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ARTICLE

Codon Usage of Chloroplast Gene *rbc*L in *Laurencia sensu lato* (Rhodophyta) species

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ABSTRACT

Recent advances in molecular biology make it possible to sequence not only genes or genomes, but also to understand codon dynamics. For the organelle genes of these organisms, a small set of preferred codons are used for encoding proteins. For the first time, this paper treats the divergence of synonymous codon usage and its bias in the *rbc*L gene within the *Laurencia* complex of red algae. We observed that the synonymous codon preference biases in *rbc*L are large and differ among species. A clear distinction in codon usage between genera is evident: the genera *Dasya* and *Delesseria* use a set that fluctuated between 53 and 58 codons. Whereas, in the genera *Ceramium, Chondrophycus*, *Chlamydomonas*, *Chlorella*, *Laurencia s.s*, *Osmundea*, and *Palisada* codon usage indicates a higher restriction fluctuating between 40 to 51 codons. *Laurencia* complex genera and other representative algae showed a defined composition pattern, with lower percentage values of NNC/G (7-24.9%). *Dasya* and *Delesseria* showed a selective pattern tendency because of high percentage values of NNC/G (54-55%). The estimated codon bias parameters were tested to infer systematic relationships and match suitable codons with the NNC / G codon percentages. Cluster analysis based on Codon Usage supports phylogenetic relationships between *Chondrophycus*, *Palisada*, *Laurencia*, *Osmundea*, and *Yuzurua* species.

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1. Introduction

All the complexity of life is organized based on the simplicity of three nitrogenous bases known as codons. Genetic code is considered degenerate, since there is more than one codon that codes for an amino acid. Of the 64 codons, three are considered stop and one start. The genome has a particular coding strategy accordingly. Synonym codon bias is reported for countless genes within the enormous diversity of known organisms, but the patterns and the biological basis of codon choice in plants and particularly in algae are not been well understood [1,2]. At the interspecific or intraspecific level, codon bias can affect gene expression, as well as their length [3]. [4], hypothesized that codon usage is genome specific and related to taxonomic order. The strength of codon usage bias in a gene can be used to make predictions about its expression level [5]. Besides helping with taxonomy and phylogeny, codon usage bias can be helpful in the detection of lateral gene transfer events [6]. [7], in the same way, the phylogeny of individual genes also considers the impact of codon bias on the configuration and strength of nodes in those phylogenies. [8], suggest that codon bias has a role in protecting protein properties that ensure thermostability in thermophilic organisms. The use of synonymous codons in bacteria, yeast, and higher eukaryotes has been extensively analyzed [9]. Recently several higher plant genes had been sequenced to be able to draw significant conclusions on their codon usage [10]. Particularly in algae, there are some published studies on codon usage of chloroplast, mitochondrial and nuclear genes in the genera *Chlamydomonas* C.G. Ehrenberg [11], *Chlorella* M. Beyerinck [12], *Gracilaria* Greville [13], *Griffithsia* C. Agardh [14], and *Porphyra* C. Agardh [15].

Two important factors, with relative significance for different species, contribute to codon bias. The first, the bias of the genome structure that produces a bias in the degenerate positions of the coding sequences. [16], and the second is the choice of coding sequences for specific codons ("so-called optimal codons") that correspond to abundant tRNAs, thus improving their translation efficiency [17]. Although most plant chloroplast genes appear to have a composition pattern of codon usage, there is evidence for weak selection acting on codon usage to enhance protein synthesis [11]. The most distinctive difference between the two patterns of codons usage is that selection favors C at the third position of NNY (Y=C+T) synonymous groups, whereas the composition bias is towards T [18]. A second less noticeable difference between the two patterns is that selection favours T at the third position of four-fold degenerate groups so that, even though the composition bias results in a high T content, it is increased further by selection [11,18]. Both of these features appear to be an adaptation to match the 31 tRNA genes of the chloroplast that are available for translation [11], pointed out that selection acts with different intensities in different lineages and is correlated with the levels of gene expression. In general, selection seems to be stronger in algae than in higher plants, but within algae the selection is strongest in green algae [19]. The intensity of the preference varies widely, being higher in Chlamydomonas, intermediate in *Odontella sinensis* (Greville) Grunow but weak in *Porphyra purpurea* (Roth) C. Agardh.

Base usage bias on synonymous sites is also correlated with gene activity level [20] probably as a response to the RNAs transference content bias that mediates translation. [21] considered that the chloroplast genome of *Marchantia polymorpha* L. has 31 preferred codons, and that these preferred codons correspond to the 31 tRNA species encoded by the liverwort chloroplast genome, which are nearly the same as the tRNA species encoded in the tobacco chloroplast [22]. [23], carried out similar studies in algae groups and predicted the number of the tRNAs species in chloroplast and mitochondrial genes. Since the codon frequency of chloroplast genes is related to the concentrations of tRNA species in the chloroplast, the tRNAs encoded by the organelle genome appear to be adequate to support protein synthesis by acting as are acceptors for all codons [23].

Many explanations for extreme codon bias have been presented, for example: including mRNA stability, optimization of mRNA secondary structure, refinement of codon interpretation context, and optimization of codon-anticodon energy interaction [10,24]. However, how these or other factors operate differently in monocots and dicots is not obvious, as is that in algae. Both the underlying driving force that results in the extreme codon bias found in some monocot genes and its utility in plant metabolism and development are unclear at this time.

The objective of this article is to have a more general appreciation of codon bias in a segment of red algae (the *Laurencia sensu lato* complex) and to compare this codon usage with other representatives of algae to explore the usefulness of codon usage for systematic studies of the complex.

2. Materials and Methods

All of the *rbc*L nucleotide sequences included in this study were obtained from the National Center for Biotechnology Information (NCBI) database and around 30 percent of the sequences were obtained in the present work). Several Codon Bias measures were calculated for inter-generic and inter-specific levels: 1) Relative use of synonymous codons (RSCU). RSCU values are a measure of the use of codons seen with respect to the desired value if there was an equivalent use of synonymous codons, they were calculated as in [25], 2) Association on the basis of the use of codons. The codon use model of the *rbc*L gene of different taxa was compared by examining unweighted pair groups with arithmetic mean (UPGMA) using distances calculated using the methods of [26] using the SPSS v.13.0 program, which compares genes solely on the basis of codon usage similarity. 3) *Scaled* X^2 (Schi^2). This is a measure of codon bias derived from the deviation from equal usage of degenerate codons. It is calculated as in [27] for each taxon. 4) *GC Percentages.* GC percentages of the whole gene were calculated using the DnaSP program [28], 5) *Effective Number of Codons (ENC)*. This measure quantifies the "effective" number of codons that are used in a gene. It is calculated as in [29], 6) Codon Bias Index (CBI) is a dimension of the deviation from the similar use of synonymous codons and is calculated as in [30].

Inter-generic statistically significant differences were determined for the following parameters: RSCU, Schi^2, percentages of total codons encoded by NNC/G, ENC, CBI, Used Codons and Preferred Codons by a Nonparametric Kruskal-Wallis ANOVA Test, with 95% of confidence. In cases in which statistically significant differences were found, an SNK *post hoc* comparison test was carried out. Both analyses were executed in the statistical package SPSS v.13.0.

3. Results and Discussion

The codon usage guide of the *rbc*L gene was estimated and are presented in (Table 1) for two green algal genera: *Chlamydomonas* and *Chlorella*, and five red algae genera of the *Laurencia* complex: *Chondrophycus* (J. Tokida *et* Y. Saito) D.J. Garbary *et* J.T. Harper, *Laurencia* Lamouroux *sensu stricto*, *Osmundea* Stackhouse, *Palisada* K.W. Nam and *Yuzurua* (K.W. Nam) Martin-Lescanne (Figure 1). The results obtained indicated that the chloroplast genes of green algae and *Laurencia* complex representatives have a preference for codons ending in A or U. *rbc*L sequences in green algae are rich in A and T [31,32], because of this, the genes located in regions rich on those nucleotides showed

Table 1. Comparison of Codon Bias Usage in *rbc*L for green algae and *Laurencia* complex with emphasis in preferred codons.

Amino Acid[a]	Chlamydomonas[b]	Chlorella	Laurencia s.s	Osmundea	Palisada	Chondrophycus	Yuzurua
Preferred codon							
Leu [6]	UUA/CUU/CUA	UUA/CUU	UUA/CUU/CUA	UUA	UUA	UUA/CUA	UUA/CUA
Arg [6]	CGU	CGU	CGU/AGA	CGU/AGA	CGU	CGU/AGA	CGU/AGA
Ser [6]	UCU/UCA	UCU/UCA/AGU	UCU/UCA	UCU/UCA	UCU/UCA	UCU/UCA	UCU/UCA
Val [4]	GUU/GUA	GUU/GUA	GUU/GUA	GUU/GUA	GUU/GUA	GUU/GUA	GUU/GUA
Pro [4]	CCU/CCA	CCU/CCA	CCU/CCA	CCU/CCA	CCA	CCU/CCA	CCU/CCA
Thr [4]	ACU/ACA	ACU/ACA	ACU/ACA	ACU/ACA	ACU/ACA	ACU/ACA	ACU/ACA
Ala [4]	GCU/GCA	GCU/GAU	GCU/GCA	GCU/GCA	GCU/GCA	GCU/GCA	GCU/GCA
Gly [4]	GGU	GGU	GGU/GGA	GGU/GGA	GGU	GGU/GGA	GGU/GGA
Ile [3]	AUU/AUC	AUU/AUC	AUU	AUU	AUU	AUU	AUU
Tyr [2]	UAC	UAC/UAU	UAU	UAU	UAU	UAU	UAU
His [2]	CAC	CAC/CAU	CAU	CAU	CAU	CAU	CAU
Gln [2]	CAA/CAG	CAA	CAA	CAA	CAA	CAA	CAA
Asn [2]	AAC	AAC/AAU	AAU	AAU	AAU	AAU	AAU
Lys [2]	AAA	AAA	AAA	AAA	AAA	AAA	AAA
Asp [2]	GAU/GAC	GAU/GAC	GAU	GAU	GAU	GAU	GAU
Glu [2]	GAA	GAA	GAA	GAA	GAA	GAA	GAA
Cys [2]	UGU	UGU	UGC	UGU	UGU	UGU	UGU
Phe [2]	UUC	UUC/UUU	UUU	UUU	UUU	UUU	UUU
Trp [1]	UGG	UGG	UGG	UGG	UGG	UGG	UGG
Met [1]	AUG	AUG	AUG	AUG	AUG	AUG	AUG

[a] Number of synonymous codons for each amino acid are shown in brackets. [b] References: *Chlamydomonas* (Campbell and Gowri, 1990) in reference [11], *Chlorella* (Yoshinaga *et al*., 1988) in reference [12]; all others from GenBank.

a clear tendency to choose codon ending in A or T. This is consistent with [19] suggestion that composition bias in the chloroplast genome of plants is the predominant factor influencing codon bias.

Differences of the inter-generic-preferred codon values varied from 3 to 6 between green algae genera, 0 to 5 between *Laurencia* complex representatives and 1 to 10 between green and red genera (Table 2).

Table 3 shows the codon usage guide of the *rbc*L gene for red algal genera in different families inside the order Ceramiales: Ceramiaceae (genus *Ceramium*), Dasyaceae (*Dasya*), Delesseriaceae (*Delesseria*), and five genera in the Rhodomelaceae (*Chondrophycus*, *Laurencia s.s*., *Osmundea*, *Palisada*, *Yuzurua* - the *Laurencia* complex). Chloroplast genes of *Ceramium*, *Laurencia* complex and green algal genera have a preference for codons ending in A or U, thus showing a similar preferred codon as in the genera of the *Laurencia* complex and other red algae genus like *Gracilaria* [33]. However, *Dasya* and *Delesseria* showed a preference for codons ending in C or G. [30] pointed out that *psbA* has a high C content at the third position of specific synonymous groups, and the pattern of codon usage of the plant *psbA* is also seen in the genes for the chloroplast of the green algae Chlamydomonas, showing that preference is much more common in this organism [30].

The differences of the inter-generic-preferred codons values varied from 3 to 18 between the genera *Ceramium*, *Dasya* and *Delesseria*, and varied from 0 to 22 between *Laurencia* complex representatives and these red algae (Table 4). As we can see, the preferred codon differences values obtained from the comparison of *Laurencia* complex representatives *versus* the other red algal genera

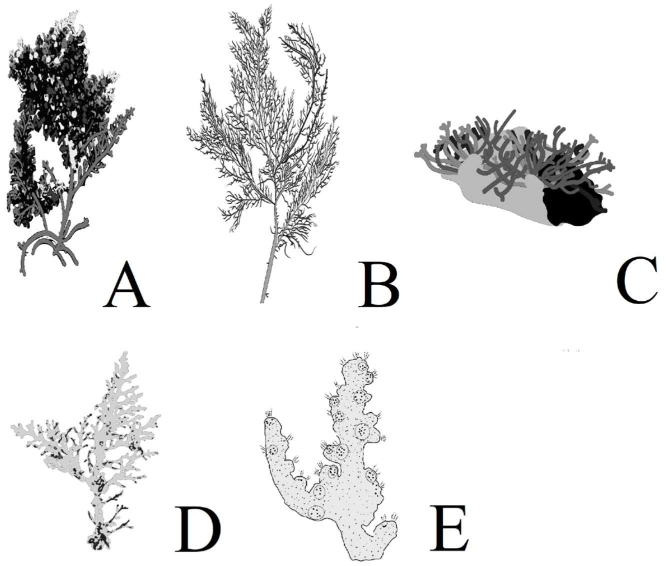

Figure 1. Habit of *Chondrophycus* (**A**), *Laurencia* (**B**), Osmundea (**C**), *Palisada* (**D**), *Yuzurua* (**E**).

Table 2. Comparison of Codon Bias Usage in *rbc*L for green algae and *Laurencia* complex with emphasis in differences on preferred codons.

Differences in preferred codons:c	Chlamydomonas **b**	Chlorella	Laurencia s.s.	Osmundea	Palisada	Chondrophycus	Yuzurua
Chlamydomonas	-	3	7	9	10	8	6
Chlorella	6	-	9	9	9	9	4
Laurencia s.s.	7	5	-	2	5	1	1
Osmundea	5	3	0	-	3	0	1
Palisada	5	1	0	0	-	0	3
Chondrophycus	5	4	0	1	4	-	0
Yuzurua	8	9	2	0	0	0	-

[b]References: *Chlamydomonas* (Campbell and Gowri, 1990) in reference [11], *Chlorella* (Yoshinaga *et al*., 1988) in reference [12], all others from GenBank. [c]Number of preferred codons used by this species which are not used by the other species.

Table 3. Comparison of Codon Bias Usage in *rbc*L for red algae and *Laurencia* complex with emphasis in preferred codons.

Amino Acid[a]	Ceramium	Dasya	Delesseria	Laurencia s.s.	Osmundea	Palisada	Chondrophycus	Yuzurua
Preferred codon								
Leu [6]	CUA/UUA	CUA/UUG/CUU/CUG	CUA/UUG/CUG	CUA/UUA/CUU	UUA	UUA	CUA/UUA	CUA/UUA
Arg [6]	AGA/CGU	CGC	AGA/CGC/CGA/CGG	AGA/CGU	AGA/CGU	CGU	AGA/CGU	AGA/CGU
Ser [6]	UCU/UCA	UCU/UCC	UCU/UCA	UCU/UCA	UCU/UCA	UCU /UCA	UCU/UCA	UCU/UCA
Val [4]	GUA/GUU	GUA/GUG	GUA/GUG	GUA/GUU	GUA/GUU	GUA/GUU	GUA/GUU	GUA/GUU
Pro [4]	CCA/CCU	CCU	CCU/CCG	CCA/CCU	CCA/CCU	CCA	CCA/CCU	CCA/CCU
Thr [4]	ACU/ACA	ACU	ACU/ACA/ACC	ACU/ACA	ACU/ACA	ACU/ACA	ACU/ACA	ACU/ACA
Ala [4]	GCA/GCU	GCA/GCC/GCG	GCU/GCC/GCG	GCA/GCU	GCA/GCU	GCA/GCU	GCA/GCU	GCA/GCU
Gly [4]	GGA/GGU	GGA/GGG	GGA/GGG/GGC	GGA/GGU	GGA/GGU	GGU	GGA/GGU	GGA/GGU
Ile [3]	AUU	AUU/AUA	AUU/AUA	AUU	AUU	AUU	AUU	AUU
Tyr [2]	UAU	UAU	UAU	UAU	UAU	UAU	UAU	UAU
His [2]	CAU	CAU	CAU	CAU	CAU	CAU	CAU	CAU
Gln [2]	CAA	CAG	CAG	CAA	CAA	CAA	CAA	CAA
Asn [2]	AAU	AAC	AAU	AAU	AAU	AAU	AAU	AAU
Lys [2]	AAA	AAG	AAG	AAA	AAA	AAA	AAA	AAA
Asp [2]	GAU	GAU	GAU	GAU	GAU	GAU	GAU	GAU
Glu [2]	GAA	GAG	GAG	GAA	GAA	GAA	GAA	GAA
Cys [2]	UGU	UGC	UGC	UGC	UGU	UGU	UGU	UGU
Phe [2]	UUU	UUU	UUC	UUU	UUU	UUU	UUU	UUU
Trp [1]	UGG	UGG	UGG	UGG	UGG	UGG	UGG	UGG
Met [1]	AUG	AUG	AUG	AUG	AUG	AUG	AUG	AUG

[a] Number of synonymous codons for each amino acid are shown in brackets.

Table 4. Comparison of Codon Bias Usage in *rbc*L for red algae and *Laurencia* complex with emphasis in differences on preferred codons.

II. Differences in preferred codons:[c]	***Ceramium*[b]**	Dasya	Delesseria	Laurencia s.s	Osmundea	Palisada	Chondrophycus	Yuzurua
Ceramium	-	14	9	0	0	0	0	0
Dasya	14	-	3	13	16	18	15	14
Delesseria	18	11	-	17	19	22	18	11
Laurencia s.s	2	13	11	-	2	5	1	1
Osmundea	0	14	11	0	-	3	0	1
Palisada	0	13	11	0	0	-	0	3
Chondrophycus	0	14	12	0	1	4	-	0
Yuzurua	0	15	18	2	0	0	0	-

[b] References: *Ceramium*, *Dasya* and *Delesseria* sequences were taken from GenBank. [c] Number of preferred codons used by this species, wich are not used by the other species.

were higher than values obtained from the comparison of *Laurencia* complex *versus* green algae genera. The preferred codon differences values obtained from the comparison of *Laurencia* complex representatives and the values obtained from the comparison of green algae genera were similar.

The parallelism in the codon use patterns for the *rbc*L gene of representatives of Chondrophycus**,** *Laurencia s.s.*, *Osmundea* and *Palisada* are showed in Tables 5 and 6. In addition to the elemental peculiarities of codon usage additional Codon Bias parameters were estimated at the generic level: the total number of codons used to encode the polypeptide, the preferred codon, Schi^2, ENC, CBI and the percentage of total codons encrypted by NNC/G (Table 7). The Nonparametric Kruskal-Wallis ANOVA Test showed inter-generic significant differences (p<0.05) for all parameters estimated. Because of these differences we can conclude that the codon usage patterns of *Ceramium, Chondrophycus*, *Chlamydomonas, Chlorella, Dasya, Delesseria, Laurencia s.s*, *Osmundea* and *Palisada* genera are different. [34] confirmed that the estimated Codon Bias parameters might be useful for systematic studies when mixed with the group of favorite codons and the percentage of NNC / G codons.

Table 5. Codon Bias on *rbc*L gene for twenty-five representatives of the *Laurencia s.s.*

Taxa, GenBank Accession Number/ Amino Acida	**Leu [6]**	**Arg [6]**	**Ser [6]**	**Val [4]**	**Pro [4]**	**Thr [4]**	**Ala [4]**	**Gly [4]**	**Ile [3]**	**Tyr [2]**	**His [2]**	**Gln [2]**	**Asn [2]**	**Lys [2]**	**Asp [2]**	**Glu [2]**	**Cys [2]**	**Phe [2]**	**Trp [1]**	**Met [1]**
Laurencia aldingensis Saito & Womersley. JF810351.	UUA CUA	CGU	UCU	GUA GUU	CCA	ACU ACA	GCA GCU	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGC	UUU	UGG	AUG
L. arbuscula Sonder. AF465810.	UUA CUA	CGU	UCU	GUA GUU	CCA CCU	ACU ACA	GCA GCU	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
L. brongniartii J. Agardh. EF061654.	UUA CUA	CGU	UCU	GUA GUU	CCA	ACU ACA	GCA GCU	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGC	UUU	UGG	AUG
L. caraibica P.C. Silva. EF658642.	UUA	CGU	UCU	GUA GUU	CCA	ACU ACA	GCA GCU	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGC	UUU	UGG	AUG
L. caduciramulosa Masuda et Kawaguchi. FJ904933.	UUA	CGU	UCU	GUA GUU	CCA	ACU ACA	GCA GCU	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
L. catarinensis Cordeiro-Marino & Fujii. AF465808.	UUA	CGU	UCU	GUA GUU	CCA	ACU ACA	GCA GCU	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
L. complanata (Suhr) Kützing. AF465813.	UUA	CGU	UCU	GUA GUU	CCA	ACU ACA	GCA GCU	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
L. filiformis (C. Agardh) Montagne. AF465818.	UUA	CGU	UCU	GUA GUU	CCA	ACU ACA	GCA GCU	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
L. flexuosa Kützing. AF465815.	UUA	CGU	UCU	GUA GUU	CCA	ACU ACA	GCA GCU	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGC	UUU	UGG	AUG
L. intricata J.V. Lamouroux. EF658644.	UUA	CGU	UCU	GUA GUU	CCA	ACU ACA	GCA GCU	GGU	AUU	UAU	CAC	CAA	AAU	AAA	GAU	GAA	UGC	UUU	UGG	AUG
L. marilzae Gil-Rodríguez, Sentíes et M.T. Fujii. EF686003.	UUA	CGU AGA	UCU UCA	GUA GUU	CCA CCU	ACU ACA	GCA GCU	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGC	UUU	UGG	AUG
L. majuscula (Harvey) A.H.S. Lucas. EF686000.	UUA	CGU	UCU	GUA GUU	CCA	ACU ACA	GCA GCU	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
L. pyramidalis Bory de Saint-Vincent ex Kützing. FJ785316.	UUA CUA	CGU AGA	UCU UCA	GUA GUU	CCA CCU	ACU ACA	GCA GCU	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
L. natalensis Kylin. AF465816.	UUA	CGU	UCU	GUA GUU	CCA	ACU ACA	GCA GCU	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
L. obtusa (Hudson) J.V. Lamouroux. AF281881.	UUA	CGU	UCU	GUA GUU	CCA	ACU ACA	GCA GCU	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGC	UUU	UGG	AUG
L. oliveirana Yoneshigue. JF810352.	UUA	CGU	UCU	GUA GUU	CCA	ACU ACA	GCA GCU	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
L. pacifica Kylin. AY588411.	UUA	CGU	UCU	GUA GUU	CCA	ACU ACA	GCA GCU	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUC	UGG	AUG
L. rigida J. Agardh. AY920852.	UUA	CGU	UCU	GUA GUU	CCA	ACU ACA	GCA GCU	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG

Taxa, GenBank Accession Number/ Amino Acida	Leu [6]	Arg [6]	Ser [6]	Val [4]	Pro [4]	Thr [4]	Ala [4]	Gly [4]	Ile [3]	Tyr [2]	His [2]	Gln [2]	Asn [2]	Lys [2]	Asp [2]	Glu [2]	Cys [2]	Phe [2]	Trp [1]	Met [1]
L. translucida Fujii & Cordeiro-Marino. AF465805.	UUA	CGU	UCU UCA	GUA GUU	CCU CCA	ACU ACA	GCA GCU	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
L. venusta Yamada. EF061655.	UUA	CGU AGA	UCU	GUA GUU	CCA	ACU ACA	GCA GCU	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
L. viridis Gil-Rodríguez & Haroun. EF685999.	UUA	CGU AGA	UCU	GUA GUU	CCA	ACU ACA	GCU GCA	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGC	UUU	UGG	AUG
L. cf. kuetzingii. FJ785322.	UUA	CGU AGA	UCU UCA	GUA GUU	CCA CCU	ACU ACA	GCA GCU	GGU GGA	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGC	UUU	UGG	AUG
L. cf. macdermidiae. FJ785314.	UUA	CGU AGA	UCU UCA	GUA GUU	CCA CCU	ACU ACA	GCA GCU	GGU GGA	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGC	UUU	UGG	AUG
L. cf. mariannensis. FJ785313.	UUA	CGU AGA	UCU UCA	GUA GUU	CCA CCU	ACU ACA	GCA GCU	GGU GGA	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGC	UUU	UGG	AUG
L. cf. nidifica. FJ785315.	UUA	CGU AGA	UCU UCA	GUA GUU	CCA CCU	ACU ACA	GCA GCU	GGU GGA	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGC	UUU	UGG	AUG

[a] Number of synonymous codons for each amino acid are shown in brackets.

Table 6. Codon Bias of *rbc*L gene in representatives of genera *Chondrophycus*, *Osmundea*, *Palisada* and *Yuzurua*.

Taxa, GenBank Accession Number/ Amino Acida	Leu [6]	Arg [6]	Ser [6]	Val [4]	Pro [4]	Thr [4]	Ala [4]	Gly [4]	Ile [3]	Tyr [2]	His [2]	Gln [2]	Asn [2]	Lys [2]	Asp [2]	Glu [2]	Cys [2]	Phe [2]	Trp [1]	Met [1]
Chondrophycus cartilagineus (Yamada) Garbary & Harper. AF489859.	UUA CUA	CGU AGA	UCU UCA	GUA GUU	CCA CCU	ACU ACA	GCA GCU	GGU GGA	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
C. intermedius (Yamada) Garbary & J.T. Harper. DQ787585.	UUA CUA	CGU AGA	UCU UCA	GUA GUU	CCU CCA	ACU ACA	GCU GCA	GGU GGA	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
C. tronoi (E. Ganzon-Fortes) K.W. Nam. AF489864.	UUA CUA	CGU AGA	UCU UCA	GUA GUU	CCA CCU	ACU ACA	GCA GCU	GGU GGA	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
Chondrophycus cf. undulatus FJ785307.	UUA CUA	CGU AGA CGC	UCU UCA	GUA GUU	CCU CCA	ACU ACA	GCU GCA	GGU GGA	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
Chondrophycus sp. 1. FJ785309.	UUA CUA	CGU AGA CGC	UCU UCA	GUA GUU	CCU CCA	ACU ACA	GCU GCA	GGU GGA	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
Chondrophycus sp. 2. FJ785310.	UUA CUA	CGU AGA CGC	UCU UCA	GUA GUU	CCU CCA	ACU ACA	GCU GCA	GGU GGA	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
Chondrophycus sp. 3. FJ785311.	UUA CUA	CGU AGA	UCU UCA	GUA GUU	CCU CCA	ACU ACA	GCU GCA	GGU GGA	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
Osmundea blinksii (Hollenberg & I.A. Abbott) K.W. Nam. AY172575.	UUA	CGU	UCU UCA	GUU GUA	CCU CCA	ACU	GCU GCA	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
O. hybrida (A.P. de Candolle) K.W. Nam. FJ785317.	UUA	CGU	UCU	GUA	CCA	ACU ACA	GCU GCA	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG

Taxa, GenBank Accession Number/ Amino Acid[a]	Leu [6]	Arg [6]	Ser [6]	Val [4]	Pro [4]	Thr [4]	Ala [4]	Gly [4]	Ile [3]	Tyr [2]	His [2]	Gln [2]	Asn [2]	Lys [2]	Asp [2]	Glu [2]	Cys [2]	Phe [2]	Trp [1]	Met [1]
O. osmunda (S.G. Gmelin) K.W. Nam & Maggs. AF281877.	UUA	CGU AGA	UCU	GUU GUA	CCA	ACU ACA	GCU GCA	GGU	AUU	UAU	CAU	CAA	AAC	AAA	GAU	GAA	UGU	UUU	UGG	AUG
O. pinnatifida (Hudson) Stackhouse. AF281876.	UUA	CGU AGA	UCU	GUA GUU	CCA	ACU ACA	GCU GCA	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
O. ramosissima (Oeder) Athanasiadis. AF281880.	UUA	CGU	UCU	GUU GUA	CCA	ACU ACA	GCU GCA	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
O. spectabilis (Postels & Ruprecht) K.W. Nam var. spectabilis. AY172574.	UUA	CGU AGA	UCU UCA	GUU GUA	CCU CCA	ACU ACA	GCU GCA	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
O. spectabilis var. diegoensis (E.Y. Dawson) K.W. Nam. AY172572.	UUA CUA	CGU AGA	UCU UCA	GUU GUA	CCU CCA	ACU ACA	GCU GCA	GGU GGA	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
O. sinicola (Setchell & N.L. Gardner) K.W. Nam. AY588407.	UUA	CGU AGA	UCU	GUU GUA	CCU CCA	ACU ACA	GCU GCA	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
O. splendens (Hollenberg) K.W. Nam. AY172576.	UUA	CGU	UCU UCA	GUU GUA	CCU CCA	ACU	GCU GCA	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
O. truncata (Kützing) K.W. Nam & Maggs. AF281879.	UUA	CGU AGA	UCU	GUU GUA	CCU CCA	ACU ACA	GCU GCA	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
Palisada corallopsis Sentíez, Fujii & Díaz. EF061646.	UUA	CGU AGA	UCU	GUA GUU	CCA CCU	ACU ACA	GCU GCA	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
P. flagellifera (J. Agardh) K.W. Nam. EF061647.	UUA CUA	CGU AGA	UCU	GUA GUU	CCA CCU	ACU ACA	GCU GCA	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
P. furcata (Cordeiro-Marino et M.T. Fujii) Cassano et M.T. Fujii. GU330226.	UUA CUA	CGU AGA	UCU	GUA GUU	CCA CCU	ACU ACA	GCU GCA	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
P. patentiramea (Montagne) Cassano, Sentíes, Gil-Rodríguez & M.T. Fujii. AF489862.	UUA CUA	CGU AGA	UCU UCA	GUA GUU	CCA CCU	ACU ACA	GCU GCA	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
P. perforata (Bory de Saint-Vincent) K.W. Nam. EF658641.	UUA CUA	CGU AGA	UCU UCA	GUA GUU	CCA	ACU ACA	GCU GCA	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
P. thuyoides (Kützing) Cassano, Sentíes, Gil-Rodríguez & M.T. Fujii. EF685998.	UUA CUA	CGU AGA	UCU	GUA GUU	CCA	ACU ACA	GCU GCA	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
P. cf. cruciata. FJ785319.	UUA CUA	CGU AGA	UCU UCA	GUA GUU	CCA CCU	ACU ACA	GCU GCA	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
P. cf. robusta. FJ785321.	UUA	CGU AGA	UCU UCA	GUA GUU	CCA CCU	ACU ACA	GCU GCA	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
Yuzurua poiteaui (J.V. Lamouroux) Martin-Lescanne. EF061653.	UUA CUA	CGU AGA	UCU	GUA GUU	CCA CCU	ACU ACA	GCU GCA	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
Y. poiteaui (J.V. Lamouroux) Martin-Lescanne) var. gemmifera (Harvey) M.J. Wynne. EF061649.	UUA CUA	CGU AGA	UCU	GUA GUU	CCA CCU	ACU ACA	GCU GCA	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG
Yuzurua sp. (as P. papillosa-3). AY172577.	UUA CUA	CGU AGA	UCU UCA	GUA GUU	CCA CCU	ACU ACA	GCU GCA	GGU	AUU	UAU	CAU	CAA	AAU	AAA	GAU	GAA	UGU	UUU	UGG	AUG

[a] Number of synonymous codons for each amino acid are shown in brackets.

Table 7. Inter-generic Codon Bias measures, G/C percentages of red and green algae and results of the Kruskal-Wallis ANOVA Test (p-values).

Genera	SChi2	ENC	CBI	Used Codons	Preferred Codons	Percent of Codons % NNG/C
Ceramium	0.83	36.53	0.59	49	26	7.0
Chondrophycus	0.89	34.96	0.65	50	28	7.0
Chlamydomonas	1.51	28.96	0.72	40	30	24.9
Chlorella	1.17	31.19	0.68	44	33	19.5
Dasya	0.15	55.43	0.20	53	27	55.0
Delesseria	0.21	56.48	0.29	58	33	54.0
Laurencia s.s.	0.86	35.45	0.62	50	29	10.0
Osmundea	0.86	34.67	0.65	51	27	7.0
Palisada	0.81	35.71	0.61	51	24	8.0
Yuzurua	0.74	35.97	0.60	54	28	7.0
p-values	0.0001	0.0003	0.0004	0.0002	0.0001	0.00025

The Schi^2, a measure supported by the differences between the registered number of codons and those expected from equal usage of codons, fluctuated between 0.15 and 1.51, and inter-generic analyses showed significant statistical differences, however *Laurencia s.s.,* and *Osmundea* showed similar Schi^2 values. Once more this data confirms that all the genera have different codon usages (Table 7).

ENC values, that measure the actual number of codons used in a gene ranged between 28.96 and 56.48, also showed significant inter-generic statistical differences. The previous results express that more than one codon is used for each amino acid. In other words, the inter-generic synonymous codon usage by each amino acid was different. Interestingly *Dasya* and *Delesseria* have the highest inter-generic ENC, closer to the maximum codon usage value (61 codons number) than any other genus analysed (Table 7).

CBI values, a measurement of the variation of the equal use of synonymous codons, which fluctuated between 0.20 and 0.72, and also showed significant inter-generic statistical differences, although *Chondrophycus* and *Osmundea* showed similar CBI values. These results confirm that the inter-generic synonymous codon usage is unequal (Table 7). The CBI values obtained are comparable to those reported by other authors for *Porphyra*, another red algal genus [27]. We must emphasize that *Dasya* and *Delesseria* have the closest values to the minimum Codon Bias Index (CBI = 0).

Values for Used Codons, which fluctuated between 40 and 58, also showed statistically significant differences at the generic level, except for four genera (*Laurencia s.s.* and *Chondrophycus*, *Osmundea* and *Palisada*) that showed similar Used Codons values (Table 7). The values of the Preferred Codons varied between 26 and 33, and also showed statistically significant differences, but four genera (*Chondrophycus* and *Yuzurua*, *Chlorella* and *Delesseria*) presented similar Used Codons values However, the species *Agarophyton* tenui*stipitatum* (C. F. Chang et B.-M. Xia) Gurgel, J. N. Norris & Fredericq indicated limited codon bias as he used all codon combinations and in most cases relatively low bias.

The percentages of total codons encoded by NNC/G showed statistically significant differences and reflects that all the studied genera recorded lower values (between 7 and 29.4), except for *Dasya* and *Delesseria*, which showed the highest NNG/C codon percentage (Table 7), recommend that the genome has been exposed to high GC pressure throughout evolution. These results support earlier data depicted in Tables 1 and 2, where the majority of the genera have a clear codon usage preference for codons ending in A or U, except *Dasya* and *Delesseria*. The G+C content values obtained are comparable to those reported for other eukaryotic representatives like *Cyanophora* Korshikov, *Cyanidium* Geitler, *Cyanidioschyzon* P. De Luca, R. Taddei & L. Varano, *Gracilaria, Guillardia* D.R.A. Hill & R. Wetherbee*, Mesostigma* Lauterborn*, Nephroselmis* F. Stein*, Odontella* C. Agardh and *Porphyra* [19,33]. The genomic GC content is strongly correlated with the average amino acid composition of proteins. Further, it has been established that a link exists between the third position GC content and gene expression level and has been applied to organism systematics [18,32,35].

Table 8 shows the inter-specific Codon Bias measures and G/C percentages of the *Laurencia* complex representatives. Results obtained suggest a differential codon usage pattern in the inter-specific level in *Chondrophycus, Laurencia s.s.*, *Osmundea*, *Palisada* and *Yuzurua* species. This is consistent with Grantham´s hypothesis for codon usage [4], suggesting that the genetic code is used differently by different kinds of taxa. According to [34] from cormophytes to thallophytes to algae to cyanobacteria, they all fit the general hypothesis that an organism has a unique codon pattern, with closely related organelles having similar patterns.

Table 8. Inter-specific Codon Bias measures and G/C percentages of *Laurencia* complex.

Taxa	SChi2	ENC	CBI	Used Codons	Preferred Codons	Percent of Codons % NNG/C
Chondrophycus cartilagineus	0.89	33.01	0.62	50	27	7.4
C. intermedius	0.88	35.40	0.63	50	28	7.0
C. tronoi	0.90	34.14	0.66	50	28	7.1
C. cf. *undulatus*	0.87	35.56	0.65	50	29	7.5
C. sp. 1	0.85	35.63	0.63	51	29	8.3
C. sp. 2	0.95	34.20	0.65	50	29	8.6
C. sp. 3	0.89	33.60	0.68	52	28	8.5
Laurencia aldingensis	0.84	35.69	0.62	53	24	12.5
L. arbuscula	0.80	36.85	0.59	50	25	8.0
L. brongniartii	0.83	36.20	0.61	49	24	12.5
L. caraibica	0.74	37.71	0.59	57	23	13.0
L. caduciramulosa	0.82	36.29	0.60	53	23	8.6
L. catarinensis	0.83	36.36	0.59	50	23	8.6
L. complanata	0.94	33.66	0.66	35	23	8.6
L. filiformis	0.87	35.51	0.62	49	23	8.6
L. flexuosa	0.83	35.67	0.62	47	23	13.0
L. intricata	0.91	34.57	0.64	47	23	17.3
L. marilzae	0.77	37.48	0.60	54	26	11.5
L. majuscula	0.81	37.17	0.57	52	23	8.6
L. natalensis	0.88	35.20	0.63	50	23	8.6
L. obtusa	0.91	34.73	0.66	49	23	13.0
L. oliveirana	0.87	34.76	0.64	49	23	8.6
L. pacifica	0.84	36.94	0.62	48	23	13.0
L. pyramidalis	0.87	35.82	0.63	51	27	8
L. rigida	0.98	34.02	0.66	48	23	8.6
L. scoparia	0.84	38.48	0.55	46	25	8
L. translucida	0.85	38.00	0.58	47	25	8

Taxa	**SChi2**	**ENC**	**CBI**	**Used Codons**	**Preferred Codons**	**Percent of Codons % NNG/C**
L. venusta	0.83	36.16	0.60	52	24	8.3
L. viridis	0.86	35.10	0.63	50	24	12.5
L. cf. *kuetzingii*	0.80	36.75	0.62	51	27	8.0
L. cf. *macdermidiae*	0.92	34.39	0.64	47	27	13.0
L. cf. *mariannensis*	0.86	35.09	0.64	49	27	10.0
L. cf. *nidifica*	0.90	33.78	0.64	52	27	8.2
Osmundea blinksii	0.78	36.24	0.66	54	24	8.3
O. hybrida	0.89	35.37	0.64	50	22	9.0
O. osmunda	0.85	34.70	0.66	51	24	12.5
O. pinnatifida	0.93	33.68	0.66	49	24	8.3
O. ramosissima	0.82	34.70	0.63	48	23	8.6
O. spectabilis var *spectabilis*	0.82	35.01	0.65	54	26	7.6
O. spectabilis var *diegoensis*	0.82	34.94	0.65	54	28	7.1
O. sinicola	0.81	34.84	0.62	52	25	8
O. splendens	0.81	35.70	0.67	54	24	8.3
O. truncata	0.92	34.85	0.65	46	25	8
Palisada corallopsis	0.86	34.27	0.63	53	25	8
P. flagellifera	0.84	34.56	0.64	49	26	7.6
P. furcata	0.83	34.55	0.63	49	26	7.7
P. patentiramea	0.81	35.46	0.61	50	27	7.4
P. perforata	0.78	36.41	0.60	51	26	7.6
P. thuyoides	0.84	34.61	0.59	48	25	8.0
P. cf. *cruciata*	0.87	34.02	0.62	48	27	8.4
P. cf. *robusta*	0.88	34.42	0.61	48	26	8.6
Yuzurua poiteaui	0.73	35.97	0.6	53	27	7.4
Y. poiteaui var. *gemmifera*	0.73	36.55	0.59	54	26	7.6
Yuzurua sp. (as *P. papillosa-3*)	0.78	35.60	0.61	49	27	7.5

RSCU values for 54 taxa (Tables 5 and 6) were subjected to cluster analysis. Clustering of the genera (Figure 2A) reveals three groups in terms of codon usage: 1) Rhodomelaceae (*Laurencia s.s.*, *Palisada, Yuzurua, Chondrophycus* and *Osmundea*) 2) Dasyaceae, Delesseriaceae and Ceramiaceae (*Dasya*, *Delesseria* and *Ceramium*) and 3) green algae (*Chlamydomonas* and *Chlorella*). These analyses clearly segregate the red and green algae groups suggesting phylogenetic relationships among these genera. [30] mention that patterns of codon usage for various organisms have been correlated to their phylogenetic relationships.

In general, cluster analysis at the intra-generic level reveals the phylogenetic relationships among '*Laurencia* complex representatives species'. The cluster results agree with previously published phylogenies [36-40]. The cluster analysis of the *Laurencia s.s.,* taxa (Figure 2B) shows six groups in terms of codon usage: 1) *L. arbuscula*,

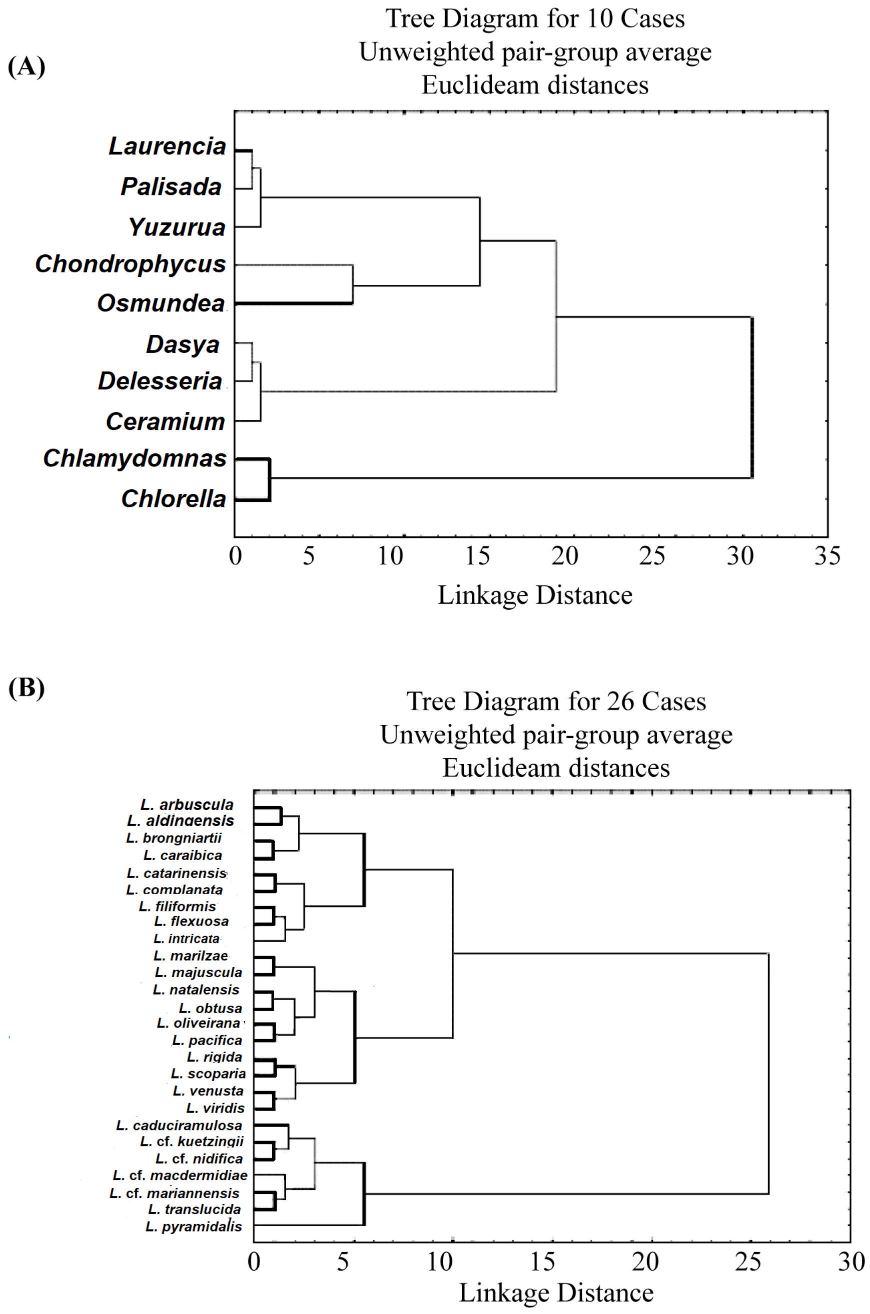

Figure 2. Cluster of general groups (A), and *Laurencia s.s.,* species (B) *rbc*L gene based on codon employment similarity as measured by the Long and Gillespie (1991) procedure in reference [34].

L. aldingensis, *L. brongniartii* and *L. caraibica*; 2) *L. catarinensis*, *L. complanata*, *L. filiformis*, *L. flexuosa* and *L. intricata*; 3) *L. marilzae*, *L. majuscula*, *L. natalensis*, *L. obtusa*, *L. oliveirana* and *L. pacifica*; 4) *L. rigida*, *L. venusta* and *L. viridis*; 5) *L. caduciramulosa*, *L. cf. kuetzingii*, *L. cf. nidifica*, *L. cf. macdermidiae*, *L. cf. mariannensis* and *L. translucida*; 6) *L. pyramidalis*.

In the case of the *Osmundea* taxa, cluster analysis (Figure 3A) shows three groups in terms of codon usage: 1) *O. blinksii*, *O. hybrida*, *O. osmunda* and *O. pinnatifida*; 2) *O. ramosissima* and *O. spectabilis* var *diegoensis*; 3) *O. sinicola*, *O. splendens*, *O. spectabilis* var. *spectabilis* and *O. truncata*.

For the *Palisada* species, cluster analysis (Figure 3B) shows four groups in terms of codon usage: 1) *P. perforate*, *P. corallopsis* and *P. flagellifera*; 2) *P.*

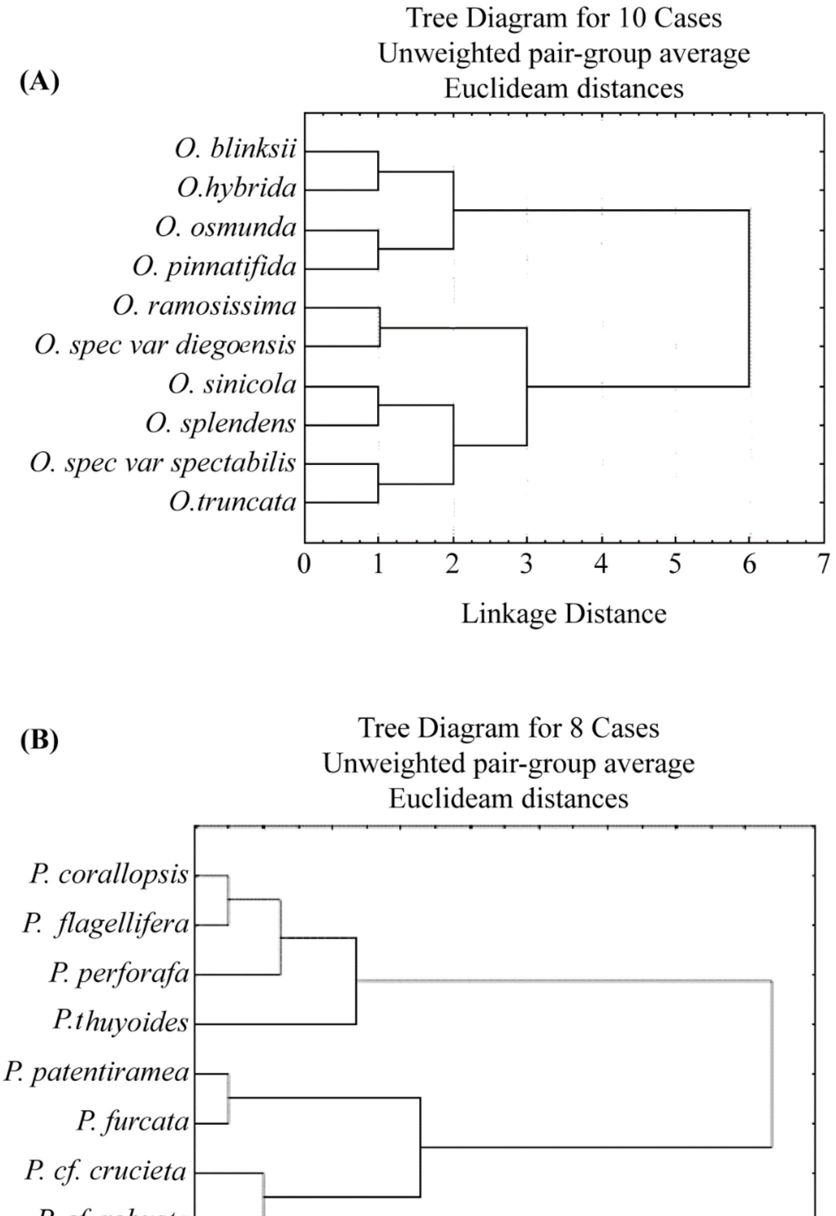

Figure 3. Cluster of *Osmundea* (A) and *Palisada* (B) genera *rbc*L gene based on codon employment similarity as measured by the Long and Gillespie (1991) procedure in reference [34].

thuyoides; 3) *P. patentiramea* and *P. furcata*; 4) *P. cf. cruciata* and *P. cf. robusta*.

Moreover, clustering of the *Chondrophycus* taxa (Figure 4A) shows four groups in terms of codon usage: 1) *C. cf. undulatus*, *Chondrophycus* sp. 1; 2) *Chondrophycus* sp. 2 and *Chondrophycus* sp. 3; 3) *C. intermedius* and *C. cartilagineus*; 4) *C. tronoi*.

In addition, the cluster analysis for the *Yuzurua* taxa (Figure 4B) indicates two groups in terms of codon usage: 1) *Y. poiteaui* and *Yuzurua* sp. (as *P. papillosa*-3); 2) *Y. poiteaui* var. *gemmifera*.

It is obvious that the evolution of codon bias over all chloroplast lineages is an abstract and active process. It is possible that several factors are associated in the decision of selective restrictions on codon bias [41]. In the present work we found that the synonymous codon preference biases in *rbc*L are large and differ even among species. A clear distinction between analyzed genera codon usage is evident: for instance, *Dasya*, *Delesseria* and *Yuzurua* use a set that fluctuated between 53 and 58 codons. However, in

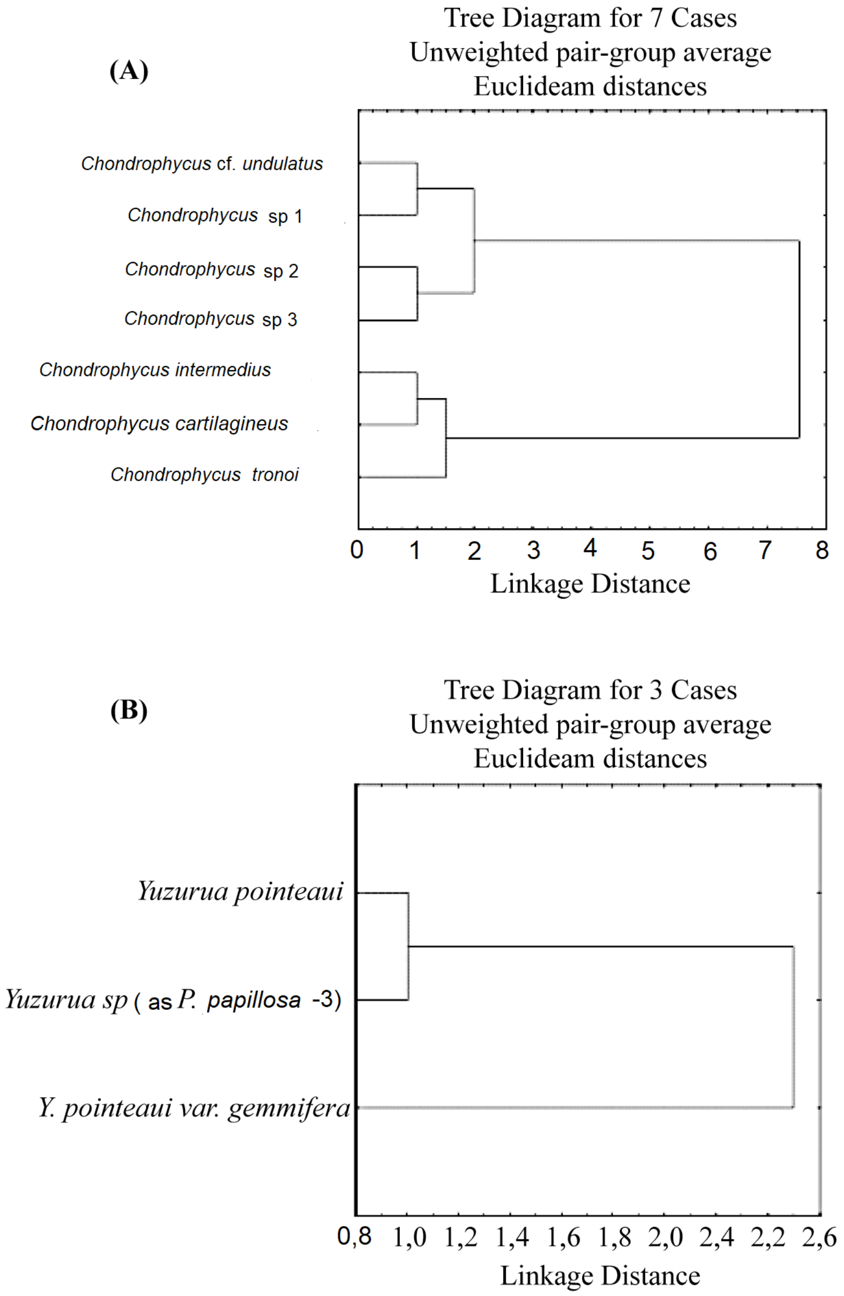

Figure 4. Cluster of *Chondrophycus* (A) and *Yuzurua* (A) genera *rbc*L gene based on codon employment similarity as measured by the Long and Gillespie (1991) procedure in reference [34].

Ceramium, Chondrophycus, *Chlamydomonas*, *Chlorella*, *Laurencia s. s.*, *Osmundea* and *Palisada*, codon usage is more restricted with an average that fluctuated between 40 and 51 codons. Genera of the *Laurencia* complex and other red and green algal representatives showed a defined composition pattern, with lower percentage values of NNC/G (7-24.9 %). *Dasya* and *Delesseria* showed a selective pattern tendency, because of the presence of high percentage values of NNC/G (54-55%). The Codon Bias parameters estimated could be useful for systematics studies, when handled with the favorite codon set and the percentage of NNC / G codons.

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Authors' Contribution

RC and JD-L conceived and designed the experiments, analyzed the data, prepared figures, and reviewed all manuscript drafts. AA prepared figures and led the manuscript write up. LNG and GV-S contributed to data analysis, provided reagents, supplies, analysis tools, and reviewed all drafts for this manuscript. JRC-A worked on conceptualization and figure revisions. All authors have read and agreed to the version of the manuscript submitted.

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