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LC/ESI-MS^n and ^{1}H HR-MAS NMR analytical methods as useful taxonomical tools within the genus *Cystoseira* C. Agardh (Fucales; Phaeophyceae)

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ABSTRACT

Species of the genus *Cystoseira* are particularly hard to discriminate, due to the complexity of their morphology, which can be influenced by their phenological state and ecological parameters. Our study emphasized on the relevance of two kinds of analytical tools, (1) LC/ESI-MS^n and (2) ^{1}H HR-MAS NMR, also called *in vivo* NMR, to identify *Cystoseira* specimens at the specific level and discuss their taxonomy. For these analyses, samples were collected at several locations in Brittany (France), where *Cystoseira baccata*, *C. foeniculacea*, *C. humilis*, *C. nodicaulis* and *C. tamariscifolia* were previously reported. To validate our chemical procedure, the sequence of the ITS2 has been obtained for each species to investigate their phylogenetic relationships at a molecular level. Our study highlighted the consistency of the two physico-chemical methods, compared to "classical" molecular approach, in studying taxonomy within the genus *Cystoseira*. Especially, LC/ESI-MS^n and phylogenetic analyses converged into the discrimination of two taxonomical groups among the 5 species. The occurrence of some specific signals in the ^{1}H HR-MAS NMR spectra and/or some characteristic chemical compounds during LC/ESI-MS^n analysis could be regarded as discriminating factors. LC/ESI-MS^n and ^{1}H HR-MAS NMR turned out to be two relevant and innovative techniques to discriminate taxonomically this complex genus.

The genus *Cystoseira* C. Agardh (1820) is composed of forty-seven validated species, among nearly three hundred of taxa listed in the AlgaeBase at present [1]. It belongs to the family Sargassaceae, and in Europe, it shows the biggest species richness within this family. Currently, more than thirty species have been identified in the Mediterranean Sea, while only ten species have been reported along the Atlantic coasts of continental Europe [1–3], among which are the most common *Cystoseira baccata* (S.G. Gmelin) P.C. Silva, *C. foeniculacea* (Linnaeus) Greville, *C. humilis* Schousboe ex Kützing, *C. nodicaulis* (Withering) M. Roberts and *C. tamariscifolia* (Hudson) Papenfuss. Most of the chemical studies on *Cystoseira* species have been led on exclusively Mediterranean taxa and/or in Mediterranean sites [4,5]. Despite the growing number of scientific publications focusing on some specific taxa in the last decades, their classification remains unclear. Concerning the five Atlantic species cited before, many taxonomical changes have occurred since their first description. Between three and nine synonymous are recorded for each taxon [1,2]. Misclassification due to the high morphological variability of these taxa, observed throughout the seasons [6], and linked with multiple environmental conditions as described for the genus *Sargassum* [7], could explain these taxonomic ambiguities.

In order not to be influenced by morphological variability, recent researches in taxonomy frequently include molecular analyses. Molecular tools have proven themselves to be valuable in a taxonomical context, such as samples identification and species phylogenetic relationships establishment. Among the order Fucales (in which is found the genus *Cystoseira*), phylogeny has become more and more investigated during the last decade. The phylogeny of both Fucaceae and Sargassaceae families has been studied using mitochondrial, chloroplastic, and nuclear markers [7–18]. Within the family Sargassaceae, recent works have proven the usefulness of nuclear ribosomal DNA (and more particularly the Internal Transcribed Spacers—*ITS*) to establish phylogenetic relationships [9,12,13].

Abbreviations: HR-MAS NMR, High Resolution Magic Angle Spinning Nuclear Magnetic Resonance; *ITS2*, Internal Transcribed Spacer 2; LC/ESI-MS^n, Liquid Chromatography/ElectroSpray Ionization Multistage Mass Spectrometry; MCA, Multiple Correspondence Analysis; ppm, parts per million.

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However, the selection of efficient molecular markers is a crucial step in such studies. A molecular marker can be helpless in studying phylogeny within some taxa, because of a too poor informational variability, resulting in identical sequences or the impossibility of their alignment. Furthermore, some characteristic biomolecules in brown macroalgae (such as polyphenols) are also known to decrease the quality of DNA, making the sequences harder to get than what could be expected [19]. Considering these problematic issues, and the cost of molecular methods, other approaches, such as chemical investigations, could be performed to discuss taxonomy.

Considering the genus *Cystoseira*, few phylogenetic studies of Phaeophyceae have been led [9,14–16], whereas many authors have investigated their chemotaxonomy. Actually only fragmental information has been obtained from phylogenetic studies concerning the genus, and the most exhaustive works have been realized taking account of chemical criteria. Most of them have focused on the characterization of lipophilic content, and notably the identification of several kinds of terpene derivatives that could be regarded as chemotaxonomic markers [20,21]; for a complete review see [4,5,22]. The classification of all taxa has been discussed according to the chemical composition of the algae and assumed biosynthetic pathways of the isolated molecules.

In addition, a novel chemotaxonomic approach has recently proven itself useful to study the genus *Turbinaria* (also included within the family Sargassaceae) from the Pacific Ocean. Thus, Le Lann et al. [23] used NMR to produce *in vivo* chemical signals of pieces of thalli. In this study, without any precise investigation about the chemical composition of the studied algae, a statistical analysis of the spectra obtained using *in vivo* HR-MAS NMR permitted the efficient separation of two close species, *Turbinaria conoides* and *T. ornata*, which are hard to discriminate using the only classical taxonomical criteria (involving morphological features).

The aim of the present work was then to evaluate the relevance of two innovative analytical techniques for taxonomical purposes, in the case of 5 species of the genus *Cystoseira*, present along the coasts of Brittany (France). We used Liquid Chromatography-Electrospray Ionization Multiple-Stage Mass Spectrometry (LC/ESI-MS^n) to analyze lipophilic extracts, and *in vivo* ^{1}H HR-MAS NMR to observe the global chemical profile of each taxon. Both techniques were then employed to distinguish and classify five species of the genus *Cystoseira*. The sequences of the *ITS2*, commonly informative in phylogenetic studies within the Fucales, were obtained for each species and used as a reference step to validate and discuss the results of both analytical methods. The powerfulness of both techniques was evaluated following two major criteria: their ability to distinguish the species, and their capacity to establish relevant taxonomic boundaries, in comparison to the phylogenetic study data.

1. Materials and methods

1.1. Sample collection and preparation

Investigations in chemical and molecular taxonomy within the genus *Cystoseira* was permitted by a dual sampling strategy.

Specimens of the five *Cystoseira* species – *C. baccata*, *C. foeniculacea*, *C. humilis*, *C. nodicaulis* and *C. tamariscifolia* – were firstly sampled on September 2008 in Penmarc'h (47°48′N, 4°22′W), in order to get fresh material for chemical investigations. A preliminary work was realized to find out the position of individuals on the intertidal and subtidal zones. We localized about 250 points where some *Cystoseira* species could be observed (unpublished data). Among them, 3 sample collection spots were then randomly chosen for each taxon, so as to get triplicates in chemical analyses, and to face the maximum variability on the field, considering their morphology and phenology. The choice was made to collect only apical axes, for two reasons: (i) among the five species, two of them (*C. foeniculacea* and *C. humilis*) do not have a real primary axis and (ii) this sampling is much less destructive as *Cystoseira* species are known to seasonally drop and regenerate their ultimate axes [24]. The second series of algal material was sampled on December 2008 on other sites to get optimal quality of tissues for DNA extraction. These particular date and sites were chosen in order to get the more reproductive parts possible from samples in the aim of maximizing the quantity of DNA available for extraction. Consequently *C. baccata* and *C. tamariscifolia* were sampled on the low shore of Porspoder (48°29′N, 4°46′W), *C. foeniculacea* and *C. nodicaulis* on the intertidal zone of Portsall (48°34′N, 4°42′W), and *C. humilis* in the rocky pools from the upper shore of Plougonvelin (48°21′N, 4°42′W). The five resulting samples were submitted to physico-chemical and molecular analyses. In addition, specimens of other Sargassaceae occurring in Brittany: *Bifurcaria bifurcata* R. Ross, *Halidrys siliquosa* (L.) Lyngb. and *Sargassum muticum* (Yendo) Fensholt, were collected on early April 2009 at Penmarc'h. These extra-generic specimens permitted to study the position of *Cystoseira* species from Brittany within the phylogenetic tree of the family Sargassaceae. For both sampling series, the algal material was cleaned up from its possible epiphytes. Samples were either freeze-dried or conserved in silica gel, to be used for physico-chemical analyses or molecular analyses, respectively. A small part of the freeze-dried material (top axis) was used for ^{1}H HR-MAS analyses. The rest was ground to powder for extraction.

1.2. Species morphological discrimination

On the field, the identification of the *Cystoseira* species was carried out according to the key given by Cabioc'h et al. [24]. We particularly focused on the following criteria: absence/presence of a primary axis, axes organized in a plan or not, and absence/presence of tophules. Drawings of the shape of ultimate axes of the *Cystoseira* species are presented at Fig. 1. Maximal attention was paid to distinguish *C. foeniculacea* from *C. humilis*, for two reasons. First, these two species are highly morphologically related; both are cespitous, and moreover, the first, differing by its kind of ramification (in a plan), is not easy to distinguish from *C. humilis* when its axes are beginning to regenerate (C. Jégou, pers. obs.). Secondly, the key proposed by Cabioc'h et al. [24] uses deduction to identify *C. humilis*, so that no particular morphological characteristic makes it clearly different.

1.3. Chemical investigations

1.3.1. LC/ESI-MS^n fingerprinting

The powder obtained from each collected sample was used for a sequence of extractions using three kinds of mixtures of dichloromethane and methanol as solvent: (2:1, v/v) the first time, and after that the powder was re-used with (1:1) mixture, then (1:2), and finally (1:1) again for 7 times. Every extraction consisted in addition of 500 mL solvent, beginning with 30 min of sonication (Sonicater 88155, Bioblock Scientific, Illkirch, France), and lasted about 4 h with agitation on a universal shaker (SM-30, Edmund Bühler GmbH, Hechingen, Germany). The 10 extracts obtained from each sample were then pooled, and evaporated under vacuum (Laborota 4000, Heidolph Instruments GmbH & Co.KG, Schwabach, Germany). The final extract was cleaned using Solid Phase Extraction (Strata C18-E, Phenomenex Inc., Torrance, CA, USA). First elution was realized with water, resulting in the loss of salt and polar organic compounds such as sugars or phlorotannins. Finally, non-polar compounds were eluted using a mixture

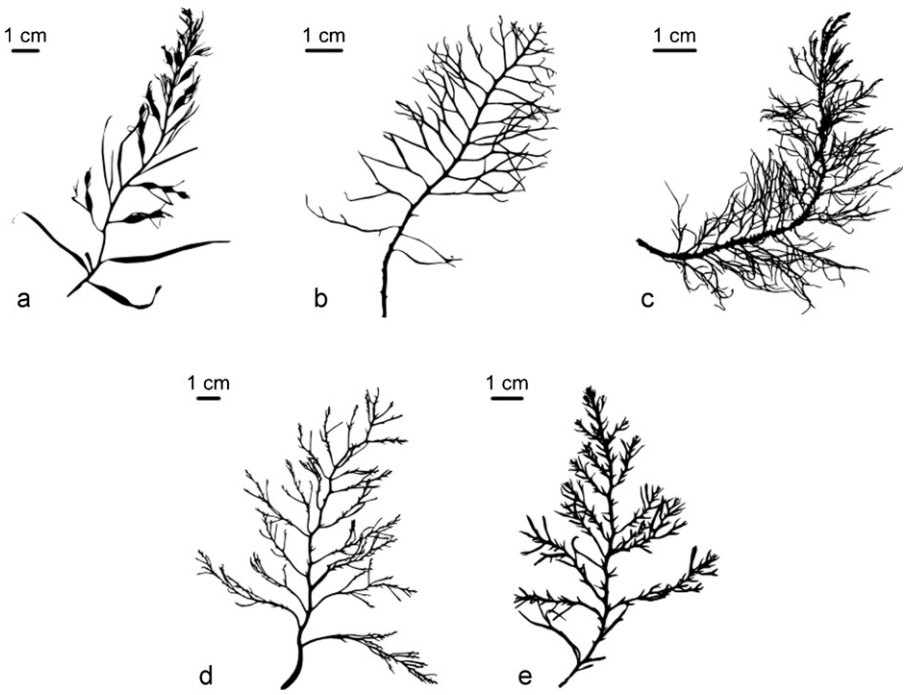

Fig. 1. Drawings of the ultimate axes of the *Cystoseira* species, obtained from samples used in this study: (a) *C. baccata*, (b) *C. foeniculacea*, (c) *C. humilis*, (d) *C. nodicaulis* and (e) *C. tamariscifolia*.

of dichloromethane and methanol (1:1). These "cleaned" samples were analyzed using LC/ESI-MS^n coupled also with a Diode Array Detector (DAD) and an Evaporative Light Scattering Detector (ELSD). For this experiment, a LaChrom Elite HPLC (VWR-Hitachi, Fontenay-sous-Bois, France) composed by a L-2130 quaternary pump, a L-2200 autosampler and a L-2300 column oven was used. Detection was performed with a L2455 DAD and an ELSD (Chromachem model, Eurosep, Cergy Pontoise, France) coupled to an ion trap mass spectrometer fitted with an ESI interface (Esquire 6000, Bruker Daltonik, GmbH, Bremen, Germany). Operating conditions for MS analysis were: dry temperature, 350 °C; capillary voltage, 4000 V; nebulizer, 50 psi; dry gas, helium at 12 L/min. Ion trap full-scan analysis was conducted from *m*/*z* 50 to 1200 with an upper fill time of 200 ms. Approximately 1 mg of each "cleaned" sample was dissolved in 1 mL of a mixture of methanol/dichloromethane (1:1, v/v) and a 10 μL sample volume was injected in the chromatographic system. Compounds were separated using an analytical reversed-phase column (Gemini C_6-Phenyl, 250 mm × 3 mm, 5 μm, Phenomenex) maintained at 30 °C. For this separation, a binary mobile phase was used: eluent A was constituted by 1% (v/v) of formic acid in water and eluent B by 1% (v/v) of formic acid in acetonitrile. After a preliminary step of optimization, the gradient of elution was set as follow: (i) a start with 20% of B and an increasing up to 65% of B in 20 min (linear ramp), (ii) an isocratic step with 65% of B during 15 min, (iii) a linear ramp until 100% of B in 5 min (linear ramp). This composition was finally maintained during 15 min until re-equilibration of the system to the initial conditions (5 min). The flow rate was fixed at 0.5 mL/min. Finally 15 + 5 chromatograms (3 + 1 per species) were obtained. Considering the polarity-decreasing gradient used for this study, we paid attention to the retention time interval from 10 up to 40 min. Out of this range, non-discriminating molecules such as sterols and fatty acids could be found. Possible terpene derivatives were the most likely to be detected in the chromatograms from 10 to 40 min. The mass over charge ratio (*m*/*z*) of the ions detected at a precise retention time in positive and/or negative modes was eventually used to discriminate compounds with similar retention time.

1.3.2. In vivo NMR spectroscopy

All HR-MAS NMR spectra were acquired on a DRX 500 spectrometer (Bruker BioSpin, Wissembourg, France) equipped with an indirect HR-MAS $^1H/^{31}P$ probehead with gradient Z at 25 °C. A typical proton ^1H HR-MAS NMR spectrum consisted of 64 scans was performed with presaturation of the water peak. Each spectrum was phased and baseline-corrected using a polynomial function. We used around 5 mg of algal axis that was placed in a 4 mm zirconium oxide MAS rotor. Approximately 30 μL of D_2O was added into the rotor with the algal sample for ^2H field locking. The sample was placed in a rotor spinning around an axis, which is oriented at the so-called "magic angle" of 54°7 with respect to the magnetic field B_0. Best homogenization was obtained at a spinning rate of 5000 Hz. This resulted in a high-resolution NMR spectrum approaching the ones obtained with liquid samples, making spectra analysis possible. A statistical tool was applied to confront the spectra obtained through the NMR analysis. First, a matrix of absence/presence of the observed signals was realized to summarize the *in vivo* chemical diversity of the samples, from a qualitative point of view. We did not restrict our observations to a certain range of chemical shifts, as many signals varying among samples occurred from 1 up to 8 parts per million (ppm).

Proceeding manually in the construction of the matrix permitted to evaluate the correspondence of NMR signals between the spectra. We used the multiplicity (*i.e.* the shape of the signal, from singlets to multiplets) in addition to the chemical shift values to characterize a unique signal, as for some molecules chemical shifts are known to slightly depend on pH [25]. Consequently, we were able to find out several chemical signals shared by different species.

To analyze the qualitative data, a Multiple Correspondence Analysis (MCA) was carried out to highlight the differences and resemblances in the spectra. The matrix was used as input for the

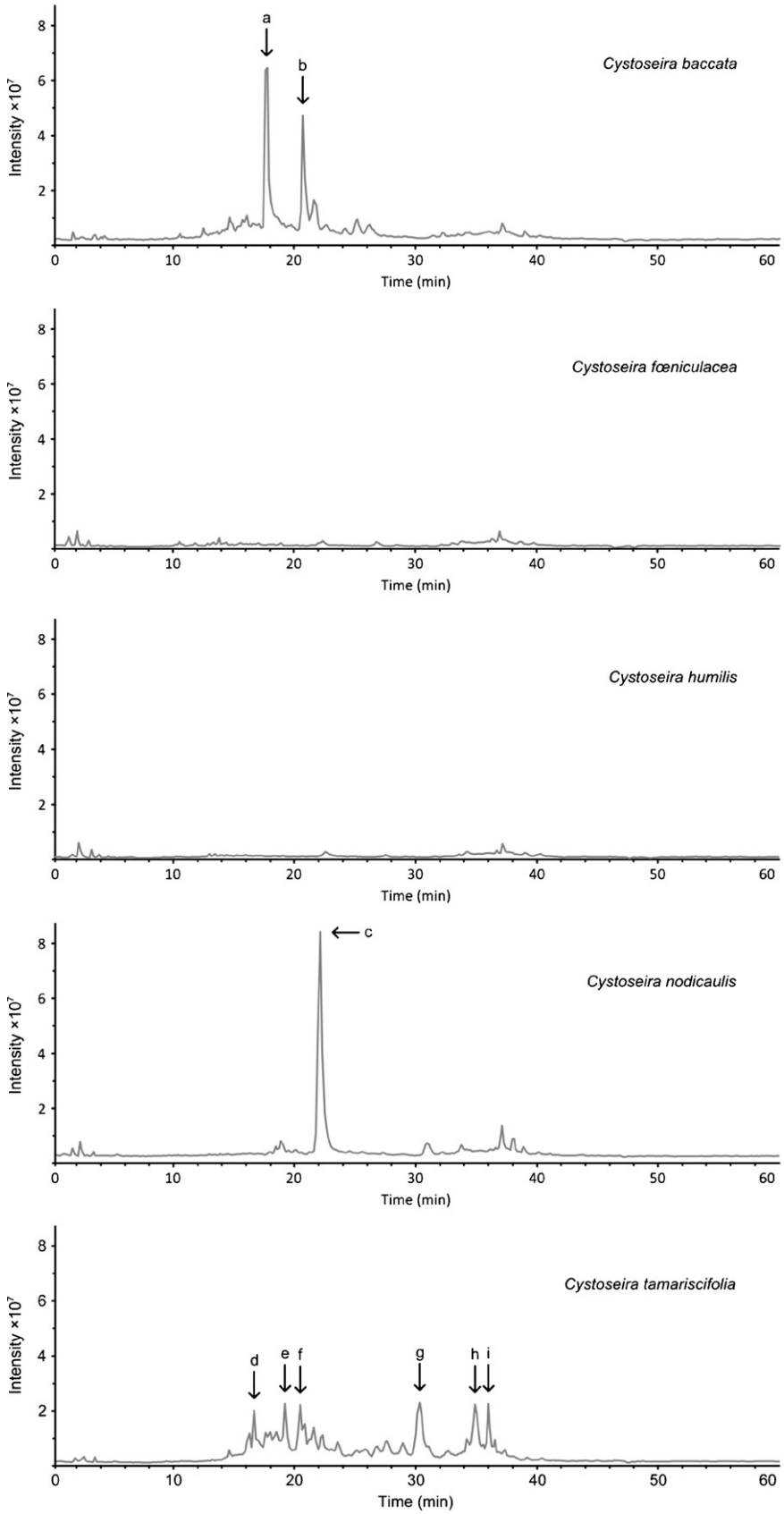

Fig. 2. Chromatograms obtained from the extracts of the *Cystoseira* species (+MS detection). The letters indicate the presence of some molecules, detailed in Table 1.

Table 1
Major compounds detected in the extracts of the *Cystoseira* species, associated *m*/*z* ratios and fragmentation profiles. Correspondence to the signals observed in Fig. 2 is indicated. *C. foeniculacea* and *C. humilis* not shown (no signal observed in their chromatograms).

Peak	Retention time (min.)	Molecular weight (g/mol)	+MS	+MS/MS	-MS	-MS/MS
Cystoseira baccata						
a	17.8	458	M_1: 481 $[M+Na]^+$ M_2: 441$[M-H_2O+H]^+$ M_3: 423 $[M-2H_2O+H]^+$	463 $[M_1-H_2O]^+$ 423 $[M_2-H_2O]^+$ 405 $[M_3-H_2O]^+$	M'$_1$: 457 $[M-H]^-$	442 $[M'_1-CH_3]^-$ 439 $[M'_1-H_2O]^-$
b	20.8	440	M_1: 463 $[M+Na]^+$ M_2: 423 $[M-H_2O+H]^+$	n.o. 405 $[M_2-H_2O]^+$	M'$_1$: 439 $[M-H]^-$	424 $[M'_1-CH_3]^-$
Cystoseira nodicaulis						
c	22.2	472	M_1: 495 $[M+Na]^+$ M_2: 455 $[M-H_2O+H]^+$	480 $[M_1-CH_3]^+$ 477 $[M_1-H_2O]^+$ 437 $[M_2-H_2O]^+$	n.o.	n.o.
Cystoseira tamariscifolia						
d	16.7	474	M_1: 497 $[M+Na]^+$ M_2: 425 $[M-H_2O-CH_3OH+H]^+$	479 $[M_1-H_2O]^+$ 465 $[M_1-CH_3OH]^+$ 407 $[M_2-H_2O]^+$	473 $[M-H]^-$	n.o.
e	19.3	440	M_1: 463 $[M+Na]^+$ M_2: 423 $[M-H_2O+H]^+$	445 $[M_1-H_2O]^+$ 405 $[M_2-H_2O]^+$	M'$_1$:439 $[M-H]^-$	421 $[M'_1-H_2O]^-$
f	20.4	472	M_1: 495 $[M+Na]^+$ M_2: 423 $[M-H_2O-CH_3OH+H]^+$	477 $[M_1-H_2O]$ 463 $[M_1-CH_3OH]$ 405 $[M_2-H_2O]$	n.o.	n.o.
g	30.2	440	M_1: 463 $[M+Na]^+$ M_2: 423 $[M-H_2O+H]^+$	445 $[M_1-H_2O]$ 405 $[M_2-H_2O]$	439 $[M-H]^-$	n.o.
h	34.9	454	M_1: 477 $[M+Na]^+$ M_2: 423 $[M-H_2O+H]^+$	445 $[M_1-CH_3OH]$ 405 $[M_2-H_2O]$	n.o.	n.o.
i	36.0	454	M_1: 477 $[M+Na]^+$ M_2: 423 $[M-H_2O+H]^+$	445 $[M_1-CH_3OH]$ 405 $[M_2-H_2O]$	n.o.	n.o.

n.o.: no observed.

MCA realized with the statistical software R [26] and its package FactoMineR [27].

1.4. Phylogenetic validation

DNA was fully extracted from the silica-gel conserved samples using a DNEasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany), following the furnished procedure. Extracted DNA was purified using Geneclean II Kit (MP Biomedicals LLC, Solon, OH, USA). As many studies proved the usefulness of the *ITS*2 sequences to investigate phylogenetic relationships at a low taxonomic level [18], and particularly within the family Sargassaceae [7,8,12,13], we chose 5.8S BF – forward – and 25BR2 – reverse – primers to amplify the ITS2 locus of our samples according to Yoshida et al. [28]. Amplification was realized according to Stiger et al. [13]. PCR products were purified using GeneClean II Kit, and sequenced by Macrogen (Macrogen Corp., Amsterdam, The Netherlands) in forward and reverse directions, using Big Dye Terminator Method. Additional sequences from other Sargassaceae algae were retrieved from the GenBank. The secondary structure of the *ITS*2 has been taken into account, as an efficient way to align ITS2 sequences using Bioedit software [29]. A phylogenetic tree was built under Mega 4 software [30] based on the Neighbor-Joining (NJ) algorithm. Sequences of some other Sargassaceae taxa (include species of the genera *Sargassum*, *Turbinaria* and *Myagropsis*) were used to enroot the consensus tree (outgroup). The relevance of the position of each node was assessed with bootstraps, obtained with 1000 replicates.

2. Results

2.1. LC/ESI-MS^n fingerprinting

Twenty chromatograms were produced from the extracted and analyzed samples. A very good reproducibility within each species was found. The 5 chromatograms presented at Fig. 2 are representative of the chemical profile of each of the 5 species. The first information is provided by the signal/baseline noise ratio. *C. baccata*, *C. nodicaulis* and *C. tamariscifolia* have high maximal intensities (respectively up to 6.10^7, 8.10^7 and 2.10^7), while the noise intensity is for the 5 species about 2.10^6. This results in an optimal signal/noise ratio (up to 40). This highlights the presence of major compounds, illustrated by high peaks on the +MS chromatograms and checked by ELSD detection. There are 2 clear compounds for *C. baccata* (retention times: 17.8 and 20.8 min), 1 for *C. nodicaulis* (22.2 min), and 6 for *C. tamariscifolia* (16.7, 19.3, 20.4, 30.2, 34.9 and 36.0 min). On the contrary, *C. foeniculacea* and *C. humilis* have comparatively smaller intensities signals (under 1.10^7), leading to poor signal/noise ratio (about 4). For both species, there is no clear specific signal in the 10–40 min range that could be investigated any further. The weakly detected molecules are fatty residues that have not been fully eliminated during the Solid Phase Extraction process. This is the reason why no other result is presented, considering *C. foeniculacea* and *C. humilis*.

In a second time, the graphical comparison of chromatograms indicates the existence of only 4 kinds of chemical profiles. Actually, all chromatograms are significantly different to each other, except the ones of *C. foeniculacea* and *C. humilis*, which show similar shapes. The *m*/*z* ratios at selected retention times confirm the impossibility to distinguish both species thanks to the only LC/ESI-MS^n analyses of their lipophilic extracts. In the case of the three other species, MS fragmentation pattern of the major compounds are detailed in Table 1. This way, LC/ESI-MS^n separated two groups: (1) *C. foeniculacea* and *C. humilis*, species showing no intense peaks and (2) *C. baccata*, *C. nodicaulis* and *C. tamariscifolia*, with several specific molecules associated.

2.2. In vivo NMR analyses

Twenty spectra, with little intraspecific chemical diversity, were obtained from the samples. Among them, only 5 spectra are presented and highlighted the interspecific chemical diversity (Fig. 3). Some clear signals, permitting unambiguous identification, can be

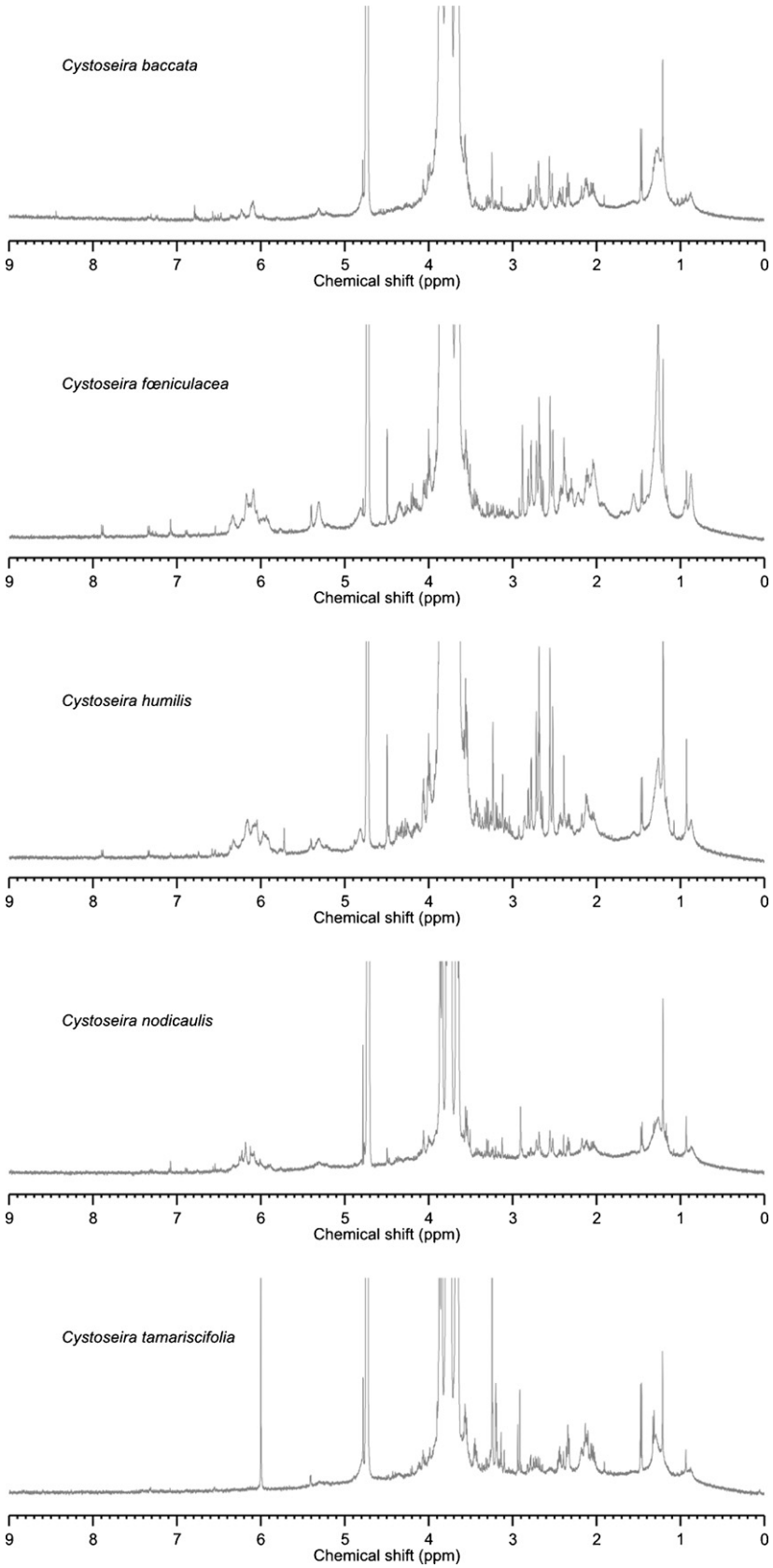

Fig. 3. ^{1}H HR-MAS NMR spectra of *Cystoseira* specimens collected in Brittany.

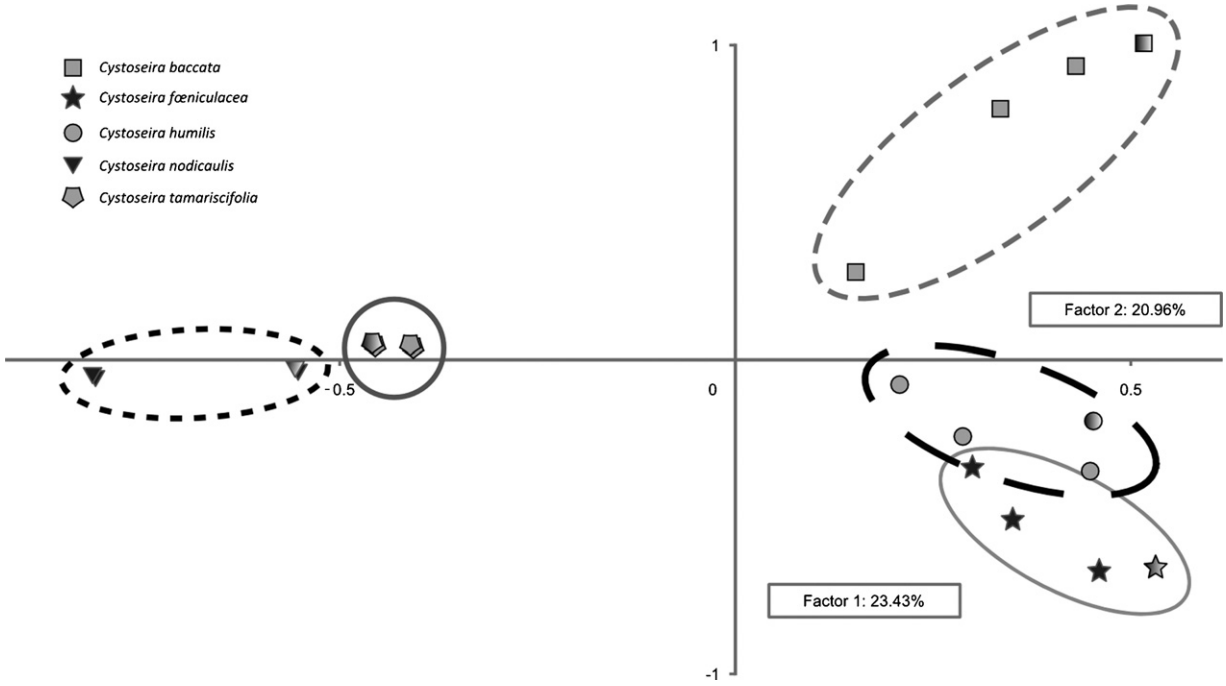

Fig. 4. Multiple Correspondence Analysis: projection of the 20 spectra obtained from the *Cystoseira* samples. Samples collected at Penmarc'h in plain color (15 points), others in shades of grey (5 points).

observed in the spectra of *C. nodicaulis* and *C. tamariscifolia*. As an example, for the first species, it is a singlet at 2.91 ppm. For the latter, one peak at precisely 6.00 ppm was characteristic of the species. *C. foeniculacea* and *C. humilis* are altogether characterized by the occurrence of two doublets of equal intensity at 7.90 and 7.36 ppm, among a wide variety of common signals. *C. baccata* shows the most important chemical diversity, but the presence of many signals permits constant discrimination to the other species. However, the absence of particular signals in the spectra of *C. foeniculacea* and *C. humilis*, associated with a slight intraspecific chemical diversity within both species, makes impossible their absolute discrimination using *in vivo NMR*.

All the information from NMR spectroscopy is compiled in the MCA graph (Fig. 4). In this scatter plot, clusters highlight the chemical domain for each species. The closer two points are on this plot, the closer their chemical composition as observed on their spectra is.

This statistical analysis highlights three issues about the ^{1}H HR-MAS NMR data: (1) *C. foeniculacea* and *C. humilis* are not different considering their global chemical composition; (2) among the five species, only four chemical profiles are observed: *C. nodicaulis*, *C. tamariscifolia*, *C. baccata*, and the couple *C. humilis*/*C. foeniculacea*; (3) *C. nodicaulis* and *C. tamariscifolia* appears as two chemically close species.

2.3. Phylogenetic analysis

Including gaps, the sequences of the five *Cystoseira* species plus 8 other Sargassaceae consist in 649 base pairs. Based on ITS2 sequences, the 5 *Cystoseira* species from Brittany are not monophyletic (Fig. 5); they are separated into two strongly supported clades. *C. foeniculacea* and *C. humilis* are strongly linked to each other (bootstrap of 100). *C. baccata* and *C. nodicaulis* also turn out to be phylogenetically very close species (bootstrap of 100), and form another clade with *C. tamariscifolia* and *Bifurcaria bifurcata* (bootstrap of 85).

So, the phylogenetic analysis revealed (1) a suspected polyphyly within the genus *Cystoseira*, underlined by two distinct clades in the tree: {*C. foeniculacea* + *C. humilis*} versus {*C. baccata* + *C. nodicaulis* + *C. tamariscifolia*} and (2) the strength of the relationships between *C. baccata* and *C. nodicaulis*.

3. Discussion

Both analytical methods employed in our study led to four chemical profiles among the five species. Moreover, *ITS2* sequences from the five *Cystoseira* species turned out to be all different (Fig. 5). This is not contradictory to the existence of five validated species, and concludes about the usefulness of the key identification proposed by Cabioc'h et al. [24].

3.1. Relevance of analytical methods in species discrimination

LC/ESI-MS^{n} analyses clearly indicated the presence of some major compounds in the chromatograms of *C. baccata*, *C. nodicaulis* and *C. tamariscifolia* (Fig. 2). These were characteristic of each species, as between the taxa, the "peaks" detected by the mass spectrometer differed by their retention time (*i.e.* polarity) and their *m*/*z* ratio (Table 1). Due to their chromatographic and MS data, these compounds could be identified as meroditerpenoids. These metabolites issued from mixed biosynthesis (mevalonate and shikimate pathways) were generally constituted by a diterpenic side-chain linked to a toluquinol moiety. Meroditerpenoids were commonly found as main constituents in the extracts of species belonging to the genus *Cystoseira* [4,5]. Based on their systematic occurrence in lipophilic extracts, considering different sampling sites and different sampling periods, we can suggest their use as chemotaxonomic markers of the *Cystoseira* species in Brittany. Further investigations on the mass spectral data in the chromatograms of *C. baccata* clearly identified the two major compounds detected (peaks **a** and **b**) as bicyclic meroditerpenoids, already described from Moroccan samples of this species (referred as compounds 1 and 2 in Mokrini et al. [31]). This identification has been unambiguously confirmed by injection of pure standards and by comparison of their retention time and mass spectrum with those of peaks **a** and **b** from this present study (Table 1). In the

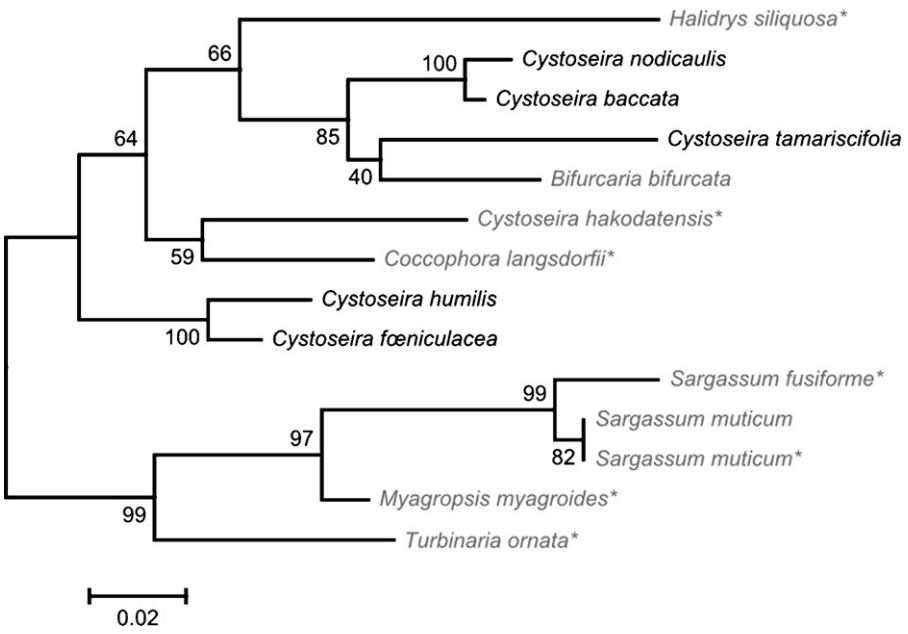

Fig. 5. Phylogenetic tree obtained using the Neighbor-Joining method on the ITS2 sequences. *Cystoseira* species in black, others in grey. * indicates sequences retrieved from the GenBank.

case of *C. nodicaulis*, the chromatogram showed only one major compound (peak **c**) while for *C. tamariscifolia* six main compounds (peaks **d**–**i**) were detected: their spectral data were in agreement with those of meroditerpenoid-related compounds with various levels of oxygenation and/or degrees of insaturation on the diterpenic part (*m*/*z* 474, 472, 454 and 440). For these two last species, further works are in progress to purify and elucidate the chemical structure of the main components of their extracts, and the assessment of their variations in space and time must be done to validate these compounds as chemomarkers. In the case of *C. nodicaulis*, such an analysis is of particular interest, as this species has never been the subject of any chemical study about its lipophilic content.

^{1}H HR-MAS NMR analyses managed also to give a characteristic chemical profile for these three species, with one homogeneous group per species in the Multiple Correspondence Analysis (Fig. 4). As a matter of fact, two species, *C. nodicaulis* and *C. tamariscifolia* supplied very precise information that could be regarded as discriminating factor on their spectra. For the first, a typical singlet occurred at 2.91 ppm. For *C. tamariscifolia*, an intense singlet at precisely 6.00 ppm indicated the possible occurrence of a simple phlorotannin. Glombitza et al. [32] isolated two simple phenolic compounds from this alga, *i.e.* bifuhalol and diphloretol, but none of their NMR chemical shifts did match with the ones obtained here. Identification of the products at the origin of this 6.00 ppm singlet is in progress, so as to evaluate its relevancy as a chemotaxonomic marker, at least for Breton samples of *C. tamariscifolia*.

However, neither LC/ESI-MS^{n} nor ^{1}H HR-MAS NMR was helpful to distinguish *C. foeniculacea* and *C. humilis*. This is particularly interesting and means that neither their precise lipophilic composition, nor their global chemistry differs from the one to the other. No really "original" compound is synthesized by these two species. This is in accordance with previous results dealing with chemical classification of *Cystoseira* species [22,4,5], where *C. humilis* was also characterized by the absence of lipophilic secondary metabolites.

3.2. Relevance of methods in species boundaries determination

The analysis of *ITS2* sequences highlights a possible polyphyletic status of the genus *Cystoseira* (Fig. 5). The five species of this "genus" are distributed within two separated clades. The first comprises *C. foeniculacea* and *C. humilis* while the second clade is composed, among other *Sargassaceae* species, of *C. tamariscifolia*, *C. baccata*/*C. nodicaulis*, and indicates a close relationship between the two latter species, which is in accordance with Rousseau et al. [15] where the same topology was observed using the LSU marker. So, it is necessary to check the taxonomic validity of the genus *Cystoseira*, including taxa from the Mediterranean Sea, Indian and Pacific Oceans. As we particularly focused on terpene derivatives throughout their mass spectral data and retention times using LC/ESI-MS^{n} analyses, at first sight this method can hardly be employed to determine species boundaries within the three species displaying clear information. At this step, the information summarized in Table 1 is only a way to distinguish species. Complementary investigations are required to make comparisons between the structures of the detected metabolites. However, LC/ESI-MS^{n} results can be regarded as absolutely consistent with the possible polyphyletic status of the genus *Cystoseira* highlighted with the molecular approach. The resulted phylogenetic tree clearly separates two strongly supported *Cystoseira* groups: "terpene derivatives producers" (3 species) versus "non lipophilic secondary metabolites producers" (2 species). The taxonomical value of this chemical separation is strengthen by the fact that *Bifurcaria bifurcata* R. Ross and *Halidrys siliquosa* (L.) Lyngb. are also known to be terpene producers [33,34,35], and are located in the tree with the *Cystoseira* species producing terpene derivatives (Fig. 5). Piatelli [22] proposed a classification of the genus based on the production of terpenes. According to him, *C. foeniculacea* can now be added to the "Chemical Group I", that includes all *Cystoseira* taxa which never synthesize lipophilic secondary metabolites.

We consider relevant to go on with chemical investigations for *C. nodicaulis*, in order to complete the chemical classification of the *Cystoseira* species. Regarding the close phylogenetic relationship between both species, it will be particularly interesting to evaluate whether *C. nodicaulis* will join the same group as *C. baccata*: the "Chemical Group V" (defined by Piatelli [22]), characterized by the presence of cyclic meroditerpenoids. The distinction of species using the terpene derivatives production criterion is taxonomically correct for the five Breton species of the genus *Cystoseira*. Investigations could be led to check whether the consistency between

chemical groups and phylogeny could be extended to the whole Sargassaceae family.

Concerning the ^{1}H HR-MAS NMR results, except the previously discussed similarities in *C. foeniculacea* and *C. humilis*, the clearest two others linked species revealed by the close position of the samples of both species in the MCA are *C. nodicaulis* and *C. tamariscifolia* (Fig. 4). This result is due to the presence of several signals shared by both species (used as input for MCA, data not shown). This is then contradictory to the phylogeny assessed by the *ITS*2 sequences, which revealed a stronger link between *C. baccata* and *C. nodicaulis*. A possible explanation of this phenomenon is that the two species must have in common particular cell structures, called iridescent bodies, which have been described in *C. tamariscifolia* (referred as *C. ericoides* by Pellegrini [36]) and *C. amentacea* (referred as *C. stricta* by Pellegrini [36,37]). These structures are responsible for the Purple-to-Green color of the thalli when immersed, often used as an identification criterion in taxonomy. Looking at *C. nodicaulis*, despite iridescence has also been described [38,39], to our knowledge no study has investigated the cellular origin of this phenomenon. Nevertheless, we can reasonably assume that it is the same as described for the other iridescent *Cystoseira* species. Iridescent bodies have been the subject of some electronic microscopy works. Despite this, the composition of these multi-layered objects has not been elucidated yet. For *Cystoseira amentacea* var. *stricta* Montagne, Pellegrini [36] concluded to a "proteic, polysaccharidic, and lipidic" composition. As these iridescent bodies are present throughout all the cells of the secondary axes, the molecules composing it must have particularly intense NMR signals, actively participating to the overall chemical spectra of the samples. This may be at the origin of the similarity of the chemical composition of both species underlined by the MCA results.

3.3. Interests of the developed methods

The two analytical methods appeared to be useful to discriminate the *Cystoseira* species in Brittany. Chemical composition turned out to be useful for such purpose. Furthermore, both techniques were robust face to spatiotemporal variations (unpubl. data). The inclusion of samples coming from different sites, collected at different periods of the year, did not change the result of the study. It seems that, for the five *Cystoseira* species studied, the chemical composition is constitutive for these organisms. However, among the five species, two of them, *C. foeniculacea* and *C. humilis*, could never be separated, due to a common chemical composition, notably characterized by the absence of "real" major lipophilic secondary metabolites. Nevertheless, consistent information was highlighted to describe both taxa. Considering (1) their high morphological similarities, (2) their very close chemical composition, in a whole, or more precisely concerning their lipophilic molecules, and (3) their tight relationship apart from the other *Cystoseira*, the two species form a highly homogeneous taxonomical duet. If the two chemotaxonomic methods did not detect a difference between both species, at least they revealed the strength of their relationships, confirmed by their phylogeny.

LC/ESI-MS^n proved itself to be a valuable technique for investigating the taxonomy of the genus *Cystoseira*. For such a method, results are easy to obtain and provide accurate information about the chemical composition of the extracts. It is a precious preliminary step to investigate secondary metabolites content and taxonomy, in the case of the genus *Cystoseira*.

For taxonomical purposes, the two methods display many advantages, compared to molecular techniques, in terms of manipulation time and cost. They can lead, depending on the species studied, to unambiguous identification of samples, within a few hours concerning ^{1}H HR-MAS NMR, and one day with LC/ESI-MS^n. This is to be compared to the time necessary for obtaining sequences from samples (more than one week at least, from the extraction of DNA to the obtaining of the phylogenetic trees). Moreover, molecular studies are frequently dependent on many factors that can increase the time gap before getting the sequences. Phylogenetic studies also require high-quality tissues for DNA extraction, e.g. receptacles with male gametes, so as to extract the maximum DNA possible. The use of strictly vegetative tissues can be useless, so the sampling should be done in accordance to the seasonality in the reproduction of the species studied. In addition, some metabolites (polysaccharides, phlorotannins . . .) can disturb the amplification of DNA during PCR. All these factors shall be taken into consideration before choosing a method or another. Finally, the major advantage for these techniques is the amount of supplementary information obtained, beyond the simple taxonomically oriented work. Indeed, these techniques also bring new data on the chemical composition within the algae, which is crucial when a screening of new molecules is started. As a comparative point of view, ^{1}H HR-MAS NMR, as a non destructive technique, can be valuable in order to get a "^{1}H identity card" of a biological sample, and LC/ESI-MS^n is crucial to investigate further on the chemical composition of the extracts. Both techniques appeared relevant in preliminary studies on the search of natural products, so a complementary purification and identification process is being developed to determine the nature of the chemical compounds occurring in the species of the genus *Cystoseira*.

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