Cíntia Iha

**Estudo filogenômico e caracterização
genômica organelar de algas gracilarioides
(Gracilariaceae, Rhodophyta)**

Phylogenomic study and organellar genomic characterization
of gracilarioids seaweeds (Gracilariaceae, Rhodophyta)

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Tese apresentada ao Instituto de Biociências da
Universidade de São Paulo, para a obtenção do
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Colaborador: Dr. Heroen Verbruggen
(The University of Melbourne, Australia)

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Prof^a. Dr^a. Mariana Cabral de Oliveira
Orientadora

*Dedico esta tese a todas as pessoas que
direta ou indiretamente me ajudaram,
influenciaram ou permitiram que este
trabalho fosse realizado.*

*Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveler, long I stood
And looked down one as far as I could
To where it bent in the undergrowth;*

*Then took the other, as just as fair,
And having perhaps the better claim,
Because it was grassy and wanted wear;
Though as for that the passing there
Had worn them really about the same,*

*And both that morning equally lay
In leaves no step had trodden black.
Oh, I kept the first for another day!
Yet knowing how way leads on to way,
I doubted if I should ever come back.*

*I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I—
I took the one less traveled by,
And that has made all the difference.*

The Road Not Taken – Robert Frost

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Resumo

A família Gracilariaceae está globalmente distribuída e inclui várias espécies economicamente importantes. Atualmente, cinco gêneros são reconhecidos em Gracilariaceae: *Gracilariophila* (parasita), *Curdiea*, *Melanthalia*, *Gracilaria* e *Gracilariopsis*. Algumas espécies de *Gracilaria* foram taxonomicamente transferidas para *Hydropuntia*. Entretanto, esse gênero é bastante controverso. Técnicas de sequenciamento de alta performance (HTS) levaram a um aumento de estudos usando genomas organelares completos, que têm sido usados para inferir relações filogenéticas em Rhodophyta e na investigação de outros aspectos dos genomas de algas vermelhas, incluindo sentença gênica e transferências horizontal de genes (HGT). O HTS também facilitou a busca por plasmídeos extracromossômicos e sua influência nos genomas organelares por HGT. Nós utilizamos HTS para montar e anotar genomas organelares (mitocôndrias e cloroplastos) de sete espécies de Gracilariaceae usando a plataforma Illumina HiSeq 2500 e recebemos sequências de 31 amostras Gracilariaceae da Dr. Goia Lyra que foram montadas, anotadas e incluídas em nossas análises. Utilizamos esses dados, combinados com genomas publicados, para inferir filogenias e comparar a arquitetura do genoma dessas espécies representando as principais linhagens em Gracilariaceae. Os genomas mitocondrial e plastidial são altamente conservados na sentença gênica e a variação ocorreu principalmente em regiões onde foram encontradas inserções de sequências derivadas de plasmídeos (PDS), similares aos plasmídeos extracromossômicos conhecidos de algas vermelhas. Nos genomas mitocondriais, as inserções de PDS estavam em duas regiões onde a direção da transcrição muda: entre os genes *cob* e *trnL* e os genes *trnA* e *trnN*. As inserções de PDS no genoma do cloroplasto estavam em posições diferentes, mas geralmente encontradas entre os genes *psdD* e *rrs*. O operon bacteriano *leuC/leuD* foi encontrado nos genomas dos cloroplastos de *Gracilaria tenuistipitata*, *G. chilensis*, *M. intermedia* e também no plasmídeo de *G. vermiculophylla*. As árvores filogenéticas mostram duas origens diferentes de *leuC/leuD*: os genes encontrados no cloroplasto e no plasmídeo estavam próximos de proteobactérias, e os genes codificados no núcleo estavam próximos de Viridiplantae e cianobactérias. Gracilariaceae pode ser um bom modelo para estudar o impacto de PDS na evolução de genomas devido à presença frequente de inserções PDS em genomas organelares. Nossas análises filogenéticas demonstraram histórias evolutivas similares entre cloroplasto e mitocôndria. No entanto, a filogenia de cloroplasto foi melhor resolvida com valores máximos de Bootstrap em todos os ramos. Nossa amostragem taxonômica corrobora a presença de três linhagens principais: *Melanthalia/Curdiea*, *Gracilariopsis* e *Gracilaria*. *Melanthalia intermedia* aparece como grupo-irmão do clado monofilético incluindo *Gracilaria* e *Gracilariopsis*, que foram resolvidos como gêneros monofiléticos. Além disso, baseado nas características da arquitetura do genoma organelar, os gêneros *Gracilariopsis* e *Gracilaria* se distinguem pela perda do gene plastidial *petP* em *Gracilaria* e pela posição de rearranjo do gene *trnH* no genoma mitocondrial. Nós não encontramos evidências para a permanência o gênero *Hydropuntia* como originalmente proposto.

Abstract

Gracilariaceae is a worldwide distributed family that includes numerous economically important species. Currently, five genera are recognized in the Gracilariaceae: *Gracilariophila* (parasitic), *Curdiea*, *Melanthalia*, *Gracilaria*, and *Gracilariopsis*. Some species of *Gracilaria* were taxonomically transferred to *Hydropuntia*. However, this genus is quite controversial. High-throughput sequencing (HTS) techniques has led to an increase in studies using complete organellar genomes, which have been used to infer phylogenetic relationships in Rhodophyta and the investigation of other aspects of red algal genomes, including gene synteny and horizontal gene transfers (HGT). HTS also facilitated the search for extrachromosomal plasmids and its influence in the organellar genomes by HGT. We applied HTS to assemble and annotate organellar genomes (mitochondria and chloroplast) from seven species of Gracilariaceae using Illumina HiSeq 2500 platform. We also received raw reads of 31 samples of Gracilariaceae from Dr. Goia Lyra that were analysed and included in our work. We used these data, combined with published genomes, to infer phylogenies and compare the genome architecture of these species representing the main lineages in Gracilariaceae. The mitochondrial and chloroplast genomes were highly conserved in gene synteny among the species, and variation mainly occurred in regions where insertions of plasmid-derived sequences (PDSs) were found, which were similar to known red algae extrachromosomal plasmids. In mitochondrial genomes, the PDS insertions were in two regions where the transcription direction changes: between *cob* and *trnL* genes, and *trnA* and *trnN* genes. PDS insertions in chloroplast genome were in different positions, but generally found between *psdD* and *rrs* genes. The bacterial *leuC/leuD* operon was found in *Gracilaria tenuistipitata*, *G. chilensis*, *M. intermedia* chloroplasts genomes, and also in *G. vermiculophylla* extrachromosomal plasmid. Phylogenetic trees show two different origins of *leuC/leuD*: genes found in chloroplasts and plasmids were close to proteobacteria, and genes encoded in the nucleus are close to Viridiplantae and cyanobacteria. Gracilariaceae may be a good model to study the impact of PDS in genome evolution due to the frequent presence of these sequences inserted in organellar genomes. Our phylogenetic analyses demonstrated similar evolutionary histories between the chloroplast and mitochondrial genomes. However, chloroplast phylogeny was better resolved with full support. Our taxonomical sampling supports the presence of three main lineages: *Melanthalia/Curdiea*, *Gracilariopsis* and *Gracilaria*. *Melanthalia intermedia* was sister to a monophyletic clade including *Gracilaria* and *Gracilariopsis*, which were resolved as monophyletic genera. Furthermore, the characteristics of organellar genome architecture, *Gracilariopsis* and *Gracilaria* genera are also supported by the loss of the plastid gene *petP* in *Gracilaria* and the rearrangement position of the gene *trnH* in the mitochondrial genome. Beside this, we found no support for the genus *Hydropuntia* as originally proposed.

Introduction

The Gracilariaceae family

The family Gracilariaceae Nägeli (1847) used to belong to the order Gigartinales F.Schmitz in Engler (1892), but based on morphological characteristics specific to the Gracilariaceae species, the family was elevated to the level of order (Fredericq and Hommersand 1989a). Therefore, the order Gracilariales Fredericq & Hommersand (1989a) was determined to contain only one family Gracilariaceae. The fundamental characteristics of the order and family are based on vegetative structure, such as the development of a pseudoparenchymatous thallus and presence of pit-plugs with a single plug cap and a cap membrane (Pueschel and Cole 1982; Fredericq and Hommersand 1990a) and characteristics of the female reproductive structure: i) a supporting cell that carries a two-celled carpogonial branch flanked by sterile branches; ii) the fertilized carpogonium fusing with its supporting cell to form the fusion cell, which cuts off several gonimoblast initials; iii) secondary fusions between gonimoblast derivatives and cells of gametophytic tissues (Fredericq and Hommersand 1989a, 1990a; Gurgel and Fredericq 2004). Moreover, molecular studies including species of Gracilariales or with higher taxonomical level have confirmed the monophyly of Gracilariales (Lyra et al. 2015; Yang et al. 2015; Lee et al. 2016a).

The genera of Gracilariaceae are characterized mainly by the morphology and ontogenetic development of the female and male reproductive structures (Fredericq and Hommersand 1990b). These characteristics correspond to the spermatangia distribution and cystocarp structure (Fredericq and Hommersand 1989a; Lyra et al. 2015). The use of the distribution of spermatangia to species separation was initially proposed by Yamamoto (1978), who recognized three basic types: (i) *chorda*-type, considered the most primitive, it is characterized by the distribution of spermatangia in a layer on the surface of the thalli; ii) *textorii*-type, in which the spermatangia are found in shallow conceptacles in the cortex; (iii) *verrucosa*-type, in which the spermatangia are located in deep conceptacles that penetrate the subcortex (Yamamoto 1978; Bellorin 2002; Lyra et al. 2015). Two distribution patterns were added posteriorly: the *symmetrical*-type, in which the spermatangia located in the superficial cell layer are interrupted by sterile cortical cells; and the *henriquesiana*-type, in which the spermatangia are produced in deep conceptacles composed of several cavities that open through a single pore, causing slight elevation of the thallus surface (Bird and McLachlan 1984; Yamamoto 1984; Bellorin 2002). *Symmetrical*-type has not been used anymore and is considered a variation of *chorda*-type. The characteristics of the cystocarp structure are based on the presence or absence of the nutrient tubular cells and its location in the cystocarp cavity, and cystocarp completely filled or not by gonimoblast (Fredericq and Hommersand 1990b).

Currently, five genera are recognized in the Gracilariaceae: *Gracilariophila* Setchell & H.L. Wilson (Wilson 1910), which is parasitic; *Curdiea* Harvey (1855), *Melanthalia* Montagne (1843), *Gracilaria* Greville (1830), and *Gracilariopsis* Dawson (1949), which are benthic and free-living

(Lyra et al. 2015; Guiry and Guiry 2018) (Table 1). Two parasitic genera used to be recognized in Gracilariaceae: *Gracilariophila* (hereafter *Gh.*), with two species; and the *Congracilaria* Yamamoto (1986), with the only species *C. babae* Yamamoto (1986) (Table 1). Both genera are adelphoparasites, which evolved from the most recent common ancestor with their host (Setchell 1918; Goff et al. 1996; Hancock et al. 2010). *Gracilariophila* had cystocarps lacking nutrient tubular cells linking gonimoblast and pericarp, and superficial spermatangia. *Congracilaria* had cystocarps with nutrient tubular cells linking gonimoblast and pericarp, and spermatangia organized in conceptacles (Fredericq and Hommersand 1990b). *Gracilariophila oryzoides* Setchell & H.L. Wilson (Wilson 1910) is the most common species of *Gracilariophila* and it is mainly parasitic of *Gracilariopsis andersonii* (Grunow) E.Y. Dawson (1949) (Hancock et al. 2010). *Congracilaria babae* was mainly parasitic of *Gracilaria salicornia* (C. Agardh) E.Y. Dawson (1954) (Yamamoto 1986). However, based on molecular analysis of DNA sequences from the nuclear, mitochondrial and plastid genomes and anatomical observations, *C. babae* was transferred to *Gracilaria* (Ng et al. 2014).

The genera *Melanthalia* and *Curdiea*, with a few species each, are restricted to Oceania, especially to southern Australia, Tasmania, and New Zealand. They were also found in Antarctica (Gurgel and Fredericq 2004). *Melanthalia* is represented currently by four species, and *Curdiea* by eleven species (Guiry and Guiry 2018) (Table 1). The cystocarp structure of both genera is characterized by presenting a cavity completely filled by gonimoblasts, the absence of nutrient tubular cells, carposporangia in long lines. However, *Melanthalia* cystocarp presents thick-walled sterile gonimoblast tissue, which is absent in *Curdiea*. The spermatangia are unknown and tetrasporangia are cruciately divided and are located between raised cortical filaments (nemathecia) (Fredericq and Hommersand 1990b).

The genus *Gracilaria* is the most diverse, with about 186 species (Guiry and Guiry 2018) (Table 1). Most species are concentrated in the tropical region, with diversity decreasing towards the poles (Plastino and Oliveira 1994). *Gracilaria* is characterized primarily based on the female and male reproductive structures. The cystocarp structure presents nutrient tubular cells in both pericarp and floor of the cystocarp cavity, which is not completely filled by gonimoblast. The spermatangia are always produced in conceptacles, which may be simple, shallow or deep (*textorii*, *verrucose* and *henriquesiana*-type, respectively) (Fredericq and Hommersand 1990b; Bellorin 2002).

Gracilariopsis is the second larger genus with 22 species (Guiry and Guiry 2018) (Table 1). The genus was separated from *Gracilaria* by Dawson (1949) based on anatomical details of the cystocarp, which lack nutrient tubular cells (Dawson 1949; Fredericq and Hommersand 1989b). However, Papenfuss (1966) observed that the nutrient tubular cells may be present or not in some *Gracilaria* species, thus *Gracilariopsis* was reduced to a taxonomic synonym under *Gracilaria*. *Gracilariopsis* has been widely accepted after the morphological and taxonomical studies based on *Gp. lemaneiformis* (Bory de Saint-Vincent) E.Y. Dawson, Acleto & Foldvik (1964) (Fredericq and Hommersand 1989b). In addition to the absence of the nutrient tubular cells in the cystocarps (Fredericq and Hommersand 1989b, Liao and Hommersand 2003), *Gracilariopsis* was recognized as a

distinct genus based on the male reproductive apparatus. The spermatangial cells developed on the surface layer (*chorda*-type) on the branches, close to the apices (Fredericq and Hommersand 1989b). This characteristic was observed by Yamamoto (1975), but he kept *Gracilaria* and *Gracilariopsis* merged and proposed three subgenera to *Gracilaria* based on the three main types of spermatangia development (Yamamoto 1978).

Some species of *Gracilaria* were taxonomically transferred to the genus *Polycavernosa* C.F. Chang et B.M. Xia (1963). However, the morphological characteristics that defined *Polycavernosa* included the species *P. urvillei* (Montagne) Xia & Abbott (1987), which was the type of the genus *Hydropuntia* Montagne (1842). Therefore, as *Hydropuntia* was the oldest name, all species of *Polycavernosa* were transferred to *Hydropuntia* (Wynne 1989). This genus is still quite controversial, dividing the opinion of researchers about its existence. The separation of *Gracilaria* was made based on characteristics of the reproductive system: the nutrient tubular cells connect to the gonimoblasts only at the floor of the cystocarp, and the spermatangia are produced in deep conceptacles composed of several cavities (Fredericq and Hommersand 1990b, Liao and Hommersand 2003), the *henriquesiana*-type (Bird and McLachlan 1984; Bellorin 2002). The genus *Hydropuntia* did not have general acceptance since some authors considered compound conceptacles as a variation of the simple *verrucosa* conceptacle (Bird and McLachlan 1984; Wynne 1989; Bellorin 2002). In addition, some species presented compound and simple conceptacles mixed in the same specimen (Bird et al. 1986; Abbott et al. 1991; Plastino and Oliveira 1997). However, some authors considered *Hydropuntia* as a distinct genus (Wynne 1989; Liao and Hommersand 2003; Gurgel and Fredericq 2004).

Table 1 - Genera of Gracilariaceae, number of currently recognized species and notes about currently taxonomical situation. Data were obtained from Guiry and Guiry (2018).

Genus	Species number	notes	Synonym font
<i>Congracilaria</i> Yamamoto, 1986	0	Parasite. Synonym of <i>Gracilaria</i>	Ng et al. 2014
<i>Curdiea</i> Harvey, 1855	11	Currently accept genus	
<i>Gracilaria</i> Greville, 1830	186	Currently accept genus	
<i>Gracilariophila</i> Setchell & H.L.Wilson, 1910	2	Parasite. Currently accept genus	
<i>Gracilariopsis</i> E.Y.Dawson, 1949	22	Currently accept genus	
<i>Hydropuntia</i> Montagne, 1842	0	Synonym of <i>Gracilaria</i>	Lyra et al. 2015
<i>Melanthalia</i> Montagne, 1843	4	Currently accept genus	

All these genera of Gracilariaceae presented a limitation in relation to the taxonomically informative characters used in the species identification, leading to discussions on the taxonomic condition of these genera. The high number of species, the wide geographic distribution and the presence of cryptic species contribute to the taxonomic confusion (Oliveira 1984; Plastino and Oliveira 1994). In addition, the recognition of species, or genera, is a complex task since it usually requires fertile plants and is only possible after microscopic analysis and the study of characteristics related to the ontogeny of spermatogenesis and to the development of post-fertilization carpogonia (Oliveira 1984; Fredericq & Hommersand 1989a). The addition of molecular studies with a

combination of morphological and anatomical data was important to the development of systematics of Gracilariaceae.

Molecular phylogenetic studies that included *Melanthalia* and/or *Curdiea* supported the monophyly of the genera (Bird et al. 1992, Bellorin et al. 2002, Gurgel and Fredericq 2004, Lyra et al. 2015). The recognition of *Gracilariopsis* as a distinct genus was also supported by molecular analysis (Bird et al. 1992, 1994; Bellorin et al. 2002; Gurgel et al. 2003, Lyra et al. 2015). However, molecular studies with Gracilariaceae were controversial in relation to the permanency of *Hydropuntia* (Bellorin et al. 2002). One of the first phylogenetic analyses with Gracilariaceae was done combining parsimony, distance and maximum-likelihood (ML) analysis using nuclear-encoded small subunit ribosomal gene (SSU rDNA) indicated that *Gracilaria* and *Hydropuntia* might be distinct taxa (Bird et al. 1992). However, the sampling of this research was very limited (Bird et al. 1992). This study also suggested that *Gracilaria chilensis* C.J.Bird, McLachlan & E.C.Oliveira (1986) would represent another group (Bird et al. 1992). Posteriorly, a phylogenetic analysis with SSU rDNA and internal transcribed spacer (ITS) regions combining parsimony, distance, and ML, with a larger dataset, resulted that species bearing *henriquesiana*-type spermatangia (*Hydropuntia*) and *Gracilaria* were not phylogenetically separated (Bellorin et al. 2002). A while later, another molecular study including the chloroplast ribulose 1,5-bisphosphate carboxylase large subunit gene (*rbcL*), which was considered to provide better resolution for inferring species level, and Bayesian analysis, beside the molecular markers and phylogenetic methods previous reported, determined a major clade including *Gracilaria* (most of the *Gracilaria* species – called *Gracilaria sensu stricto*), *Hydropuntia* complex and a new unnamed genus containing *G. chilensis* (Gurgel and Fredericq 2004). This major clade was called *Gracilaria sensu lato*. They considered that these three lineages should be distinct genera (Gurgel and Fredericq 2004). The largest and most well-resolved phylogeny of Gracilariaceae to date showed that *Hydropuntia* is not a monophyletic genus and some species with *henriquesiana*-type spermatangia were grouped in the *Gracilaria sensu stricto* clade (Lyra et al. 2015). Furthermore, the morphological features used to distinguish both genera overlap in some cases. Therefore, they concluded that *Hydropuntia* should be synonymized to *Gracilaria* (Lyra et al. 2015).

Economic importance

There are species of Gracilariaceae that have great economic importance, being highly relevant in biotechnology, food, pharmaceutical, and textile industries, due to the wide applicability of the hydrocolloids (agar-agar) present in their cell walls (Zemke-White & Ohno 1999). In addition to the production of agarans, pharmaceutical studies of *Gracilaria* species, including with Brazilian species, show the presence of molecules with antiviral, anti-inflammatory and antihypertensive properties (Smit 2004, Vanderlei et al., 2011, Amorim et al., 2012).

The species of Gracilariaceae that are indicated as ideal for the cultivation are generally terete and wherefore most of the studies are focused on terete species (Bellorin et al. 2002). Economically species must have a high content of agar-agar, fast growth and be easy to reproduce vegetatively.

Therefore, precise taxonomic identification of species selected for cultivation is important, specially considering the occurrence of cryptic species (Santelices & Doty 1989).

Gracilariaceae terete species are cultivated in several parts of the world and currently represent the main source for the production of agar-agar (McHugh 2003). In Brazil, *Gracilaria* is the most economically important genus. In the 1970s, the exploitation of these agarophytes was made by collecting biomass directly from natural banks on the Northeast coast. The biomass was sold for the production of colloids, which resulted in a decrease of these reserves along the coast. Sea cultivation attempts were made, but they achieved little success due to the lack of basic knowledge about the species selected for cultivation (Carneiro 2011). Currently, with incentives to sea aquaculture, some *Gracilaria* species have been used in pilot experiments in the Northeast coast and better results are being obtained (Bezerra & Marinho-Soriano 2010, Carneiro 2011). Therefore, the interest in studies with Brazilian species for industrial-scale production has increased (Marinho-Soriano et al., 2007; Carneiro 2011).

High-throughput sequencing

Since the advent of the DNA sequencing, molecular studies become highly important to understand the complexity and diversity of the life. Sequencing of complete or nearly complete genomes is a key step in better understanding the biology of living organisms. The first genomes were sequenced using the traditional chain-terminated Sanger DNA sequencing method (Sanger 1977) in automatic sequencers, which were posteriorly called first generation sequencing (Dijk et al. 2014a). Only a single fragment, between 700-900 base pairs (bp), previously amplified by polymerase chain reaction (PCR) and cloned, was sequenced in each capillary of the sequencer, which had 96 capillaries. For the study of genomes, this method is highly costly and time-consuming (Kim et al. 2014a). For example, the Human Genome Project (International Human Genome Sequencing Consortium 2004) spent more than 10 years to publish the complete genome at a cost of about US\$ 3 billion (Dijk et al. 2014a). It was clear that faster, higher throughput, and cheaper technologies were required for genome sequencing (Dijk et al. 2014a). In the last two decades, new DNA sequencing technologies have arisen decreasing the time and costs, making it possible to obtain larger molecular datasets. These new technologies were first called Next Generation Sequencing, but nowadays are better called High-Throughput Sequencing (HTS).

The number of genomic studies has increased rapidly since of the last decade thanks to HTS (Chan and Ragan 2013). HTS allows the generation of millions of DNA reads – the HTS output – in a single process, without the need to go through cloning steps and in some cases without the need for PCR before entering the sequencer (Dijk et al 2014a). A range of technologies is now available for use in HTS. Among these are several short-read sequencing platforms, which produce much shorter reads than traditional Sanger sequencers, as well as some long-read platforms. For short-read sequencing, Illumina is the dominant system in the HTS market (Reuter et al. 2015; Oliveira et al. 2018), which possess different types of sequencing instruments with a large range of output quantity, from low-

throughput to very large output (up to 900 Gb in Illumina HiSeq X) (Goodwin et al. 2016). Error rates are very low for all these platforms, compared with other technologies. PacBio and MinION technologies can produce long sequence reads but produce a lower yield and generally have a higher error rate than Illumina (Goodwin et al. 2016, Oliveira et al. 2018). They are typically used to complement the more accurate and higher-throughput Illumina methods for assembly and genome finishing (Oliveira et al. 2018).

Before sequencing, the HTS platforms require that a DNA library be constructed for each sample. A DNA library is a collection of DNA fragments each containing oligonucleotide adapters at their ends that are compatible with the sequencing system (Head et al. 2014). Library protocols consist of four main steps: DNA fragmentation, size selection, adapter ligation, and final DNA library product quantification for sequencing (Head et al. 2014). Illumina sequencing method is based on sequencing by synthesis, which each nucleotide is identified during the sequencing after its incorporation into an elongating strand (Goodwin et al. 2016). The sequencing process involves clonal amplification of adaptor-ligated DNA fragments on the flow cell, which is a glass slide with adaptors on the surface, where the DNA fragments are linked (Bentley et al. 2008; Reuter et al. 2015; Goodwin et al. 2016). After this process of amplification of each DNA fragment in clonal clusters, the sequencing by synthesis is performed (Goodwin et al. 2016).

The advantage of HTS is the wide range of its application to generate different types of output. For example, a whole-genome sequencing (WGS) including the total genomic DNA of the organism, which include organelle DNA, plasmid DNA, nuclear DNA, and genomes from endosymbionts. It is also possible to sequence a set of different organisms in the same run, using the multiplexing approach, in which the DNA fragments of each sample is ligated to a short sequence tag (called index or barcode) and the reads of each sample are latter identified by bioinformatic tools (Oliveira et al. 2018). The biggest challenge about HTS technologies is the high demand for bioinformatic tools capable of sorting, analyzing and managing large amounts of data (Dijk et al. 2014a; Oliveira et al. 2018). Each HTS run produce millions of sequences reads that require specific computational analysis workflows to produce interpretable results. Individual steps in a workflow can be carried out by different programs, with customizable sets of features (Oliveira et al. 2018).

Genomic studies in red algae

The HTS techniques have transformed many areas of biology, including the study of algae, allowing large-scale approaches in the evolutionary investigation, systematics, biodiversity, genetic population, gene expression, and epigenetic (Kim et al. 2014a; Bhattacharya et al. 2015; Oliveira et al. 2018). At the time of writing of this thesis, eight studies of nuclear genome have been performed so far for red algae. The extremophilic unicellular red alga *Cyanidioschyzon merolae* P.De Luca, R.Taddei & L.Varano (1978) was the first complete algal genome (Matsuzaki et al. 2004). Two other unicellular red algae had their nuclear genomes sequenced: the mesophilic *Porphyridium purpureum* (Bory) K.M.Drew & R.Ross (1965) (Bhattacharya et al. 2013) and another extremophilic red alga

Galdieria sulphuraria (Galdieri) Merola (Merola et al. 1982), which live in hot, toxic metal-rich, acidic environments (Schönknecht et al. 2013). For the seaweeds, two Bangiophyceae, *Pyropia yezoensis* M.S.Hwang & H.G.Choi in Sutherland et al. (2011) (Nakamura et al. 2013) and *Porphyra umbilicalis* Kützinger (1843) (Brawley et al. 2017); and three Florideophyceae, including two Gracilariaceae, *Gracilariopsis lemaneiformis* (Zhou et al. 2013) and *Gp. chorda* (Lee et al. 2018), and, the carrageenan-producing macroalga, *Chondrus crispus* Stackhouse (1797) (Collén et al. 2013).

The known nuclear genomes of Rhodophyta are relatively small compared to other eukaryotic strains (Matsuzaki et al., 2004; Collén et al., 2013; Lee et al. 2018). The smaller known genome of a red algae is *Galdieria sulphuraria*, with 13 Mb (Schönknecht et al. 2013) and *Chondrus crispus* has the largest sequenced genome, so far, with around 105 Mbp (Collén et al. 2013). The draft genomes of the two *Gracilariopsis* are 97 Mb and 92 Mb length to *Gp. lemaneiformis* (Zhou et al. 2013) and *Gp. chorda* (Lee et al. 2018), respectively. The reduced genome is probably because the apparent absence of polyploidization and majority of monoexonic genes (88% in *C. crispus*) (Collén et al. 2013; Lee et al. 2018). The knowledge about the genomes of red algae is highly important because this group presents great economic importance, as exemplified above for gracilarioid algae, and, also have great evolutionary importance since they originated through secondary endosymbiosis the complex chloroplasts of other eukaryotic lineages: Heterokonta, Alveolata, Cryptophyta and Haptophyta (Kim et al. 2014). Therefore, red algae genes are spread throughout the Eukarya Tree of Life and the genetic and genomic knowledge of red algae are crucial for understanding the evolution of Eukaryotes (Collén et al. 2013; Kim et al. 2014).

The use of HTS techniques has led to an increase in studies using complete organellar genomes. Dozens of chloroplast and mitochondrial genomes have been published in GenBank in the last years. At the time of the writing of this work, there were available eight chloroplasts and eleven mitochondrial genomes of Gracilariaceae species in Genbank (Table 2). Most of these are from economically important crop species, e.g. *Gracilariopsis chorda* (Yang et al. 2014b), *Gp. lemaneiformis* (Zhang et al. 2012, Zhang et al. 2016), *Gracilaria firma* Chang & Xia (1976) (Ng et al. 2017), *G. tenuistipitata* var. *liui* Zhang & Xia (1988), which was the first complete chloroplast genome published of Florideophyceae (Hagopian et al. 2004). The family was also investigated in the parasitic relationship between the parasite *Gracilariophila oryzoides* and its host *Gp. andersonii* using mitochondrial genomes (Hancock et al. 2010). Organellar genomes have been used to infer phylogenetic relationships in Rhodophyta, first for the study of higher taxonomical levels (Janouškovec et al. 2013, Yang et al. 2015, Lee et al. 2016a) and more recently for order and families (Costa et al. 2016, Díaz-Tapia et al. 2017), using phylogenomics approaches, which is the study of evolutionary relationships based on the comparison of data analyzes at the genomic scale (Chan & Ragan 2013).

Table 2 – Mitochondrial and chloroplast genomes from Gracilariaceae published in GenBank.

Mitochondrion									
Species	INSDC	Size (Kb)	GC%	Protein	rRNA	tRNA	Other RNA	Intron	Reference
<i>Gracilaria changii</i> (B.M.Xia & I.A.Abbott) I.A.Abbott, J.Zhang & B.M.Xia	KX980031	25,729	27.7	25	3	24	-	1	Song et al. 2017
<i>Gracilaria chilensis</i> C.J.Bird, McLachlan & E.C.Oliveira	KP728466	26,898	27.6	25	3	26	-	1	Lee et al. 2015
<i>Gracilaria chilensis</i>	MF401962	26,897	27.6	25	3	25	-	1	Liu et al. unpublished
<i>Gracilaria chouae</i> Zhang & B.M.Xia	MF351970	25,224	28.2	25	3	20	-	1	Zhang and Liu unpublished
<i>Gracilaria salicornia</i> (C.Agardh) E.Y.Dawson	KF824534	25,272	28.4	25	3	21	-	1	Campbell et al. 2014
<i>Gracilaria salicornia</i>	KT373903	25,915	28.6	25	3	24	-	1	Song et al. 2016
<i>Gracilaria vermiculophylla</i> (Ohmi) Papenfuss	KJ526627	25,973	28.1	25	3	22	-	1	Chi et al. unpublished
<i>Gracilariophila oryzoides</i> Setchell & H.L.Wilson	KX687879	25,184	28.1	25	3	21	-	1	Salomaki and Lane 2016
<i>Gracilariopsis andersonii</i> (Grunow) E.Y.Dawson	KX687878	27,011	28	25	3	21	-	1	Salomaki and Lane 2016
<i>Gracilariopsis chorda</i> (Holmes) Ohmi	KC875851	26,534	27.6	26	3	24	-	1	Yang et al. 2014b
<i>Gracilariopsis lemaneiformis</i> (Bory de Saint-Vincent) E.Y.Dawson, Acleto & Foldvik	JQ071938	25,883	27.5	26	3	21	-	1	Zhang et al. 2012
Chloroplast									
<i>Gracilaria chilensis</i>	KT266788	185,637	29.3	205	3	30	2	1	Lee et al. 2016b
<i>Gracilaria chilensis</i>	MF401963	185,640	29.3	202	3	30	2	1	Liu et al. unpublished
<i>Gracilaria firma</i> Chang & Xia	KX601051	187,001	28.1	216	3	30	2	1	Ng et al. 2017
<i>Gracilaria salicornia</i>	KF861575	179,757	28.8	204	3	30	2	1	Campbell et al. 2014
<i>Gracilaria tenuistipitata</i> var. <i>liui</i> Zhang & Xia	AY673996	183,883	29.2	206	3	30	2	1	Hagopian et al. 2004
<i>Gracilariopsis chorda</i>	KX284722	182,459	27.4	203	3	30	2	1	Lee et al. 2016a
<i>Gracilariopsis lemaneiformis</i>	KU179794	182,505	27.4	208	3	30	2	1	Zhang et al. 2016
<i>Gracilariopsis lemaneiformis</i>	KP330491	183,013	27.4	203	3	31	2	1	Du et al. 2016

Organellar genomes have been also facilitated the investigation of other aspects of red algal genomes, including gene synteny and horizontal gene transfers (HGT). For example, sequences derived from previous reported Rhodophyta plasmids (e.g. Goff and Coleman 1990, Villemur 1990a,

1990b, Moon and Goff 1997) were observed in chloroplast genomes in Rhodophyta (Lee et al. 2016b). Furthermore, HGT from a bacterial plasmid, which contained the genes *leuC* and *leuD*, were also reported in the chloroplast genomes of *Gracilaria tenuistipitata* var. *liui* (Hagopian et al. 2004, Janouškovec et al. 2013) and *G. chilensis* (Lee et al. 2016b).

Plasmids are extrachromosomal DNA molecules, autonomously replicating, generally circular, AT-rich, double-stranded and may have active open reading frames (Goff and Coleman 1990, Moon and Goff 1997, Lee et al. 2016b). They were first found in some species of Rhodophyta in a study of delimitation of species and populations of Gracilariaceae, using endonuclease digests of chloroplasts genomes (Goff and Coleman 1988). Up to now, only five species contain one or more extrachromosomal plasmids published in GenBank (Table 3), most of them are Gracilariaceae species (Goff and Moon 1990, Villemur 1990a, Lee et al. 2016b) which means that this family is interesting to investigate the evolution of plasmids in red algae and their relationship with organellar and nuclear genomes. These genetic elements have no known function in these seaweeds, conferring no obvious phenotype to species possessing them (Moon and Goff 1997). They occur in apparently in high proportions in the genomic DNA and may be present in high copy number per cell. Their exact cellular location is not yet known but may be reside in the nucleus (Goff and Coleman 1990). Previous hybridization studies indicated that plasmid sequences were not present integrated in the nuclear and organelles genomes. Also, these studies indicated that these plasmids are autonomously replicated, each possessing its own origin of replication site (Goff and Coleman 1990). However, analysis of complete mitochondrial and chloroplast genomes suggests that plasmid sequences behave as parasitic elements that spread plasmid-derived DNA regions in organellar genomes (Lee et al. 2016b).

Table 3 - Information of red algae plasmids available in GenBank.

Plasmid	Accession number	Organism	Sequence length	GC %	Topology	N° of ORFs	Reference
Gch3937	AF034721	<i>Gracilaria chilensis</i>	3,937	25	Circular	5	Goff and Moon, unpublished/Lee et al. 2016b
Gch7220	AF034719	<i>Gracilaria chilensis</i>	7,220	24.9	Circular	16	Goff and Moon, unpublished/Lee et al. 2016b
Gch_Gc2	X51317	<i>Gracilaria chilensis</i>	3,827	24.9	Circular	1	Villemur 1990b
Gro4059	AF034717	<i>Gracilaria robusta</i>	4,059	26.5	Circular	2	Goff and Moon, unpublished/Lee et al. 2016b
Gro4970	AF034718	<i>Gracilaria robusta</i>	4,970	25.8	Circular	7	Goff and Moon, unpublished/Lee et al. 2016b
Gle4293	AF034720	<i>Gracilariopsis lemaneiformis</i>	4,293	26.8	Circular	5	Goff & Coleman 1990
Gle_GL3.5	X56441	<i>Gracilariopsis lemaneiformis</i>	3,491	25.9	Linear	2	Goff & Coleman 1990
Pp6427	AF002711	<i>Pyropia pulchra</i>	6,427	35.5	Circular	5	Moon & Goff 1997
Pp6859	AF002712	<i>Pyropia pulchra</i>	6,859	32.9	Circular	7	Moon & Goff 1997
pPT4	FJ956744	<i>Pyropia tenera</i>	1,820	41.7	Circular	2	Choi et al. 2001

Objectives

General

Considering the relative ecological and economic importance of Gracilariaceae, our study aimed obtain organellar genomes of representative species of the main lineages of Gracilariaceae with HTS and use these data to investigate the evolution of the family using organellar genomic architecture and a phylogenomic approach.

Specific

- Characterize new mitochondrial and chloroplast genomes of Gracilariaceae, expanding the number of different lineages within the family;
- Evaluate the evolutionary history of the family using phylogenomics;
- Compare the genomic architecture of each of the organelles, including an investigation of plasmid derived sequences.

Conclusion

High-throughput sequencing is a very powerful technique to obtain organellar genomes from whole-genome sequencing of algae. We could obtain all the organellar genomes from the reads of each sample. Even when it was not possible to close the circular genome, we could extract most of the genes used for phylogenomics analysis. Furthermore, we could also extract the plasmid genomes from the reads of some species, which improved our understanding about the influence of the plasmid-derived sequences in the organellar genomes. Beside this, all the nuclear genomes are available to future research. Therefore, with one single run (or two, including Dr. G. Lyra database) we obtained a large and high-quality data for this research and, possible, many other future projects.

Organellar genomes are attractive to investigate the evolution of the organisms. Our phylogenies are the first using larger gene content matrices (23 genes for the mitochondrial and 187 genes for the chloroplast) for Gracilariaceae and had the highest supports and congruent results between chloroplast and mitochondrial phylogenomics. Furthermore, chloroplast tree had full support for all those nodes, which corroborates the use of chloroplast genomes as a promising resource to resolve red algal phylogenies. Our taxonomical sampling represents the main lineages that have been discussed in Gracilariaceae systematics literature and supports the presence of three main lineages: *Melanthalia/Curdia*, *Gracilariopsis* and *Gracilaria*. Furthermore, the characteristics of organellar genome architecture, *Gracilariopsis* and *Gracilaria* are also supported by the loss of the plastid gene *petP* in *Gracilaria* and the rearrangement position of the gene *trnH* in the mitochondrial genome. Beside this, we found no support for the genus *Hydropuntia* as originally proposed.

The genomic architecture is useful to understand the nature and source of change that occurred during the evolution of organisms. Organellar genomes are highly conserved in Rhodophyta, but also have variable regions that can be useful for comparisons at species or population levels, e.g. the plasmid insertion regions. Plasmids have been recognized as mobile elements. Their origin and the process of horizontal gene transfer into organellar genomes of red algae remain unclear (Lee et. al. 2016b). Moreover, Gracilariaceae is a good model to study the impact of PDS in genome evolution due to the significant presence of these sequences in organellar genomes.

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