Natural Products among Brown Algae: The Case of *Cystoseira schiffneri* H$_{AMEL}$ (Sargassaceae, Phaeophyceae)

Abdelkader Ben Salem,[a,b] Graziano Di Giuseppe,[c] Andrea Anesi,[b] Saoussen Hammami,*[a] Zine Mighri,[a] and Graziano Guella[b,d,e]

[a]Research Unit of Applied Chemistry and Environment (UR13ES63), Faculty of Sciences of Monastir, Avenue of the Environment, 5000 Monastir, University of Monastir, Tunisia, e-mail: h_saoussen@yahoo.fr

[b]Laboratory of Bioorganic Chemistry, Department of Physics, University of Trento, 38123 Povo, Trento, Italy

[c]Unit of Zoology-Anthropology, Department of Biology, University of Pisa, 56126 Pisa, Italy

[d]Biophysical Institute, CNR, 38123 Povo, Trento, Italy

[e]Centre for Agriculture, Food and Environment, University of Trento, via Mach 1, 38010 San Michele all Adige, Trento, Italy

A chemotaxonomic study on the marine brown alga *Cystoseira schiffneri* collected from the Tunisian marine coast allowed us to identify kjellmanianone (1) and a new isololiolide derivative named schiffnerilolide (2). The structure elucidation and the assignment of relative configurations of the isolated natural products were based on advanced mass spectrometric and nuclear magnetic resonance techniques. This outcome suggested a close phylogenetic relationship of *C. schiffneri* with brown algae belonging to genus *Sargassum* C. A$_{GARDH}$. Molecular characterization using the nuclear small subunit rRNA (SSU rRNA) gene (18S) sequence as genetic marker was made. Pigment analysis showed a significant seasonal change of carotenoids, in particular of fucoxanthin and fucoxanthinol. Also galactolipids, the main constituents of the thylakoid membranes, showed remarkable seasonal changes.

Keywords: Brown algae, *Cystoseira schiffneri*, Molecular characterization, Phytochemical investigation, Lipid analysis.

Introduction

Since ancient times, natural product research interest was directed towards marine flora considered as an interesting reservoir of secondary metabolites having several beneficial effects. Marine brown algae are prolific producers of natural products with various biotechnological and pharmacological applications.[1]

Representatives of the families Sargassaceae and Dictyotaceae constitute the main producers with 2/3 of all the secondary metabolites from brown algae. Sargassaceae is a family composed by the recent merge of the two old Sargassaceae and Cystoseiraceae.[2]

The genus *Cystoseira* C. A$_{GARDH}$ (Fucales, Fucophyceae) is a taxon with a worldwide distribution. Most of the species are frequently occurring along the Mediterranean and the adjoining Atlantic coast.[3]

Several species of *Cystoseira* were not well-defined morphologically and the characters are not easily differentiated since they presented the same morphological variability overlooked for other Sargaccaceae genera as *Sargassum*.[4]

Previous investigations on marine algae showed the utility of some secondary metabolites as chemotaxonomic markers and additional tools that can be useful to decipher the identification and phylogeny of several seaweeds.[5] Recently, molecular techniques have been applied to resolve taxonomic issues.[5] The most common ones are nuclear ribosomal markers, as small subunit (SSU) and large subunit (LSU) rRNA genes, Internal Transcribed Spacers (ITS 1 and 2),[5 – 8] plastid markers, as the large-chain gene of the Ribulose-1,5-bisphosphate carboxylase (*rbcL*) and the photosystem I coding *psa*A gene[9][10] and mitochondrial (mt) markers, as 23S gene.[11][12]

The present work is a further extension of our chemical and biological investigations of *Cystoseira schiffneri* brown alga aimed to provide a basis of a delineation system based on a comprehensive overview of *Cystoseira* and *Sargassum* relationships. Moreover, phytochemical investigation of crude extracts from *C. schiffneri* collected from the Tunisian coast, resulted in the isolation and the identification of a new isololiolide derivative (named schiffnerilolide)

together with the previously reported kjellmanianone. Carotenoids and membrane lipids profiles of *C. schiffneri*, including their main seasonal changes, were also determined.

Results and Discussion

Isolation of Pure Compounds

This is the first report on the phytochemical investigation of MeOH crude extract deriving from *C. schiffneri* collected from the Tunisian coast leading to the isolation and structure elucidation of racemic (±)-kjellmanianone (1) together with the new isololiolide derivative named schiffnerilolide (2) (*Fig. 1*).

Kjellmanianone and isololiolides represent the chemotaxonomic markers of *Sargassum* genus and because of the relationship existing between *Cystoseira* and *Sargassum* genera, the genetic marker 18S were used to increase knowledge about *C. schiffneri*. A comparative analysis between our results and those reported by [12] has been established. Unfortunately, we cannot determine the phylogenetic relationships within the genus *Cystoseira*; in fact, the only species of *Cystoseira* of which is available in the GenBank/EMBL databases the genetic marker used in the present study (18S) and which it could be used as comparison (*Cystoseira hakodatensis* (Y_{ENDO}) F_{ENSHOLT}), does not belong to the genus (current name: *Stephanocystis hakodatensis* (Y_{ENDO}) D_{RAISMA}, B_{ALLESTEROS}, F. R_{OUSSEAU} & T. T_{HIBAUT}).[12]

The structure of kjellmanianone (1) was established by comparison of its spectroscopic data with those reported in the literature.[13] Kjellmanianone, a member of the cyclopentanoid class of antibiotics, was firstly isolated by [13] from MeOH extract of *Sargassum miyabei* Y_{ENDO} (Syn. *Sargassum kjellmanianum* Y_{ENDO}). Isolation of kjellmanianone from both MeOH and AcOEt extracts confirmed that it was not an artifact of extraction. In our hands, this compound was unambiguously proved to be racemic. In fact, from one side its specific optical rotation $[a]_D$ was found vanishing small from the other side the ^{1}H-NMR spectra (in $CDCl_3$) taken by subsequent additions of chiral NMR complexing agent $Yb(tfc)_3$ to kjellmanianone showed pairs of diasteroisotopic signals in exactly 1:1 ratio as highlighted in *Fig. 2*.

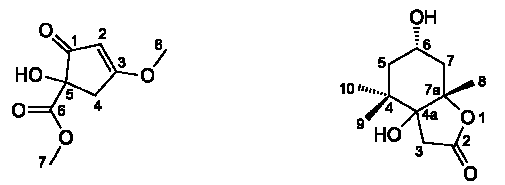

Figure 1. Secondary metabolites isolated from *Cystoseira schiffneri.*

Our finding cast doubts on the previous isolation[13] of optically active (+)-(*S*)-kjellmanianone from marine algae *Sa. kjellmanianum*. The very low positive value of optical rotation here reported ($[a]_D$ = +1.6, *c* = 1.8, $CHCl_3$) strongly suggests that it exists in Nature only as natural racemic metabolite.[13] Curiously, several groups of synthetic organic chemists have been attracted by the enantioselective total synthesis of this compound, thus establishing the (+)-(*S*) and (–)-(*R*) absolute relationships of kjellmanianone enantiomeric forms.[14 – 21]

Compound 2 was unambiguously identified as a new derivative of isololiolide on the basis of 1D- and 2D-NMR experiments and in comparison with the literature data of isololiolide.[15] The ^{13}C-NMR spectrum displayed eleven C-atom signals, three methyls, two methylenes, one O-bearing methine and three quaternary sp^3 C-atoms and an ester-carbonyl function (*d*(C) 175.0, C(2)). The ^{1}H-NMR spectrum displayed two *doublet*s with high geminal coupling constants appearing at *d*(H) 2.41 (*d*, 1 H, *J* = 18, H_a–C(3)) and 2.99 (*d*, 1 H, *J* = 18, H_b–C(3)) indicating the presence of a methylene group directly linked to the carbonyl. These data suggested that compound 2 is a new metabolite derived from isololiolide. The significant long range correlations H_a–C(3)/C(4a); H–C(9)/C(4a); H–C(10)/C(4a); H_b–C(5)/C(4a); H_a–C(3)/C(7a); H_a–C(3)/C(2); H_b–C(3)/C(2); H–C(8)/C(4a); H–C(8)/C(7a); H–C(9)/C(5); H–C(10)/C(5) and H_a–C(7)/C(5) allowed to confirm the location of a OH group on C(4a) (*d*(C) 80.0 ppm) of the five membered lactone and to propose the structure of the new isololiolide derivative named schiffnerilolide (2). Although the relative configuration of the equatorial OH group at C(6) with respect to the methyl at C(7a) was clearly related to isololiolide skeleton, we were unable to establish the configuration of the OH group at C(4a) due to the absence of clear-cut NOE correlations maps among the relevant H-atoms within the molecule. Worth of note, isololiolide was often found in brown algae and is thought to derive from the degradation of fucoxanthin,[22][23] being this carotenoid a marker of the phylum brown algae.

SSU rRNA Gene Sequence

The SSU rRNA gene sequence of *C. schiffneri* determined in this study is available from the GenBank/EMBL databases under the accession number KJ499463. The nuclear SSU rRNA gene sequence of *C. schiffneri* is 1.781 kb in length and has a GC

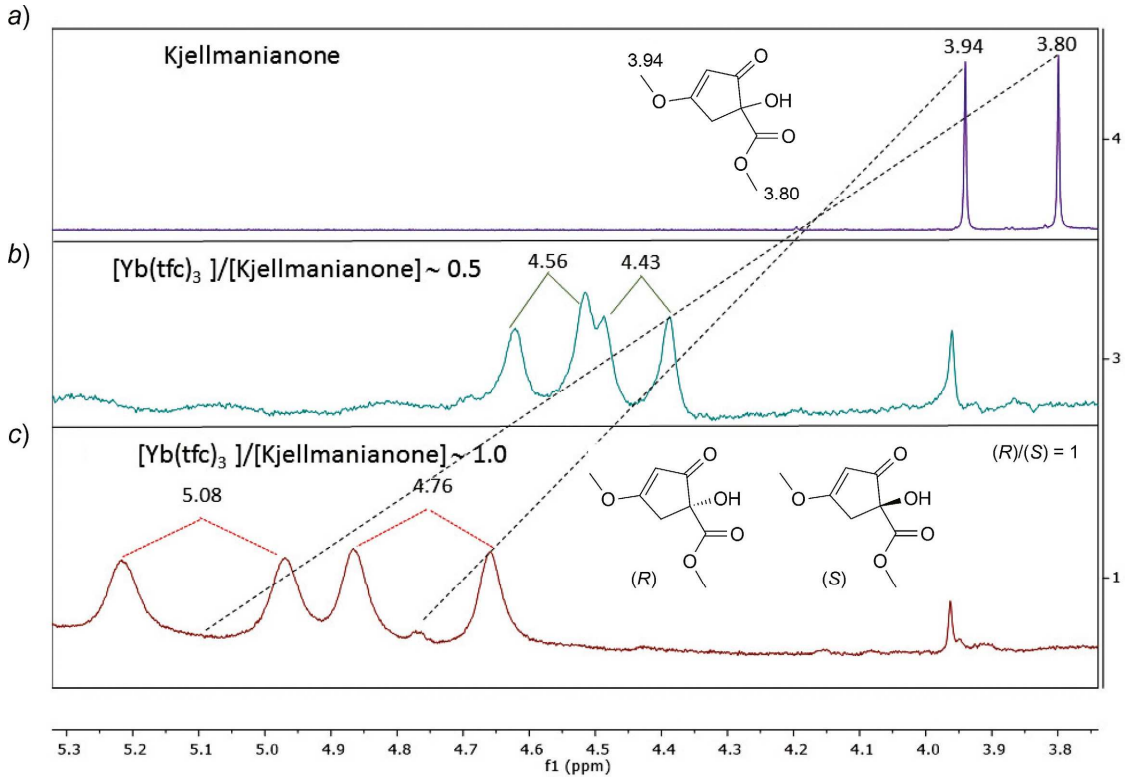

Figure 2. NMR Spectra in $CDCl_3$ of the MeO regions of pure kjellamanianone *a*), after addition of half an equiv. molar amount *b*), and an equiv. molar amount of $Yb(tfc)_3$ *c*).

content of 48.9%. The new sequence of *C. schiffneri* differs from the only homologous sequence of relative taxon available in the Genbank/EMBL databases (*St. hakodatensis*, as *C. hakodatensis*, accession number: AB011425) for 15 nucleotide mutations.

Chlorophylls, Carotenoid and Lipid Profile Analysis

Carotenoids and chlorophylls were determined and their relative amount quantified by using HPLC-VIS/ESI-MS analysis of crude extracts with chromatograms recorded at 470 and 665 nm, respectively. *Cystoseira schiffneri* pigments consisted essentially of *b*-carotene, xanthophylls (oxygenated carotenoids), and chlorophylls. Ten pigments were identified and quantified: five chlorophylls (chlorophyll A, chlorophyll A isomer, pheophytin A 13 allomer, pheophytin A and pheophytin A isomer), five xantophylls (fucoxanthin, fucoxanthin isomer, zeaxanthin, fucoxanthinol and fucoxanthinol isomer) and *b*-carotene (*Fig. 3*).

The seasonal variations of *C. schiffneri* pigment distribution was studied. Fucoxanthin has been identified as the most abundant xanthophylls in summer (76±5% of total carotenoids), while its levels significantly decreased in autumn (42±4%). Fucoxanthinol and Fucoxanthinol isomer were detected only in the autumn sample. Additionally, a slight quantitative variation of the chlorophylls content was also observed. Chlorophyll A, the most abundant chlorophyll in *C. schiffneri*, had the highest concentration in autumn (54%) compared to summer (48%); inversely, pheophytin A was higher in summer (37%) than in autumn (23%).

HPLC/ESI-MS analysis of *C. schiffneri* MeOH extract, enabled the identification and the quantification of different lipid classes characteristic of both thylakoid and plasma membranes. Chloroplast lipid comprised galactolipids, monogalactosyldiacylglycerols (MGDG) and digalactosyldiacylglycerols (DGDG) and sulfolipids (sulfoquinovosyldiacylglycerols, SQDG), while plasma membrane lipids comprehend betainesdiacylglyceryl-*N*,*N*,*N*-trimethylhomoserines (DGTS) and phospholipids phosphatidylcholines (PC).

Under our chromatographic setup, lipids eluted following the order SQDG < DGDG < MGDG < DGTS ~ PC. Neutral lipids diacylglycerols and triacyglicerols (TAG) were also detected but their data are not discussed in this paper.

The unsaturation index (UI) and the average chain length (ACL) values allowed us to determine which lipid classes are effected by seasonal changes (summer/autumn). As reported in *Table 1*, the fatty acyl chains in MGDG and DGDG classes are, on average, significantly less unsaturated and shorter in sample

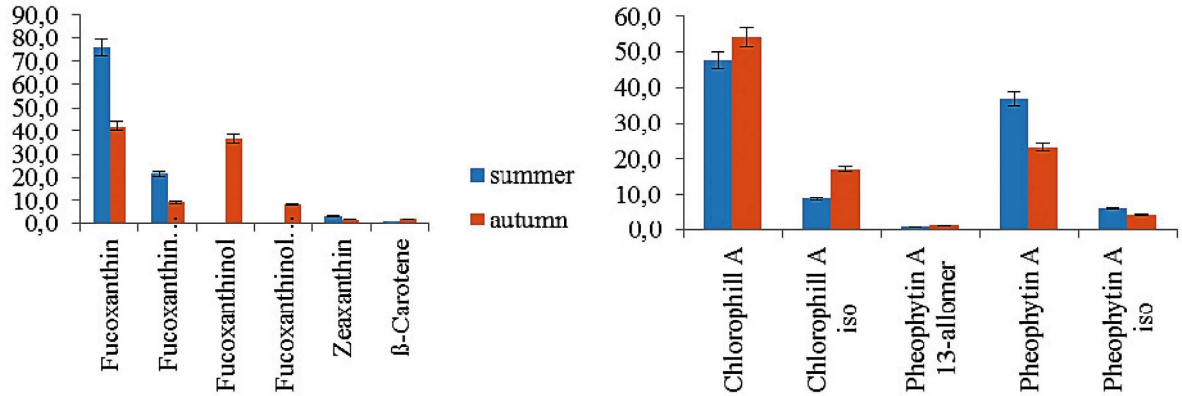

Figure 3. Seasonal variations of the relative distribution of carotenoid (left) and chlorophylls (right) in *Cystoseira schiffneri*. Error bars represent standard deviation (T5%) from three independent experiments.

Table 1. Unsaturation index (UI) and average chain length (ACL) values detected by HPLC/ESI-MS analysis for each lipid class

	UI		ACL	
	Summer	Autumn	Summer	Autumn
DGDG	4.9	5.8	36.5	37.3
MGDG	3.6	4.4	35.6	36.3
SQDG	1.0	0.9	33.2	33.2
DGTS	4.2	3.8	36.2	35.8
PC	4.4	4.7	36.5	37.2

DGDG, digalactosyldiacylglycerols; MGDG, monogalactosyldiacylglycerols; SQDG, sulfoquinovosyldiacylglycerols; DGTS, diacylglyceryl-*N,N,N*-trimethylhomoserines; PC, phospholipids-phosphatidylcholines.

collected in summer whilst the same effect is not observed in other lipid classes such as SQDG, DGTS and PC. Note of worth, the molar ratio [DGDG]/[MGDG] was almost constant (*ca.* 0.7) within the seasonal change.

The seasonal variation in both pigment and lipid profiles has been demonstrated and this is the results of changes in environmental factors such as pH, salinity, temperature and light radiation. Chloroplast lipids and pigments showed greater variation compared to plasma membrane lipids; this outcome is not surprising, as chloroplasts need to compensate changes in environmental factors to maintain their functions and prevent photosynthesis inhibition.

Conclusions

A key point tackled by this report is the phylogenetic significance of the secondary metabolites isolated from marine brown alga C. *schiffneri* collected from the Tunisian marine coast. Several authors have commented on the use- or misuse-of rDNA sequence data as the sole descriptor for establishing a taxon or for suggesting that a single molecular marker can serve to reveal phylogenetic relationships of the targeted organism.[24] Thus, on the ground of an interdisciplinary approach, we reaffirmed here the added value of polyphasic taxonomies in which molecular biological data (genotypic, rDNA) complement but do not supplant morphological information (phenotypic) or micromolecular biological data (secondary metabolites).[25] Our multidisciplinary approach allow to establish a polyphasic database wherein morphological and genetic information (SSU rRNA gene sequence)[26] have been compared with chemodiversity represented by secondary metabolites (kjellmanianone (1) and isololiolide (2)). Our study indicates that the biosynthesis of metabolites typical of *Sargassum* genus is promptly reflected by the phylogenetic position of this alga. Seasonal changes of carotenoids and lipids profile of the brown alga C. *schiffneri* were also investigated.

Experimental Section

Plant Material

Specimens of *C. schiffneri* were harvested in Chebba region, in June 2012 and in September 2013 (Autumn season). The morphological identification was authenticated by Prof. Dr. *Lot*fi *Mabrouk*, a biologist, INSTM Sfax-Tunisia and by Prof. *Asma Hamza*, a director in Biology from the same Institute. A voucher specimen (FSM-Cys 2011) was deposited with the Herbarium of the Faculty of Sciences of Monastir, Tunisia.

Extraction Method

Freshly collected samples of the whole *C. schiffneri* algal biomass were washed in sterile distilled H_2O then dried at r.t. Seaweed powdered (1500 g) was extracted by maceration during 3 d in MeOH.

Evaporation of the solvent under reduced pressure, afforded 80 g of MeOH crude extract.

Chromatographic Study of MeOH Extract

About 40 g of MeOH crude extract were subjected to flash column chromatography on SiO_2 using hexane with increasing amounts of AcOEt followed by AcOEt with increasing amounts of MeOH to yield 26 fractions grouped in eight cluster ($C_1 - C_8$) on the basis of thin layer chromatography (TLC) analysis.

*Isolation of Kjellmanianone (*1*)*

Fraction C_4 thus obtained was successively purified through three flash reversed-phase SiO_2 columns using MeCN/MeOH gradient to afford 2.1 mg of compound (1).

Kjellmanianone (= Methyl 1-Hydroxy-4-methoxy-2-oxocyclopent-3-ene-1-carboxylate; 1). Yellow amorphous solid. $[\alpha]_D^{20}$ ffi 0 (c = 0.8, MeOH). ^{1}H-NMR (400 MHz, CD_3OD): 2.65 (*d*, ^2J = 16, H_a–C(4)); 3.15 (*d*, ^2J = 16, H_b–C(4)); 3.74 (*s*, COOMe); 3.97 (*s*, MeO); 5.45 (*s*, H–C(2)). ^{13}C-NMR (100 MHz, CD_3OD): 42.9 (*t*, C(4)); 53.3 (*q*, COO*Me*); 60.3 (*q*, MeO); 80.4 (*s*, C(5)); 102.2 (*d*, C(2)); 173.0 (*s*, *C*OOMe); 192.4 (*s*, C(3)); 202.5 (*s*, C(1)).

^{1}H-NMR (400 MHz, $CDCl_3$): 2.75 (*dd*, ^4J = 1.2, ^3J = 17.6, H_a–C(4)); 3.18 (*dd*, ^4J = 1.2, ^3J = 17.6, H_b–C(4)); 3.80 (*s*, COOMe); 3.94 (*s*, MeO); 5.36 (*t*, ^4J = 1.2, H–C(2)); ^{13}C-NMR (100 MHz, $CDCl_3$): 40.6 (*t*, C(4)); 53.5 (*q*, COO*Me*); 59.2 (*q*, MeO); 79.1 (*s*, C(5)); 101.1 (*d*, C(2)); 171.5 (*s*, *C*OOMe); 190.2 (*s*, C(3)); 199.5 (*s*, C(1)).

Measure of the Enantiomeric Purity. To a 5 m_M solution of pure 1 in $CDCl_3$ increasing amount of solid tris[3-(trifluoromethylhydroxymethylene)-d-camphorato]-ytterbium derivative ($Yb[tfc]_3$) were added until a complete separation of the *singlet* methyls due to (–)-(*R*)-1/$Yb(tfc)_3$ and (+)-(*S*)-1/$Yb(tfc)_3$ diasteroisomeric complexes was obtained. The corresponding signal integration leads to 1:1 ratio within each pair of signal (0% enantiomeric excess).

*Isolation of Schiffnerilolide (*2*)*

Fraction C_5 was successively purified through two flash columns packed with reversed-phase SiO_2 and eluted with MeCN mixed with a gradual amount of MeOH to provide 0.8 mg of compound 2.

Schiffnerilolide (= (6*S*,7a*S*)-Hexahydro-3a,6-dihydroxy-4,4,7a-trimethyl-1-benzofuran-2(3*H*)-one; 2). Yellow amorphous solid. $[\alpha]_D^{20}$ ffi 0 (c = 0.2, MeOH). ^{1}H-NMR (400 MHz, CD_3OD): 2.41 (*d*, ^3J = 18, H_a–C(3)); 2.99 (*d*, ^3J = 18, H_b–C(3)); 1.57 – 1.61 (*m*, H_a–C(5)); 2.32 – 2.37 (*m*, H_b–C(5)); 3.89 – 3.93 (*m*, H–C(6)); 1.58 – 1.62 (*m*, H_a–C(7));1.84 – 1.86 (*m*, H_b–C(7)); 1.53 (*s*, Me(8)); 1.03 (*s*, Me(9)); 1.09 (*s*, Me(10)). ^{13}C-NMR (100 MHz, CD_3OD): 21.0 (*q*, C(8)); 23.0 (*q*, C(10));27.0 (*q*, C(9)); 37.0 (*s*, C(4)); 40.9 (*t*, C(3)); 46.0 (*t*, C(7)); 47.5 (*t*, C(5)); 63.5 (*d*, C(6)); 80.0 (*s*, C(4a)); 86.5 (*s*, C(7a)); 175.0 (*s*, C(2)).

Pigments and Lipids Analysis

Two fresh samples of algae harvested in Spring and Autumn were immediately transferred in EtOH and stored at –18 °C. The algae were extracted twice in a sonicated solution of $CHCl_3$/MeOH (2:1). The original EtOH supernatant was added to the filtered solutions. We reduced the solution to dryness by rotatory evaporation to produce the crude extract which was re-dissolved into 2 ml of MeOH and stored in glass vials until chromatographic analysis.

High performance liquid chromatography/electrospray ionization-mass spectrometry (HPLC/ESI-MS) was performed on a *Hewlett–Packard Model 1100 Series* liquid Chromatograph coupled to a Photo Diode-Array detector (DAD) (*Agilent 1100 Series*; *Agilent Technologies*, Santa Clara, California) and to a *Bruker Esquire-LC* quadrupole ion-trap mass spectrometer (*Bruker-Avance*, Ballerica, Massachusetts) equipped with atmospheric pressure electrospray ion source (ESI). We set the DAD at wavelengths 215, 470, and 665 nm and recorded all spectra. Chromatographic separation was carried out at r.t. on a *ZORBAX Eclipse XDB-C8* (*Agilent Technologies*) 150 **9** 4.6 mm, 3.5 Im column. Solvent *A* was MeOH/H_2O 70:30 (ammonium acetate 12 m_M), and solvent *B* was MeOH 100% (ammonium acetate 12 m_M). The linear gradient, at a constant flow rate of 0.8 ml/min, started from 70% *B* to reach 100% *B* in 40 min, and isocratic *B* was held for 10 min to ensure the complete elution of non-polar lipids. Injection volume was 10 **1**l.

Crude extracts were analyzed separately in positive and negative ion mode by setting the capillary voltage at +4000 and –4000 kV, resp.; the scan range was 50 – 1200 m/z and the scan rate was 13000 m/z s^{-1}. Other parameters were: high purity nitrogen pressure: 35 psi; temp.: 300 °C and flow rate: 7 l/min.

The pigments identification and quantitative analyses were carried out on *Agilent Model 1100 Series* liquid chromatograph coupled both to a PDA (*Agilent 1100 Series*) and to a *Bruker Esquire-LC* quadrupole ion trap mass spectrometer equipped with atmospheric pressure ESI ion-source. Identification of individual pigments was based on a combination of retention time

and absorbance spectra compared with 23 authentic pigment standards obtained from *DHI Water & Environment* (Denmark). Their relative amounts were quantified from their peak area by use of a standard calibration curve.

The regiochemical distribution of acyl chains in galactolipids was established by MS/MS according to [27]. To improve the assignment of lipid molecular species we applied the method published[28] using HILIC chromatography in combination with precursor ion/neutral loss scanning in positive ion mode.

Lipid molecular species were relatively quantified respect to the total area of lipids belonging to the same class (*e.g.*, relative quantification of MGDG was performed with respect to total area of MGDG class). PC, DGTS, MGDG and DGDG were quantified on the dataset recorded in positive ion mode, SQDG on that obtained in negative ion mode.

The standards MGDG (18:3/18:3) and DGDG (18:3/18:3) were used to establish the ESI-MS response factors (*F*) in positive ion mode of galactolipids, according to [29], and to calculate the ratio [Σ area DGDG]/[Σ area MGDG] **9** *F* conforming to [30].

For each lipid class, the UI and the ACL were determined as follow:

$$UI_{class\ y} = \sum (\text{relative area lipid}_x \times \text{double bond number of lipid}_x) \text{ and}$$

$$ACL_{class\ y} = \sum (\text{relative area lipid}_x \times \text{acyl chain length of lipid}_x)$$

DNA Isolation, SSU rRNA Gene Amplification, and Sequencing. Total DNA was extracted from samples preserved in 96% EtOH, using the *QIAamp® DNA Micro Kit* (*Qiagen*, Milan, Italy) in accord with the manufacturer's instructions (protocol: isolation of genomic DNA from tissues) and starting from 2 to 4 mg of ground, liquid nitrogen tissue. DNA was eluted in two fractions of 50 µl, the second fraction was used in gene amplification reactions, usually diluted 10 – 100 times with *MilliQ* water (*Millipore*, Bedford, MA, USA). The nuclear SSU rRNA (18S) gene was amplified by PCR using the universal eukaryotic forward primer 18S F9 [(5′-CTGGTTGATCCTGCCAG-3′)][31] and the 18S R1513 Hypo reverse primer [(5′-TGATCCTTCYGCAGGTTC-3′)].[32] PCR amplifications were performed by adding 1 µl diluted genomic DNA to 49 µl of a reaction mixture containing 2 m_M $MgCl_2$, 250 m_M of dNTP, one unit of Taq DNA polymerase (*Polymed*, Florence, Italy), and 0.2 m_M of each primer. Amplifications were run in a *GenAmp PCR System 2400* (*Applied Biosystems*, Foster City, CA, USA). The PCR conditions consisted of an initial denaturation step at 94 °C for 3 min, 35 cycles consisting of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and elongation at 72 °C for 2 min, followed by a final extension step at 72 °C for 5 min. Amplified products were purified using *Quantum Prep PCR Kleen Spin* columns (*Bio-Rad*, Hercules, CA, USA). In order to obtain a reference sequence to be used as template for the designation of internal primers for the direct sequencing procedure, the PCR products were cloned using the *TOPO TA cloning Kit* (*Invitrogen*, Carlsbad, CA, USA) and following the manufacturer instructions. Ten randomly selected recombinant clones were sequenced using the vector-specific primers, M13 forward (5′-GTAAAACGACGGCCAG-3′) and M13 reverse (5′-CAGGAAACCAGCCCTAATTTGGACC-3′), and the sequences were compared with each other. On the basis of the produced consensus sequence, three internal primers were designated: the CysF505 forward (5′-GAGAGAGACTTAAATCCATCATCGAGG-3′), the CysR663 reverse (5′-CAAACGGCCCCGGGATACA ATG-3′), and the CysF1063 forward (5′-TGGCGGTCGTT AACTTACAGGACTCCG-3′). Sequencing of both PCR products and the plasmid DNA was performed with an *ABI Prism 310* automated DNA sequencer (*Applied Biosystems*). To minimize amplification errors, sequences of two different amplicons were compared.

Acknowledegements

This study was supported by an Erasmus Mundus-Averroes Ph.D. Grant from the *University of Trento*. The authors would like to thank *Adriano Sterni* for recording HPLC/ESI-MS data.

References

[1] J. H. Fitton, 'Brown Marine Algae: A Survey of Therapeutic Potentials', *Altern. Complement. Ther.* 2003, *9*, 29 – 33.

[2] S. Rožić, J. Puizina, I. Šamanić, A. Žuljević, B. Antolić, 'Molecular Identification of the Brown Algae *Cystoseira* spp. (Phaeophycae, Fucales) from the Adriatic Sea-Preliminary Results', *Acta Adriat* 2012, *53*, 447 – 456.

[3] B. Montesanto, P. Panayotidis, 'The *Cystoseira* spp. Communities from the Aegean Sea (NE Mediterranean)', *Mediterr. Mar. Sci.* 2001, *2*, 57 – 68.

[4] L. Mattio, C. E. Payri, '190 Years of *Sargassum* Taxonomy, Facing the Advent of DNA Phylogenies', *Bot. Rev.* 2011, *77*, 31 – 70.

[5] C. Jégou, G. Culioli, N. Kervarec, G. Simon, V. Stiger-Pouvreau, 'LC/ESI-MSn and ^{1}H HR-MAS NMR Analytical Methods as Useful Taxonomical Tools Within the Genus *Cystoseira* C. Agardh (Fucales; Phaeophyceae)', *Talanta* 2010, *83*, 613 – 622.

[6] M. C. Leclerc, V. Barriel, G. Lecointre, B. de Reviers, 'Low Divergence in rDNA ITS Sequences Among Five Species of Fucus (Phaeophyceae) Suggests a Very Recent Radiation', *J. Mol. Evol.* 1998, *46*, 115 – 120.

[7] F. Rousseau, R. Burrowes, A. F. Peters, R. Kuhlenkamp, B. de Reviers, 'A Comprehensive Phylogeny of the Phaeophyceae Based on nrDNA Sequences Resolves the Earliest Divergences', *C. R. Seances Acad. Sci., Ser. 3*2001, *324*, 305 – 319.

[8] B. J. Harvey, L. J. Goff, 'A reassessment of Species Boundaries in *Cystoseira* and *Halidrys* (Phaeophyceae, Fucales) Along the North American West Coast', *J. Phycol.* 2006, *42*, 707 – 720.

[9] G. Y. Cho, F. Rousseau, B. de Reviers, S. M. Boo, 'Phylogenetic Relationships Within the Fucales (Phaeophyceae) Assessed by the Photosystem I Coding psaA Sequences', *Phycologia* 2006, *45*, 512 – 519.

[10] L. Bittner, C. E. Payri, A. Couloux, C. Cruaud, B. de Reviers, F. Rousseau, 'Molecular Phylogeny of the Dictyotales and Their Position Within the Phaeophyceae, Based on Nuclear, Plastid and Mitochondrial DNA Sequence Data', *Mol. Phylogenet. Evol.* 2008, *49*, 211 – 226.

[11] J. A. Coyer, G. Hoarau, M. P. Oudot-Le Secq, W. T. Stam, J. L. Olsen, 'A mtDNA-Based Phylogeny of the Brown Algal Genus *Fucus* (Heterokontophyta; Phaeophyta)', *Mol. Phylogenet. Evol.* 2006, *39*, 209 – 222.

[12] S. G. A. Draisma, E. Ballesteros, F. Rousseau, T. Thibaut, 'DNA Sequence Data Demonstrate the Polyphyly of the Genus *Cystoseira* and Other Sargassaceae Genera (Phaeophyceae)', *J. Phycol.* 2010, *46*, 1329 – 1345.

[13] M. Nakayama, Y. Fukuoka, H. Nozaki, A. Matsuo, S. Hayashi, 'Structure of (+)-Kjellmanianone, a Highly Oxygenated Cyclopentenone from the Marin Alga, *Sargassum kjellmanianum*', *Chem. Lett.* 1980, *9*, 1243 – 1246.

[14] D. Boschelli, A. B. Smith, O. D. Stringer, R. H. Jenkins, F. A. Davis, 'An Asymmetric Synthesis of (+)-kjellmanianone', *Tetrahedron Lett.* 1981, *22*, 4385 – 4388.

[15] A. B. Smith, S. J. Branca, N. N. Pilla, M. A. Guaciaro, 'Stereocontrolled Total Synthesis of (T)-pentenomycins. I-III, Their Epimers, and Dehydropentenomycin I', *J. Org. Chem.* 1982, *47*, 1855 – 1869.

[16] A. B. Smith, D. Boschelli, 'Stereocontrolled Total Synthesis of (T)-Xanthocidin, Two Diastereomers, (T)-Epixanthocidin and (T)-*b*-Isoxanthocidin, and (T)-Dedihydroxy-4,5-dideoxanthocidin, a Likely Biosynthetic Precursor', *J. Org. Chem.* 1983, *48*, 1217 – 1226.

[17] F. A. Davis, L. C. Vishwakarma, J. G. Billmers, J. Finn, 'Synthesis of *a*-Hydroxycarbonyl Compounds (acyloins): Direct Oxidation of Enolates Using 2-Sulfonyloxaziridines', *J. Org. Chem.* 1984, *49*, 3241 – 3243.

[18] B.-C. Chen, M. C. Weismiller, F. A. Davis, D. Boschelli, J. R. Empfield, A. B. Smith, 'Enantioselective Synthesis of (+)-Kjellmanianone', *Tetrahedron* 1991, *47*, 173 – 182.

[19] J. Zhu, A. J. H. Klunder, B. Zwanenburg, 'Total Synthesis of (–)-Kjellmanianone from Tricyclodecadienone. A Revision of its Absolute Configuration', *Tetrahedron* 1994, *50*, 10597 – 10610.

[20] J. Zhu, A. J. H. Klunder, B. Zwanenburg, 'Stereospecific Total Synthesis of (–)-Kjellmanianone and a Revision of its Absolute Configuration', *Tetrahedron Lett.* 1994, *35*, 2787 – 2790.

[21] J. Christoffers, T. Werner, W. Frey, A. Baro, 'Straightforward Synthesis of (*R*)-(–)-Kjellmanianone', *Chemistry* 2004, *10*, 1042 – 1045.

[22] J. Klok, M. Baas, H. C. Cox, J. W. De Leeuw, P. A. Schenck, 'Loliolides and Dihydroactinidiolide in a Recent Marine Sediment Probably Indicate a Major Transformation Pathway of Carotenoids', *Tetrahedron Lett.* 1984, *25*, 5577 – 5580.

[23] D. J. Repeta, 'Carotenoid Diagenesis in Recent Marine Sediments: II. Degradation of Fucoxanthin to Loliolide', *Geochim. Cosmochim. Acta* 1989, *53*, 699 – 707.

[24] R. G. E. Murray, 'Molecular Ecology', *ASM News* 1997, *63*, 64 – 65.

[25] P. Vandamme, B. Pot, M. Gillis, P. De Vos, K. Kersters, J. Swings, 'Polyphasic Taxonomy, a Consensus Approach to Bacterial Systematics', *Microbiol. Rev.* 1996, *60*, 407 – 438.

[26] G. E. Fox, J. D. Wisotzkey, P. Jurtshuk Jr., 'How Close is Close: 16S rRNA Sequence Identity May Not Be Sufficient to Guarantee Species Identity', *Int. J. Syst. Bacteriol.* 1992, *42*, 166 – 170.

[27] G. Guella, R. Frassanito, I. Mancini, 'A New Solution for an Old Problem: The Regiochemical Distribution of the Acyl Chains in Galactolipids can be Established by Electrospray Ionization Tandem Mass Spectrometry', *Rapid Commun. Mass Spectrom.* 2003, *17*, 1982 – 1994.

[28] A. Anesi, G. Guella, 'A Fast Liquid Chromatography-Mass Spectrometry Methodology for Membrane Lipid Profiling Through Hydrophilic Interaction Liquid Chromatography', *J. Chromatogr. A* 2015, *1384*, 44 – 52.

[29] G. Flaim, U. Obertegger, A. Anesi, G. Guella, 'Temperature-Induced Changes in Lipid Biomarkers and Mycosporine-Like Amino Acids in the Psychrophilic Dinoflagellate *Peridinium aciculiferum*', *Freshw. Biol.* 2014, *59*, 985 – 997.

[30] A. Anesi, U. Obertegger, G. Hansen, A. Sukenik, G. Flaim, G. Guella, 'Comparative Analysis of Membrane Lipids in Psychrophilic and Mesophilic Freshwater Dinoflagellates', *Front. Plant Sci.* 2016, *7*, 524.

[31] L. Medlin, H. J. Elwood, S. Stickel, M. L. Sogin, 'The Characterization of Enzymatically Amplified Eukaryotic 16S-Like rRNA-Coding Regions', *Gene* 1988, *71*, 491 – 499.

[32] G. Petroni, F. Dini, F. Verni, G. Rosati, 'A Molecular Approach to the Tangled Intrageneric Relationships Underlying Phylogeny in Euplotes (Ciliophora, Spirotrichea)', *Mol. Phylogenet. Evol.* 2002, *22*, 118 – 130.