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ARTICLE

Rapid Identification of Seaweeds in Food Products by PCR Combined with ALF-RFLP and FINS Methodologies

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In the present study, two methods for the genetic identification of the most important seaweed species used for human consumption were developed. Both are carried out through PCR amplification of an 18S rRNA gene fragment. The first one is based on the phylogenetic analysis of DNA sequences (FINS), while the second is based on length polymorphism and RFLP visualized by means of an ALF system. The main novelty of this work lies in the fact that it allows genetic identification of the main commercial species of seaweed. Moreover, the developed systems can be applied to all kinds of processed products, including those that have undergone intensive transformation, as for instance canned foods. These methodologies also permit the detection of species in complex matrixes where more than one algal species is present. The methods were validated using products manufactured in a pilot plant showing correct functioning. Finally, the methods were applied to 23 commercial samples including some that had been subjected to intensive thermal treatment, allowing the detection of those that were incorrectly labeled (30%). Therefore, these molecular tools can be used for clarifying questions related to the correct labeling and traceability of commercial products that include some seaweeds in their composition.

KEYWORDS: Seaweeds; Chlorophyta; Phaeophyta; Rhodophyta; genetic identification; fragment length polymorphism analysis; PCR-RFLP; seafood; FINS; ALF

1. INTRODUCTION

Macroalgae constitute a group of organisms that are usually classified according to their chlorophyll content associated with pigments, providing them with characteristic color and permitting their division into green (Phylum Chlorophyta), brown (Phylum Phaeophyta), and red algae (Phylum Rhodophyta).

The algae form a part of numerous culinary recipes typical for vegetarian gastronomy. In some countries from Central Europe, Japan, Korea, China, Chile, and Canada algae are integrated in daily nutrition. The increasing acquaintance to their nutritive and gastronomic values has brought about a permanent increase in their culinary uses and consumption. The new feeding habits facilitate nowadays the acquisition of algae for cooking purposes. Fresh algae are a very perishable product for which reason they may be found as dehydrated, salted, lyophilized, frozen, and canned products, and even as products such as biscuits and patés.

The increase in consumer's demand during the last 50 years has surpassed the capacity of the market offer based on natural wild reserves which involves the necessity for cultivation of the main species to be consumed. The seaweed industry provides a wide variety of products that have estimated a total annual value of 5.5−6 billion American dollars (*1*).

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Most importantly, marine algal species used for human consumption belong to the genera *Porphyra* (whose commercial denomination is nori), *Laminaria* (Kombu) *Undaria* (Wakame), *Palmaria* (Dulse), and *Chondrus crispus* (Irish moss) (*1*). Besides these, other genera exist that are being strongly introduced into European markets as Ulva. The increasing variety of offered products creates the need to provide detailed information to the consumer not only on the commercial denomination but also on the scientific name of each species included in commercialized products (*2*).

However, due to the processing to which algae are subjected, the morphological characteristics are removed, and the distinctive features disappear, making it difficult or even impossible to identify the species based on direct observation. In order to accomplish and check the correctness of the information distributed on products' labels, it is necessary to develop techniques permitting species identification independently of the grade of transformation which the product might have undergone. Thus, application of molecular techniques allows accurate identification of the corresponding species included in any product.

In the literature, different works exist on the identification of algal species through the application of molecular techniques. However, none of these studies treats the main species of algae that are used for human consumption today. For instance, the work of Antoine et al. includes 5 species of commercial interest that are identified using the ITS region (*28*). The prevailing part of

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Table 1. Species Included in This Work

filum	species	commercial name	samples	location[a]
Phaeophyta	*Himanthalia elongata*	sea thong	5	ESP, GBR
	Undaria pinnatifida	Wakame	5	JAP, AUS
	Laminaria saccharina	sugar kelp	4	AN, PN
	Laminaria hyperborea	Kombu	10	IRL, ESP, FRA
	Laminaria ochroleuca		3	GBR, ESP
	Fucus vesiculosus		5	AN, PN
	Fucus spiralis	Fucus	6	IRL, ESP, FRA
	Fucus serratus		2	AN
	Fucus ceranoides		4	POR
	Sargassum fusiforme	Hiziki	6	JAP, CHI
Clorophyta	*Ulva rigida*	sea lettuce	15	ESP, GBR, POR, JAP
	Ulva lactuca		7	ESP, GBR, POR,
	Ulva intestinalis	green nori	10	ESP, GBR, POR, IRL
	Codium tomentosum	Codium tomentosum	4	ESP, GBR, POR, ZAF
Rodophyta	*Palmaria palmata*	Dulse	8	AN, PN
	Porphyra purpurea		5	GBR, IRL, FRA, ZAF
	Porphyra tenera		3	CHN, JAP
	Chondrus crispus	Irish moss	6	ESP, FRA, POR, CAN
	Mastocarpus stellatus		3	IRL

[a] Location abbreviations: AN, Atlantic North; AUS, Australia; CAN, Canada; CHN, China; ESP, Spain; FRA, France; GBR, United Kingdom; IRL, Ireland; JAP, Japan; ; PN, Pacific North; PRT, Portugal; ZAF, South Africa.

published studies treats a low number of species (*3*−*6*) or are based on phylogenetic relationships within various groups (*7*−*12*); none of these studies meet the current needs.

For these reasons in the current study, two genetic methodologies based on the amplification of the 18S rRNA fragment were developed: the ALF (automated fragment analysis) system based on PCR following LP (length polymorphism) and RFLP (restriction fragment length polymorphisms); and the FINS (forensically informative nucleotide sequencing) technique, based on polymerase chain reaction (PCR) followed by phylogenetic analysis. Unlike previous works, this tool allows the genetic identification of the main seaweeds marketed around the world, independent of the treatment applied during the elaboration process. The developed tool will be of great help in controlling the correct labeling, improving the protection of consumers' rights, and avoiding unfair competition between industry operators.

2. MATERIALS AND METHODS

2.1. Sampling and DNA Extraction. Authentic alga samples were collected from the coastal regions from several locations around the world (**Table 1**). The identification of the specimens was carried out on the basis of morphological characteristics according to different bibliographic references (*13*, *14*). Once identified, these samples were preserved at −80 °C until DNA extraction. The number of samples of each species and the common names are shown in **Table 1**. Commercial samples were obtained from local markets (**Table 2**).

In order to obtain DNA extracts of high concentration and purity, an additional step preceding extraction was introduced. The following treatments were tested once the alga was washed with distilled water: (1) freezing with liquid nitrogen; (2) drying at 65 °C; and (3) lyophilization.

Following to the previous preceding steps, two methods were tested for total genomic DNA extraction from fresh, dried, and salted samples, starting from 30 mg of powdered algae: (1) a method based on silica gel columns using NucleoSpin Plant II (Macherey-Nagel), following the supplier's protocol with minor changes; (2) extraction of DNA using the standard CTAB-PVPP method as described by Cubero et al. with slight modifications (*15*). In the case of highly processed products, DNA was extracted from a piece of 150 mg of powdered algae.

The extracted DNA was visualized on agarose gels (Sigma) at 1% in TBE buffer with 5 μg/mL ethidium bromide (Sigma) under ultraviolet light using a Molecular Imager Gel Doc XR System transiluminator and the software Quantity One, version 4.5.2 (Bio-Rad).

The DNA concentration was determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific) by measuring the absorbance at 260 nm and the purity using the ratio of 260 and 280 nm (A_{260}/A_{280}).

2.2. Amplification and Sequencing of the PCR Products. Five sequences of the 18S rRNA were downloaded from the National Center for Biotechnology Information (NCBI) (accession numbers Z14142, AB235852, DQ317002, DQ317003, and AB425966). These were aligned with Clustal W integrated in the BioEdit 7.0 program pack (*16*) for primer design. The primer MYT 18S D, 5′-CAA CCT GGT TGA TCC TGC CAG T-3′ described by Santaclara et al. (*17*) was aligned with the sequences describes above, showing a good homology for DNA amplification. The reverse primer was designed by hand and subsequently checked with the software Primer 3. The name and sequence of the reverse primer is ALGA R, 5′-GAC GGT ATC TYA TCG TCT TCG A-3′.

All of the PCR amplifications were performed in a final volume of 50 μL containing 50 ng of DNA template or 200 ng in the case of commercial products undergoing thermal treatment. Moreover, 5 μL of 10× buffer, 2 mM $MgCl_2$, 0.4 μL of 100 mM dNTP, 4 μL of a 10 μM solution of each primer, and 1 unit of Taq-polymerase (Bioline) were added. The optimal primer annealing temperature (T_a) was experimentally determined using the temperature gradient function of the Bio-Rad iCycler iQ thermocycler. The cycling program was as follows: 95 °C for 3 min, 35 cycles [95 °C for 30 s, T_a for 30 s, and 72 °C for 30 s], followed by a final extension step of 72 °C for 3 min. The PCR products were mixed with 5 μL of loading buffer and loaded on 2% agarose gels (Sigma) at 1% in TBE buffer with 5 μg/mL of ethidium bromide (Sigma), to check the correct reaction of the PCR and size of the amplicon. Electrophoretic separation was performed at 90 V for 40 min, and the resulting DNA fragments were visualized in the conditions previously described. The size of the amplified PCR products was estimated by comparison with the molecular marker of 100−1500 bp (Dominion-MBL).

Next, these PCR products were purified with the Nucleospin Extract II kit (Macherey-Nagel), according to the manufacturer's instructions. The concentration and purity of PCR products were measured by means of a NanoDrop 1000 spectrophotometer (Thermo Scientific) in the conditions described above.

PCR products were sequenced with the primers used for amplification in an automatic DNA Genetic Analyzer (ABI Prism 3130 Genetic Analyzer) using the BigDye Terminator cycle sequencing kit v1.1 (Applied Biosystems) following the manufacturer's recommendations. The resulting electropherograms were analyzed with Chromas 1.45 software (*18*) and subsequently aligned with Clustal W, included in BioEdit 7.0 (*16*).

From these sequences, two new internal primers were designed: ALG H 5′-TGC AGT TAA AAM GCT CGT AGT-3′ and ALG L 5′-GCY TGC

Table 2. Commercial Samples Analyzed with the Methodologies Developed in This Work

Product	Declared species	Identified species
Dried seaweed[a]		
Kombu	*Laminaria ochroleuca*	*Laminaria* spp
Irish moss	*Chondrus crispus*	*Chondrus crispus*
Hiziki	*Sargassum fusiforme*	*Sargassum fusiforme*
Dulse	*Palmaria*	*Palmaria palmata*
Salted fresh seaweeds[a]		
Kombu	*Laminaria ochroleuca*	*Laminaria* spp
Sugar kelp	*Laminaria saccharina*	*Laminaria* spp
Wakame	*Undaria pinnatifida*	*Undaria pinnatifida*
Sea lettuce	*Ulva* spp.	*Ulva* spp.
Sea thong	*Himanthalia elongata*	*Himanthalia elongata*
Nori	*Porphyra umbilicalis*	*Porphyra* spp
Irish moss	*Chondrus crispus*	*Chondrus crispus*
Seaweed powder[a]		
Sugar kelp	*Saccharina latissima*	*Laminaria* spp
Nori	*Porphyra purpurea*	*Porphyra purpurea*
Seaweed salad[a]		
Japanese stile	-	*Himanthalia elongata*
		Laminaria spp
		Undaria spp
In olive oil	-	*Himanthalia elongata*
		Laminaria spp
		Undaria spp
In vinaigrette	-	*Himanthalia elongata*
		Laminaria spp
		Undaria spp
Seaweed tartare[b]		
Natural	-	*Himanthalia elongata*
		Laminaria spp
		Undaria spp
Sea urchin[b]		
Sea urchin roe with seaweed	-	*Undaria* spp
Seaweed tea[a]		
Tea with kombu	-	*Laminaria* spp
Noodles[b]		
Noodles with sea lettuce	*Ulva* spp.	-
Natural seaweeds preserved in glass jar[b]		
Wakame	*Undaria pinnatifida*	*Undaria* spp
Natural sea thong	*Himanthalia elongata*	*Himanthalia elongata*
Natural seaweeds canned[b]		
Mussels with seaweeds in white pickling brine	Wakame	*Undaria* spp

[a] DNA extraction from 30 mg of powdered algae. [b] In the case of highly processed products, DNA was extracted from a piece of 150 mg of powdered algae.

TTT GAA CAC TCT AA-3′. Next, PCR products were obtained, visualized, cleaned, and sequenced in the same conditions as those reported previously. The size of these amplified PCR products was estimated by comparison with a 50 bp ladder (GE Healthcare).

2.3. Development of an Identification Methodology. *2.3.1. Development of the FINS (Forensically Informative Nucleotide Sequencing) Methodology*. Phylogenetic analyses were carried out using the software Mega 4.0 (*19*). The genetic distances among the obtained sequences were estimated using the Kimura-2 parameter evolution model (*20*), and the inference of the phylogenetic tree was carried out with the neighbor-joining method (*21*). The degree of confidence assigned to the nodes in the phylogenetic trees was estimated by bootstrapping with 2000 replicates.

Also, the identity of PCR products was confirmed by Basic Local Alignment Search Tool (BLAST) from the database of National Center for Biotechnology Information (NCBI). Similarly, the seaweeds sequences herein obtained and those present in the NCBI database were stored in a local database to carry out a local blast using the tool present in the BioEdit software (*16*).

2.3.2. Development of an Identification Methodology Based on the Automated Fragment Analysis (ALF) Combined with RFLP. In order to carry out the identification in complex samples where more than one species is present and no direct sequencing is possible, a methodology based on ALF-RFLP was developed. For the purpose, the forward primer, ALG H, was labeled with D2 dye (WellRED dyes, Sigma). The length of PCR products amplified with internal primers was determined by capillary electrophoresis in CEQ 8800 Genetic Analyzer (Beckman Coulter). This analysis allows the detection of PCR products and the estimation of the size of the amplicons calculated from a DNA size standard 400 (Beckman Coulter) labeled with WellRED dye D1.

Samples with the following composition were loaded: 2 μL of the PCR product, 0.5 μL of the DNA size standard 400, and 30 μL of sample loading solution (SLS) (Beckman Coulter). The samples were injected for 30 s at 2.0 kV and separated for 35 min at 6.0 kV.

From the sequences obtained with the primers ALG H and ALG L, of all seaweed species studied herein, restriction maps were constructed using WEBCUTTER 2.0 software (*22*). Two restriction enzymes that generate specific restriction profiles for each species were selected. Digestions were carried out separately overnight in a final volume of 20 μL containing 2 U each of enzyme and 100 ng of PCR product without purification.

Digested PCR products were visualized by the ALF system previously described.

2.4. Methodological Validation. The aim of this process was to evaluate the correct performance of the proposed methodology for all kinds of transformed foodstuffs. For this purpose, it was necessary to check whether any treatment factor to which the products are subjected during processing has influence on the correct application of the methodologies. The reference individuals (individuals of different species authenticated on the basis of their morphological traits) were used to manufacture products corresponding to the principal commercialized formats (salted, dried, and canned) in the pilot plant of CECOPESCA (Spanish National Centre of Fish Processing Technology). The products prepared contained only one or different combination of algae species. The most extreme treatment applied to the canned samples was the sterilization in a horizontal retort steel-air at 115 °C for 50 min, with 1.2 bar of overpressure (jar of 150 mL). In order to obtain salted samples, these were covered with salt and left for the elimination of the brine formed. The samples were kept in salt for 3 to 4 weeks depending on the thickness and size of the seaweeds. Afterward, the samples were desiccated at an air speed of between 60 and 90 m/min, a temperature of 25 °C, and a relative humidity of 50−55%. The time of desiccation of the samples varied depending on their thickness.

The coincidence percentage between the species identified on the basis of morphological traits and the genetic methodologies herein developed was calculated to establish the specificity of the method.

2.5. Application to Commercial Samples. Once the methods were validated, these were applied to 23 commercial products characterized by their content of one or more algal species as announced on the label. These products were purchased in supermarkets and shops from Spain with the aim of evaluating their correct labeling.

3. RESULTS AND DISCUSSION

3.1. DNA Extraction. Successful extraction of DNA from seaweeds is often problematic due to the presence of cellular components after the extraction process, mainly polysaccharides and polyphenols which interfere with DNA isolation procedures. Polysaccharides are problematic because they coprecipitate with DNA after alcohol addition during extraction and form highly viscous solutions (*23*). They are the main source of DNA contamination and can also inhibit Taq DNA polymerase activity (*24*). Polyphenols on their side inhibit protein catalysis, thus affecting DNA extraction. Therefore, the elaboration of DNA extraction protocols containing additional steps for polysaccharide and polyphenol removal is necessary.

Different methods exist in the literature for this purpose. For example, the commercial DNA extraction kits for plants that exist in many cases permit one to obtain adequate yields but are much more expensive than other methods, and the lack of information on their components does not allow any optimization. Methods such as the Chelex resin (*25*), lithium chloride (*26*), or the pectinase protocol (*27*) are used, but DNA of very low quality is obtained.

The most viable option to extract DNA from alga is the CTAB method, which yields PCR quality DNA. It is the most universal method used in seaweed DNA extraction as pointed out different authors (*5*,*6*,*8*,*11*,*28*−*30*). Most of the researchers have designed different modifications to CTAB procedures due to particular problems they have encountered with the application of the original CTAB method (*31*).

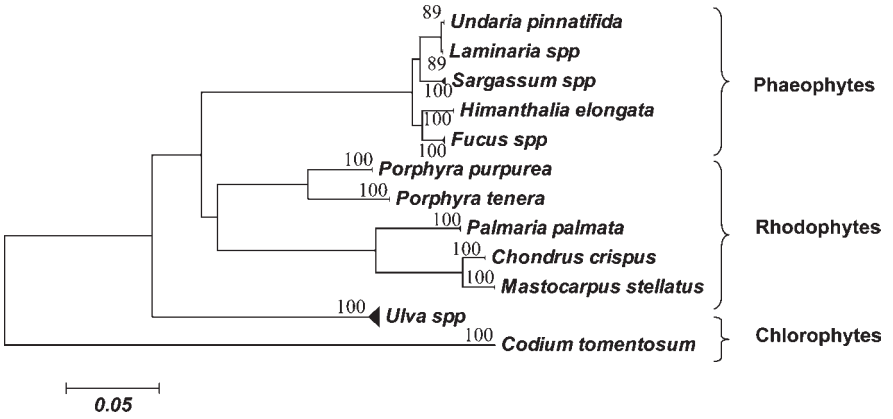

Figure 1. Phylogenetic tree showing the relationships among the studied seaweed species, carried out from the alignment of 192 bp (Primers ALG H and ALG L) of the 18S rRNA gene.

In this work, two methods were tried to evaluate DNA extraction from algae samples. The first of them was the NucleoSpin Plant II (Macherey-Nagel) and the second the CTAB-PVPP method. The second method showed better results because higher quantity and better quality of DNA were obtained according to spectrophotometric measurements (data not shown). This method eliminates the problematic compounds present in plant samples. The CTAB prevents coprecipitation of DNA with polysaccharides, and PVPP eliminates polyphenols. Therefore, the mentioned method was chosen for DNA extraction from the samples herein analyzed, for the methodological validation and for commercial samples.

Another problem often encountered during the realization of the extraction is the low DNA quantity obtained. This is most often due to the ineffective breaking of cell walls impeding the release of nucleic acids from the cell. For this reason, in this study the introduction of a preceding step previously to the DNA extraction was evaluated with the following three alternatives: freezing in liquid nitrogen, drying at 65 °C, and lyophilization. The yields of DNA extracted through these 3 operations were compared. Tissues frozen in liquid nitrogen yielded about 2-fold more DNA than those dried or lyophilized. The higher yield obtained by freezing was probably due to more effective tissue disruption.

3.2. Amplification and Sequencing of PCR Products. A region of the 18S rRNA was amplified in all studied samples (**Table 1**). This molecular marker was selected for its high degree of conservation, which facilitated the design of a primer set permitting the amplification in all of the species independently of their evolutionary distance. This allows the application of the methodology to species from different Phyla (Chlorophyta, Phaeophyta, and Rhodophyta). Moreover, interspecific variability contained in this genomic region is enough to allow genetic identification (Hd was 1), and an intraspecific one has not been detected (the intraspecific haplotype diversity (Hd) was 0). These features make the used molecular marker very suitable for the genetic identification of seaweed species.

All of the PCR products amplified showed a size between 970 and 1013 bp. The sequences obtained were submitted to the NCBI database (accession number FJ966251 to FJ966264). From the above DNA sequences two internal primers were designed (ALG H and ALG L). This primer set generated an amplicon comprising between 162 and 192 bp in all the species tested (**Figure 2**). In this way, these primers made possible the identification at least to the genus level of all species since the DNA obtained from the commercial preparations is partly degraded in fragments of approximately 200 bp (*32*, *33*). In this sense, the primer set designed in the present work was appropriate for use in such cases.

3.3. Development of an Identification Methodology. *3.3.1. Development of the FINS Method.* The FINS technique, described by Bartlett and Davidson (*34*), was evaluated because it was used for the genetic identification of species in a broad spectrum of organisms (*35*−*39*). Their main advantage is that it uses the information of all nucleotide positions of the amplified DNA fragment in comparison to RFLP or other techniques, which only assess a low number of nucleotides. The basis of this technique lays in the comparison of sequences of unknown samples with regard to sequences of pattern species. The analysis includes two steps: the calculations of genetic distances between sequences obtaining a distance matrix and the construction of a phylogenetic tree, which allow the genetic identification of species since samples belonging to the same species are grouped into the same clade. In this work, from the two distance matrices (Fragments Myt 18S D/ALGAR and ALG H/ALG L without primers), two phylogenetic reconstructions were performed using the neighbor-joining method, showing that both fragments yielded identical topologies. Samples belonging to the same species were grouped into the same cluster in both phylogenetic trees (**Figure 1**).

The bootstrap method was used to define the support of the different groups obtained in the phylogenetic tree. It has been calculated that bootstrap values higher or equal to 70% usually correspond to a probability higher or equal to 95% that the corresponding cluster is real (*40*), giving a quantitative measurement of the certainty of the assignment of a sample to a particular species. The nodes supported at species level show values higher than 89%. These values reflect the robustness of the proposed method (**Figures 1** and **2**).

These phylogenetic analyses classify the seaweeds into three large tribes: Phaeophytas (brown seaweed), Rodophytas (red seaweed group), and Clorophytas (green seaweed group). The Phaeophytas included two different orders: Laminariales, represented by the species *Undaria pinnatifida* and *Laminaria* spp., and the order Fucales with the species *Sargassum* spp., *Himanthalia elongata*, and *Fucus* spp. Among the Rodhophytas we can clearly observe two branches which represent two different classes, Bangiophyceae with the genus *Porphyra* and the class Florideophyceae which is divided into two orders: Palmariales, represented by the species *Palmaria palmata*, and Gigartinalis where *Chondrus crispus* and *Mastocarpus stellatus* are closely related. The phyllum Chlorophytas represented by *Ulva* and *Codium* are distantly related having as the only common feature their belonging to the green algae group.

An alternative to FINS is the BLAST analysis. BLAST analysis is a suitable technique to find regions of local similarity between sequences and can even be a suitable technique to identify species. This method is similar to the FINS since it uses DNA sequences and a database and the creation of local personalized

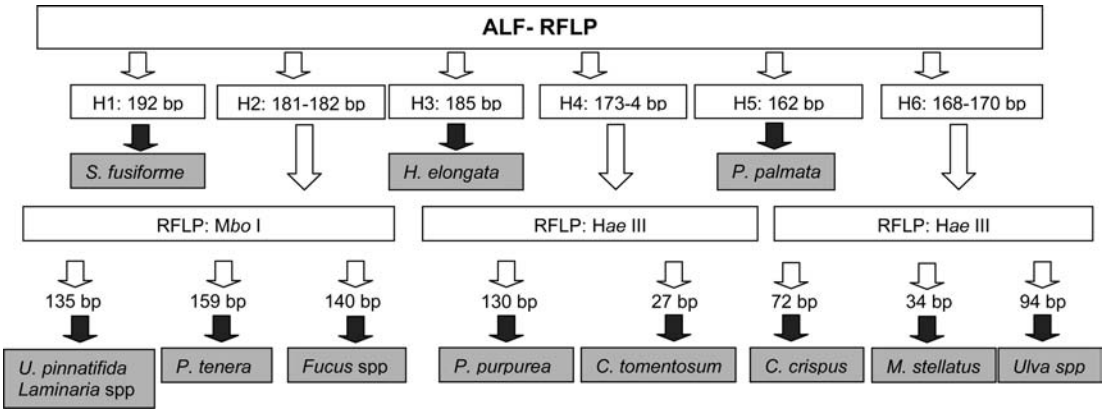

Figure 2. Diagram of the ALF-RFLP methodology used in the genetic identification of seaweed species.

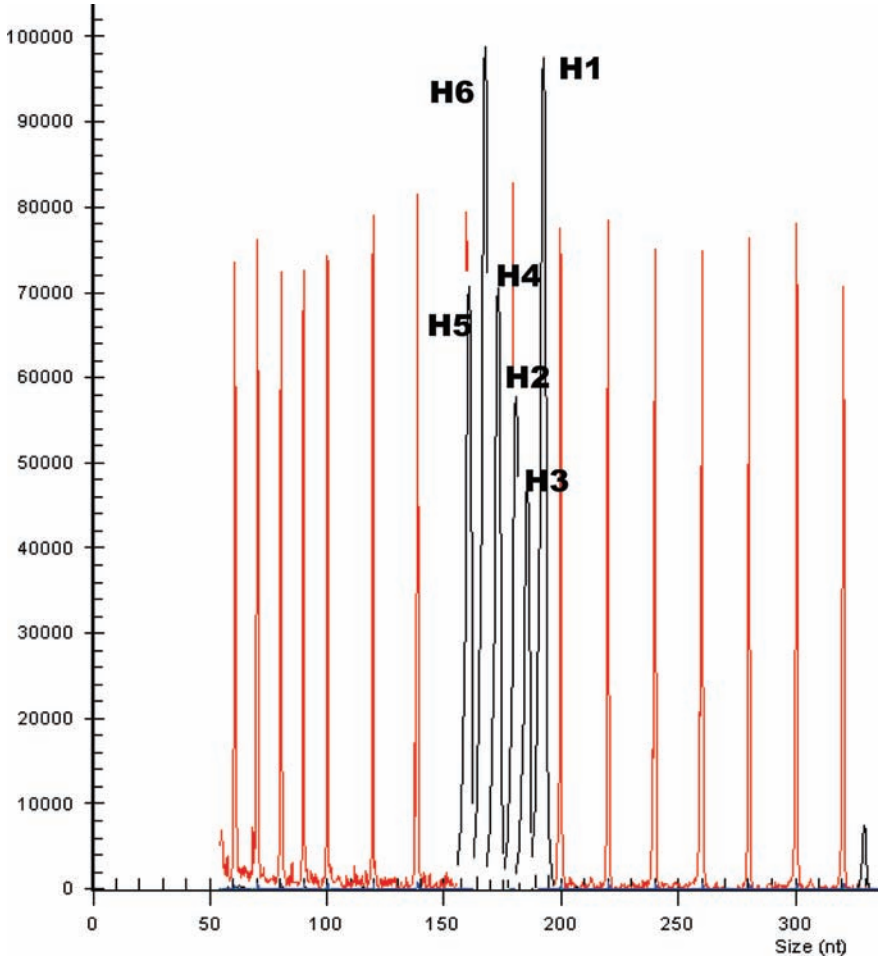

Figure 3. Electrochromatograms obtained in CEQ 8800 Genetic Analyzer (Beckman). Haplotypes of seaweeds included in this work (H: haplotype). H1, 192 bp (*S. fusiforme*); H2, 181/2 bp; H3, 185 bp (*H. elongata*); H4, 173/4 bp; H5, 162 bp (*P. palmata*); H6, 168−170 bp.

database including unpublished sequences obtained by the authors is possible. Specifically, the MEGABLAST search available at NCBI was applied in the current study to assign any seaweed DNA sequence to a particular species. The phylogenetic assignments generated by the proposed FINS technique were compared to the results obtained by BLAST. The same results of the species assignment were obtained (data not shown). Therefore, these two techniques could be used to identify the species herein studied.

3.3.2. Development of an Identification Methodology Based on the Automated Fragment Analysis (ALF) System Combined with RFLP Analysis. Capillary electrophoresis of fluorescently labeled PCR products combined with RFLP analysis was tested as a method for the identification of the all seaweed species included in this work.

The development of this methodology was necessary due to the existence of marketed products containing mixtures of different algal species, for which the species identification by sequencing would have been impossible. The ALF system besides allowing the estimation of the size of the PCR products with great precision, reproducibility, simplicity, and ease of standardization and automation makes possible the identification of one or more seaweed species in a sample through the detection of each PCR product belonging to a determined species.

The forward primer ALG H was labeled with WellRED D2 dye to make possible PCR product detection. The visualization of the PCR products obtained with the ALG H and ALG L primers by this technique permits the determination of their sizes with great precision. The ALF technique allowed detecting 6 haplotypes depending on the length polymorphism (**Figure 3**). Three of them belong to *Himanthalia elongata*, *Palmaria palmata*, and *Sargassum* spp. This polymorphism differentiation is not possible by conventional agarose gel electrophoresis, which lacks sufficient resolution.

For differentiation of the haplotypes corresponding to more than one species development of a suitable RFLP technique was necessary, a technique which has also been used in other studies of species identification of seaweeds (*28*, *41*). The restriction enzymes Mbo I and Hae III (New England Biolabs) were selected because they generated different specific restriction profiles. The PCR products obtained were digested with the selected restrictases, allowing the identification of the seaweed species. The DNA fragments generated in the digestion were loaded in the genetic analizer, and the obtained profiles matched in all cases with theorics restriction map (**Figure 4**).

In the case of products susceptible to containing mixtures of species, more than one haplotype might be detected at the first step, a result directly implying the presence of various species. If complex hapolotypes are obtained, corresponding to more than one species, the application of RFLP would permit the identification of these species.

The ALF-RFLP approach is an alternative to sequencing and offers the advantages of being simple, cheaper than other techniques such as sequencing, and being especially useful for routine analysis of a large number of samples. Moreover, it is faster than sequencing because it does not require a second thermal cycler reaction to produce sequenceable PCR products or subsequent purification of sequences. Moreover, direct sequencing is not possible in complex samples with more than one species present, and ALF-RFLP is the unique alternative to determining the identity of all of the seaweed species included in this work.

The absence of intraspecific variability in the 18S rRNA region reinforces the utility of the ALF−RFLP method since the restriction targets and are stable and characteristic of the restriction profiles for each species.

3.4. Methodological Validation. Different products were prepared simulating the conditions used by the food industry. In the pilot plant of CECOPESCA, this approach is useful to optimize the conditions of the developed methodologies and asses their correct functioning.

The standard individuals underwent several transformation processes, evaluating the influence of these variables on the genetic methods herein proposed. The results obtained from the application of the FINS and ALF-RFLP methods developed on the processed products (salted, dried, and canned) were in agreement with those from the morphological characterization. For the products that contained more than one species, such as the salads or seaweed tartare where the algae had been cut into small pieces, the possibility to carry out genetic identification through FINS was not viable. Therefore, the agreement between the results obtained by morphological characterization and from the ALF-RFLP method was checked in such cases, and a correspondence of 100% was found.

3.5. Application to Commercial Samples. The FINS and RFLP-ALF methods developed in the present study were applied to 23 commercial samples of seaweed products. The sequences obtained for all commercial samples were identical to the corresponding standard specimens.

In 69.5% of most of the products where the label reflected the species included, the labeling was correct (there was a correspondence between the species contained in the package and the species indicated). Only one incorrect label existed, which was due to an error in the scientific designation of the species: *Saccharina latissima* instead of *Laminaria saccharina*. Also 6 samples did not contain the specific name of the alga included. However, in one sample it was not possible to identify the species present due to the complexity of the matrix (noodles).

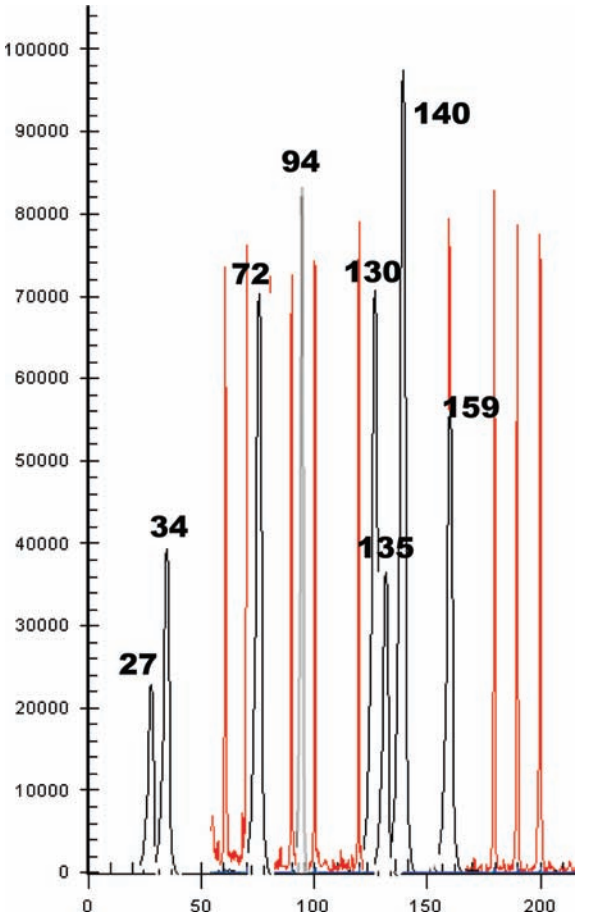

Figure 4. Electrochromatograms obtained in CEQ 8800 Genetic Analyzer (Beckman). Haplotypes of RFLP from PCR-amplified species of seaweeds included in this work. Fragment obtained from the digestion with Mbo I of PCR-amplified 135 bp (*U. pinnatifida* and *Laminaria* spp.); 159 bp (*P. tenera*); and 140 bp (*Fucus* spp.). Fragment obtained from the digestion with Hae III I of PCR-amplified 130 bp (*P. purpura*);27 bp (*C. tomentosum*); 72 pb (*C. crispus*); 34 bp (*M. stellatus*); 94 bp (*Ulva* spp.).

The specific assignments by means of both techniques FINS and RFLP-ALF were in accordance in all cases whenever these methods could be applied. As was established during the methodological validation, in commercial products containing more than one species only the RFLP-ALF was applicable.

Altogether, this work describes the development and validation of two PCR methods to detect and identify the algal species included in a wide range of foodstuffs. Among the advantages of these techniques, it is worth highlighting that these are reliable and sensitive, allowing to detect trace amounts of algae in processed products. Therefore, it can be useful for the verification of the labeling rules and the protection of the consumer's rights.

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