2021; Buchheim et al., 2017; Czech and Wolf, 2020; Heeg and Wolf, 2015; Lim et al., 2016; Lima et al. 2024; Markert et al., 2012; Plieger and Wolf, 2022; Rackevei et al., 2023; Weimer et al., 2023). Accordingly, we applied the approach from Keller et al. (2010) to all available nuclear phaeophycean 18S rDNA sequences and, in addition, to the ITS2 rDNA sequences of the Laminariales. This allowed the calculation of the presented NJ, PNJ, MP and ML sequence-structure trees, which differed from the sequence-only variants in both topology and robustness. The average bootstrap support was significantly higher for the sequence-structure variants than for the sequence-only variants. The 18S rDNA PNJ analysis revealed a significantly more robust backbone compared to the 18S rDNA NJ, MP and ML analyses. In accordance with the current multi-marker study situation, the reconstructed 18S rDNA sequence-structure trees supported the monophyly of the Asterocladales, Desmarestiales, Dictyotophycidae, Discosporangiophycidae, Ectocarpales, Fucales and Laminariales (Bringloe et al., 2020). As already shown in previous 18S rDNA analyses, the backbone of the Phaeophyceae was resolved rather insufficiently (Kawai

and Sasaki, 2000; Kawai et al., 2007; Lee and Bae, 2002; Lee et al., 2003; Tan and Druehl, 1993, 1994). In contrast to the familial phylogeny, a discussion regarding the positioning of the individual orders is therefore only useful to a limited extent, such as for the Discosporangiophycidae. The simultaneous inclusion of structural information resulted in significantly higher bootstrap support values. As a result, in contrast to previous multi-marker analyses (Phillips et al., 2008; Silberfeld et al., 2014) and our own 18S rDNA sequence-only analyses, a well-supported monophyletic Titopteridaceae/Phaeosiphoniellaceae clade was revealed. The individual groups for the 18S rDNA analyses are discussed in more detail as follows.

4.2. Discosporangiophycidae (Discosporangiales)

The well supported monophyletic order of the Discosporangiales, consisting of two sister families Discosporangiaceae and Choriostocarpaceae, was placed at the base of all phaeophycean organisms. The two identical sequences were also used in a former single marker 18S rDNA study and were concordantly positioned at the phaeophycean base (Kawai et al., 2007). This has led to a reintroduction of the Discosporangiales order (Kawai et al., 2007). Subsequent multi-marker studies supported this representation (Bringloe et al., 2020; Kawai et al., 2015; Silberfeld et al., 2014).

4.3. Dictyotophycidae/Ishigeophycidae

The moderately supported monophylum of the Dictyotophycidae, consisting of the Dictyotales and the Sphacelariales, branched off from the remaining Phaeophyceae after the Discosporangiales. This result is consistent with the previous single marker 18S rDNA study in which only the Dictyotales and not the Sphacelariales were present (Kawai et al., 2007). A single marker 18S rDNA study with both orders failed to predict the Dictyotophycidae as a monophylum (Lee and Bae, 2002). According to the NJ analyses, both orders of the Dictyotophycidae would have been the last to depart from the Phaeophyceae, along with the Fucales. Furthermore, the Fucales were intercalated as a sister clade to the Dictyotales, therefore representing a polyphylum of the Dictyotophycidae (Lee and Bae, 2002). Our study contradicts this polyphyletic presentation of the Dictyotophycidae, as the current multi-marker studies do. They presented a monophyletic origin of the Sphacelariales and the Dictyotales together with the Onslowiales and Syringodermatales (Bringloe et al., 2020; Kawai et al., 2015; Silberfeld et al., 2014). The latter two orders were not represented in our study. Moreover, the subclass of Ishigeophycidae diverged even earlier than the Dictyotophycidae (Bringloe et al., 2020; Kawai et al., 2015; Silberfeld et al., 2014), which could not be represented with our single marker 18S approach. Our study located *Ishige sinicola* (AY232600), and thus the Ishigeophycidae, as a sister group to the Ectocarpales. In contrast, multi-marker studies predicted the Ishigeophycidae as a sister group to all remaining Phaeophyceae after the divergence of the Discosporangiales (Bringloe et al., 2020; Kawai et al., 2015; Silberfeld et al., 2014), as well as the study in which the order of the Ishigeales was established based on plastid sequences (Cho et al., 2004). However, previous single marker 18S rDNA analyses revealed results consistent with our study (Kawai et al., 2007; Lee et al., 2003). Both studies used the same *Ishige sinicola* sequence as used here. In one analysis, the Ishigeaceae also formed a moderately supported clade including the Ectocarpales (Lee et al., 2003). In the other analysis, the Ishigeaceae formed an unsupported clade including the Asterocladales, Chordales, Ectocarpales and Sphacelariales (Kawai et al., 2007). In the study by Lee et al. (2003), the relevant nuclear small-subunit rDNA sequences and the morphology of *Ishige okamurae* and *Ishige sinicola* were examined in more detail, revealing a difference in 28 base pairs between the partial 18S rDNA sequences of *Ishige okamurae* and *Ishige sinicola*. Once again, however, the two sequences of *Ishige okamurae* (AY232601, AY232602) were not included in further tree analysis due to their insufficient length.

Summarized, our *Ishige sinicola* sequence is probably not incorrectly sequenced as one might expect.

4.4. Ectocarpales/Asterocladales

The monophyly of the Ectocarpales, almost fully supported in all the analyses of this study, was also often fully supported in previous 18S analyses (Kawai and Sasaki, 2000; Kawai et al., 2007; Lee and Bae, 2002; Lee et al., 2003). Along with the Asterocladales, the Ectocarpales form a sister clade to the Laminariales/Chordales clade in the most recent multi-marker summary study (Bringloe et al., 2020). An earlier multi-marker plastid gene study likewise supported monophyly of the Ectocarpales/Asterocladales clade, but additionally presented the Phaeosiphoniellales within the sister Laminariales/Chordales clade (Silberfeld et al., 2014). In our study, as in previous 18S studies (Kawai et al., 2007; Lee et al., 2003), the Asterocladales/Ectocarpales group is shown polyphyletic. Previous 18S single marker analyses could not comment on this relationship to the Asterocladales due to a significantly smaller 18S rDNA dataset in which there was no representative of this order. The Asterocladales have been represented in our study as an almost fully supported monophyletic clade, forming a sister group to the Ectocarpales, consistent with the multi-marker study in which the order of Asterocladales was first introduced (Silberfeld et al., 2011). The familial relationships within the Ectocarpales revealed in our study are consistent with those of the seven plastid gene study by Silberfeld et al. (2014). When comparing our sequence-structure analyses with our own sequence-only analyses, the simultaneous inclusion of the structure consistently yields significantly higher bootstrap values for these relationships within the Ectocarpales.

4.5. Fucales

The order of Fucales was found to be monophyletic in our study in agreement with recent multi-marker studies (Bringloe et al., 2020; Kawai et al., 2015; Silberfeld et al., 2011, 2014) and previous single marker 18S analyses (Kawai and Sasaki, 2000; Kawai et al., 2007; Lee and Bae, 2002; Silberfeld et al., 2011). Our sequence-structure analyses yielded significantly lower bootstrap values for the Sargassaceae/Fucaceae clade compared to the sequence-only analyses. The Tilopteridales included in our data set were expected to be the sister clade to the Fucales according to multi-marker studies (Bringloe et al., 2020; Kawai et al., 2015; Silberfeld et al., 2011, 2014), as the Ralfsiales and the Nemodermatales are not represented in our sample. However, neither our own sequence-only analysis nor previous single marker 18S rDNA analyses (Kawai and Sasaki, 2000; Kawai et al., 2007; Lee and Bae, 2002) indicate such a result.

4.6. Laminariales/Chordales

Starko et al. (2019) were the first to distinguish the order of the Chordales, whose representatives were previously attributed to the Laminariales by using combined plastid, mitochondrial and ribosomal genes. This was legitimized by the fact that the Akkesiphycaceae/Chordaceae/Pseudochordaceae clade was sufficiently distinct from the rest of Laminariales in terms of both morphology and phylogenetic distance (Starko et al., 2019). Thus, the Chordales very likely form the sister group to the Laminariales (Bringloe et al., 2020; Starko et al., 2019). Previous multi-marker analyses with chloroplast and mitochondrial genes were not able to differentiate the Laminariales/Chordales clade in these two distinct orders but did provide a relevant base (Kawai, 2014; Kawai et al., 2015). The family of Akkesiphycaceae was established in the study of Kawai and Sasaki (2000), for which single marker 18S rDNA analyses were performed in addition to analyses using the Rubisco large subunit gene (*rbcl*) and internal transcribed spacers (ITS1 and ITS2) of ribosomal DNA. In agreement with our study, single marker 18S rDNA analyses and *rbcl* analyses showed the Chordales clade to be

polyphyletic (Kawai and Sasaki, 2000). A non-supported Akkesiphycaceae/Chordaceae/Pseudochordaceae clade could be obtained only from the internal transcribed spacers analyses, which, in agreement with recent studies (Bringloe et al., 2020; Starko et al., 2019), has the Laminariales as a sister group. Considering our own sequence-structure analyses and sequence-only analyses, the 18S rDNA analyses provided results that contradicted the currently approved phylogeny. Analyses based on multiple plastid genes also contradicted the currently valid Laminariales/Chordales clade and presented the Laminariales/Chordales/Phaeosiphoniellales clade in which the Chordales were depicted to be polyphyletic (Silberfeld et al., 2014). In agreement with our analyses, the study by Boo et al. (1999) demonstrated that the Laminariales are a monophylum within which the resolution limit of the ribosomal 18S rDNA has been reached. In order to further unravel the phylogeny of the Laminariales, Boo et al. (1999) suggested that more informative markers are required, which would therefore involve considerably more sequential variations. Our results fully support this conclusion.

4.7. Tilopteridales/Phaeosiphoniellales

In accordance with an earlier 18S rDNA single marker study, the Tilopteridales are polyphyletic, with *Saccorhiza polyschides* as a sister taxon to a representative of the Stschapoviales (*Halosiphon tomentosus*). After its discovery, *Phaeosiphoniella cryophila* was attributed in the study of Hooper et al. (1988) to the Tilopteridaceae, the only known family of the Tilopteridales at that time (Kuhlenkamp and Hooper, 1995). The associated new family of the Phaeosiphoniellaceae was introduced for *Phaeosiphoniella cryophila* based on the chloroplastic *rbcl* gene region and nuclear 28S rDNA in a study by Phillips et al. (2008). The order Phaeosiphoniellales was introduced in the plastid multi-marker study of Silberfeld et al. (2014). Both studies thus contradict the results of our single marker 18S rDNA analyses, which consistently suggested a monophyletic origin for the Phaeosiphoniellaceae/Tilopteridaceae clade. Both the sequence-only analyses and the sequence-structure analyses uniquely revealed a phylogeny in which *Phaeosiphoniella cryophila* was intercalated within the Tilopteridaceae. The simultaneous inclusion of the structural information results in significantly higher bootstrap values compared to the sequence-only analyses, indicating that the Phaeosiphoniellaceae/Tilopteridaceae clade probably constitutes a monophylum based on the NJ, PNJ, MP and ML sequence-structure analyses. The current and comprehensive brown algae study by Bringloe et al. (2020) does not even consider the order Phaeosiphoniellales, instead referring directly to the Hooper et al. (1988) study in which *Phaeosiphoniella cryophila* was assigned to the Tilopteridaceae: Significant similarity in the thallus anatomy and unique morphological features (i.e., vestigial sexual reproduction forming intercalary oogonia and antheridia) between *Phaeosiphoniella cryophila* and *Tilopteris mertensii* (Tilopteridaceae) show their close phylogenetic affinity. The results of our study support this assessment based on the results of our sequence-structure approach.

4.8. Stschapoviales

The Stschapoviales were consistently represented as a polyphylum in our analyses. This contradicts the multi-marker study of Kawai et al. (2015) in which the order of the Stschapoviales was established based on chloroplastic and mitochondrial genes. In former 18S single marker sequence-only analyses, *Halosiphon tomentosus* was also sister to *Saccorhiza polyschides* (Tilopteridales) like in our analyses.

4.9. Ascoseirales/Sporochnales

The Ascoseirales and Sporochnales do not require any discussion due to their variable positioning and low bootstrap support. A classification based on our single marker 18S analyses is not reasonable at this point.

4.10. Desmarestiales

The Desmarestiales obtained significantly higher bootstrap support values in the sequence structure analyses compared to the sequence-only variants. The associated Desmarestiaceae obtained significantly higher bootstrap support in our ML analysis with the inclusion of structural information.

4.11. ITS2 sequence-structure analyses

The ITS2 sequence-structure overall NJ tree as well as the subset trees were not well supported. While the sequence-only trees showed more monophyletic groups than the sequence-structure trees, the backbone was also not well-supported. The low support may be due to the structural differences of the ITS2 within the Laminariales. Most Laminariales have a relatively short helix I and IV which was represented by the *Lessonia flavicans* template structure (Fig. 7). However, some Laminariales have an extraordinarily long helix I and IV for which *Pelagophycus porra* was used as a template. Although a structural homology of at least 75 % was achieved using these templates, the differences in helix lengths make it hard to properly align the sequences. A different genetic marker is needed to resolve the backbone phylogeny of the Laminariales, i.e., further research is needed. However, ITS2 inside the different genera could still be a promising molecular marker.

5. Conclusions

The fundamental study by Keller et al. (2010) has clearly shown that the simultaneous inclusion of sequence and structural information significantly improves the accuracy and robustness of NJ trees. In line with the recommendation that other methods would also benefit from such an approach (Keller et al., 2010), our study applied this not only to NJ analyses, but also to PNJ, MP and ML methods. While the phaeophyceean backbone in our 18S single marker analyses did not experience a tremendous improvement in terms of robustness and accuracy, the present monophyletic groups revealed broad consistencies compared to the current multi-marker analyses. In addition, the inclusion of the secondary structure generally results in clades with higher bootstrap support, suggesting a monophylum of the Phaeosiphoniellaceae/Tilopteridaceae clade contrary to the current state of the phylogeny. Topological differences to the current multi-marker results were also revealed in earlier 18S rDNA analyses, as well as the fact that the 18S rDNA reaches its resolution limit for the Laminariales. The sequence-structure analysis of the Laminariales using ITS2 was mostly in agreement with previous single-marker studies, however, the backbone of the tree was not well supported. This suggests that ITS2 could probably be used only to resolve the phylogeny inside the different genera. Here we suggest that other variable gene regions should be considered for further analysis. Finally, we would like to see an increase in 18S rDNA data sequencing for a more extensive taxon sampling but also an update regarding the taxonomy in all databases such as GenBank and RNA-central, as the turbulent past of brown algal phylogeny has led to extensive changes that have not always been applied retroactively.

In the age of genomes and phylogenomics, it still makes sense to continue doing single-gene analyses; after all it is individual marker genes that are concatenated in multi-gene analyses. It is important to get everything out of the individual genes and understand them better before combining them with other marker genes. In molecular systematics, it is important to understand sequence data qualitatively and not to hope that more genes will automatically give better results, regardless of how you look at the individual genes.

CRedit authorship contribution statement

Leon Berchtenbreiter: Writing – original draft, Investigation.
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Antonia Stephanie Rackevei: Writing – review & editing. **J. Mark Cock:** Writing – review & editing. **Hiroshi Kawai:** Writing – review & editing. **Matthias Wolf:** Writing – original draft, Supervision, Methodology, Investigation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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