**The emergence of molecular profiling and omics techniques in seagrass biology; furthering our understanding of seagrasses**

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**Figures and tables included:**

**Figure 1:** Illustrative diagram collectively showing the specialized traits of seagrasses, which allow for seagrasses to live a submerged life in the coastal marine environment. **Information sources:** Marbà et al. 2002; Ackerman 2006; Larkum et al. 2006; Touchette, 2007; Broderson et al. 2015; Hasler-Sheetal et al. 2015. Illustrative model concept is based on *Zosteraceae* family.

**Figure 2:** The advances in seagrass molecular profiling and omics to date. The technologies that have been utilized are shown along the bottom of the illustration, whilst types of study conducted to date are shown at the top of the illustration.

**Abstract**

Seagrass meadows are disappearing at alarming rates as a result of rising coastal development and climate change. The emergence of omics and molecular profiling techniques in seagrass research is timely, providing a new opportunity to address such global issues. Whilst these applications have transformed terrestrial plant research, they have only emerged in seagrass research within the past decade; we have observed a significant increase in the number of publications in this nascent field, and as of this year the first genome of a seagrass species has been sequenced. In this review, we focus on the development of omics and molecular profiling and the utilization of molecular markers in the field of seagrass biology. We highlight the advances, merits and pitfalls associated with such technology, and importantly we identify and address the knowledge gaps, which to this day prevent us from understanding seagrasses in a holistic manner. By utilizing the powers of omics and molecular profiling technologies in integrated strategies, we will gain a better understanding of how these unique plants function at the molecular level and how they respond to on-going disturbance and climate change events.

**1 - Introduction**

Primary productivity and nutrient recycling by seagrass meadows play major roles in the promotion and protection of coastal biodiversity (Orth et al. 2006; Cristianen et al. 2013). Equally important, their carbon sequestration capacity dwarfs that of boreal, temperate and tropical forests (McLeod et al. 2011). It has been estimated that the total productivity of seagrass meadows is approximately $29,000 US dollars per hectare per year, which is considerably more than that of terrestrial forests, grasslands and open ocean productivity (Costanza et al. 2014).

A recent meta-analysis has suggested that we are loosing a staggering 7% of global seagrass meadow coverage per year (Waycott et al. 2009), a figure that is likely to increase in future due to mounting anthropogenic and climate change pressures (Orth et al. 2006; Ralph et al. 2007; Björk et al. 2008; Waycott et al. 2009). Given the wide range of threats that have been identified for seagrass meadows (Orth et al. 2006; Björk et al. 2008; Waycott et al. 2009), it is of major concern that we still lack fundamental knowledge about the molecular biology of these plants and how they will respond to future climates. In comparison to our molecular knowledge of terrestrial plants, our understanding of seagrass molecular biology is somewhat in its infancy.

Sequencing technologies in plants have rapidly developed since the genome sequencing of the model plant *Arabidopsis thaliana* (Kaul et al. 2000). As of 2013, genomes had been sequenced for 49 plant species (Michael and Jackson. 2013). Whilst many important crop species have already had their genome sequenced (Yu et al. 2002; Jallion et al. 2007; Paterson et al. 2009; Schnable et al. 2009; Schmutz et al. 2010; PGSC 2011; Chalhoub et al. 2014; Mayer et al. 2014), it has only been of this year that the first genome of a seagrass (*Zostera marina)* has been completely sequenced (Olsen et al. 2016); it is therefore expected that we will observe increased research activity in this niche area. Whilst deciphering of the genome is invaluable, the insights offered by *de novo* transcriptomics, proteomics and metabolomics are also of high value. The *1k plant transcriptome project* by the iPlant Collaborative is one such example which has taken advantage of transcriptome sequencing. For seagrasses, several studies have made use of transcriptomics to date (Gu et al. 2012; Franssen et al. 2014; Kong et al. 2014; Olsen et al. 2016). The importance of molecular profiling and omics in plant science not only offers opportunities for bio-prospecting (Annadurai et al. 2012), but also for exploring the fundamental genetic mechanisms of plants (Mochida and Shinozaki. 2011) and projecting how species will respond to disturbance and climate change events (Ahuja et al. 2010). In this review, we discuss the current role of omics, molecular profiling and the use of genetic markers in the field of seagrass biology and how they have and will further help us to understand seagrasses in a more holistic manner. Furthermore, such information will help us to understand how seagrasses will respond to future climatic and disturbance events. We also highlight the merits and pitfalls of such techniques, and the knowledge gaps, which currently exist in seagrass biology.

**2 - Seagrasses: A unique group of plants**

Seagrasses are a polyphyletic group of marine plants belonging to the monocotolydonous lineage of the angiosperms. Seventy-two species are classified within 6 families; *Cymodoceaceae*, *Hydrocharitaceae*, *Posidonia*, *Ruppiaceae*, *Zannichelliaceae* and *Zosteraceae* (Short et al. 2011). Seagrasses evolved ca. 100 million years ago (mya) during the Cretaceous period (den Hartog 1970); recent evolutionary analysis for *Z. marina* indicates this species underwent a whole genome duplication event approximately 72-64 mya, but diverged from the monocot genera, Spirodela approximately 135-107 mya (Olsen et al. 2016). The seagrasses have feasibly experienced the most extreme evolutionary events witnessed in the angiosperm lineage (Olsen et al., 2016), they have evolved unique features to cope with survival in a saline, CO2-limited and dynamically changing marine environment due to tidal oscillations which change light availability, water flow and temperature. Figure 1 highlights the common specialized adaptive traits of seagrasses. For more detail on such specializations please refer to available literature (Ackerman. 2006; den Hartog & Kuo. 2006; Larkum. 2006; Marbà et al. 2006; Touchette, 2007).

**3 - The current status of omics and molecular profiling in seagrass biology**

Omic and molecular profiling studies have provided seagrass biologists a revolutionary approach to how seagrasses can be studied. The emergence of these approaches in seagrass biology has been relatively slow in comparison to terrestrial plants. To the best of our knowledge 31 research-based studies (excluding reviews and editorial notes) have been published since 2006, which integrate such approaches (Table 1, Figure 2). In such a short period of time, these studies have presented us with novel information on evolution, stress response, resilience and variation within and between the species studied. Studies have given us an insight into how seagrasses and land plants are similar but also dissimilar at the molecular level. Such advances are; however, majorly limited to only two species, *Z. marina* and *P. oceanica* (Table 2). These two species are geographically distributed in the Northern Hemisphere, and from a critical perspective, a wider range of global seagrass species need sequenced, especially now that technology cost has depreciated and technology has become readily accessible. Much of the current focus has been on thermal response, whilst some attention has been emphasised on light response; as such a broader approach is needed in seagrass omics, taking other important anthropogenic and climatic stressors into account. Noteworthy, in this respect we have recently observed the examination of seagrass species including the Southern Hemisphere species, *Zostera muelleri,* and the species *Cymodocea nodosa* (Table 2).

Transcriptome studies which have been completed in seagrasses to date (Table 2) have provided us with snapshots of gene expression at given times under specific conditions in species. The majority of these studies have focussed on short-term response, rather than recovery and resilience over longer periods of time. Franssen et al. (2014); however, provide a good example of an environmental response and recovery study. Transcriptomics is of course highly valuable, but without doubt deep genomic sequencing can provide more information on coding sequences as well as non-coding sequences. Such information is important for the advances of understanding genomic structure, function and evolution. Least to say, epigenetics is one area of seagrass omics that has failed to receive much attention to date (Table 2). Transposable elements, micro-RNAs (Lorenzetti et al. 2016), sRNAs, ncRNAs and other non-coding genic elements can help us to understand how coding regions of the genome are controlled and expressed under different environments, as previously shown in grape vine (Singh et al. 2012) and rice (Zhang et al. 2016). The genome of *Z. marina* and genome-wide analysis of *P. oceanica* provide details of non-coding regions and miRNAs within seagrass genomes (Barghini et al. 2015; Olsen et al. 2016); and as such this information will be most valuable for future epigenetic research in seagrass. It goes to mention, CHIP-Seq has yet to emerge in seagrass research; with the design of suitable antibodies and utilization of suitable methodology, epigenetic regulation such as histone modification can be effectively studied (Shin et al. 2012). In terms of genome complexity, the size of the *Zostera muelleri* genome has been estimated to be ~900 Mbp (Golicz et al. 2015), whilst the *Zostera marina* genome is 202.3 Mbp (Olsen et al. 2016). *P. oceanica* is suggested to exhibit a genome size that is 5 times larger than *Z. marina* (Barghini et al., 2015). Such information reveals the variation between seagrass species at the molecular level, and without doubt makes them an interesting group of plants to study, given that they are all functionally adapted to the marine coastal environment. Examination of the literature; however, reveals that several key knowledge gaps exist.

**3.1 – Seagrass light perception and response at the molecular level**

Perhaps the biggest threat known to seagrass ecosystems is direct and indirect light limitation (Ralph et al. 2007). In the past, large areas of seagrass die-off have been attributed to light limitation as a result of poor water quality (Ralph et al. 2007). Such threats are predicted to increase with increasing anthropogenic disturbance and climate change. Photo-physiology methods utilizing Pulse Amplitude Modulated (PAM) chlorophyll fluorometry have served as the most effective tools for understanding how seagrasses respond and acclimatize to varying light. PAM technology provides us with quantitative measurements (Ralph 2002; Datallo et al. 2014) and therefore comprehensive estimations of plant health. To date, only three species of seagrass; *Zostera marina* (Kong et al. 2014)*,* *Zostera muelleri* (Pernice et al. 2015; Schliep et al. 2015) and *Posidonia oceanica* (Mazzuca et al. 2009; Greco et al. 2013; Datollo et al. 2013, 2014), have been characterized using molecular datasets in relation to varying irradiance. Such studies have long been awaited, as they allow us to characterise how seagrasses use environmental light cues to control regulation and metabolism. The genome has also provided valuable insight into light perception (Olsen et al. 2016).

Dattolo et al’s. (2013) *in situ* study on the acclimation of *P. oceanica* to different water depths (i.e light levels) has identified several regulatory networks and pathways involved in response to different depth gradients and thus has provided a host of eco-genomic resources for future studies. Additionally, seagrass plasticity at the functional molecular level is evident in response to varying light. For *P. oceania* such studies are important, given that this species is rapidly disappearing in the Mediterranean (Datollo et al. 2013). Changes in photosynthesis, cellular energetic metabolism, protein turnover and stress response were most widely observed at the transcript and proteomic level. Indeed proteolysis and protein turnover have also previously been shown to up-regulate in *P. oceanica* under chronic low light in previous proteomic experiments (Mazzuca et al. 2009). Datollo et al. (2013) also noted differences in the chlorophyll binding proteins between plants occurring at different depths, suggesting photosystem complexes may re-arrange to cope with the different levels of light irradiance as similarly observed in land plants (Masuda et al. 2003). Additional work by Datollo et al. (2014) has shown that distinct light associated gene expression is linked to depth distribution. Furthermore, the photosynthetic light harvesting complex B (LHCB) genes have been found to be more abundant in *Z. marina* than in terrestrial counterparts, thereby presumably enhancing photosynthetic performance at lower irradiances in the water column (Olsen et al., 2016). Kong et al. (2016) have also recently identified light harvesting complex (LHC) genes in *Z. marina* suggesting that *LHC* genes are conserved across marine plants and land plants.

The photoreceptor and light-mediated transcription factors in *Z. marina* (Kong et al. 2014; Olsen et al. 2016) have been identified. The most significant difference in *Z. marina* compared to land plants is that only 2 phytochromes (*PHYA* and *PHYB*) have been identified, this may suggest that *PHYC* is absent in seagrasses perhaps due to a submerged lifestyle, given that this receptor has less of a role in red-light detection (Franklin et al. 2003). Additionally it has also been suggested that *PHYC* plays a role in flowering, which is of course reduced at the genic level in seagrasses (Woods et al. 2014; Olsen et al. 2016) and may therefore be associated with such functional reductions. Similarly, UV light protective UVR8 transcripts have been lost completely (Olsen et al. 2016). In *P. oceanica* photoreceptors have also been reported for blue and red wavelengths (Greco et al. 2013); suggesting the importance of these genes in perception of light quality within the water column. Additionally Kong et al. (2016) have also validated changes in expression of light harvesting complexes in response to spectral shifts. Whilst cited research (Olsen et al. 2016) gives us an idea that seagrasses may rely less on far red: red light, we suggest that further research should investigate how shallow and deep dwelling seagrass species utilize wavelengths of light differently, as key evolutionary differences may exist. Transcripts associated with chlorophyll production, pigment synthesis, binding, and the photo-protective xanthophyll cycles have also been identified (Datollo et al. 2013; Datollo et al. 2014; Kong et al. 2014; Olsen et al. 2016) suggesting that adaptation, acclimation and photo-protection are all logically regulated at the molecular level in seagrasses, and lead to changes observed at the physiological level (Ralph et al. 2002; Sharon et al. 2009). The sequencing of the chloroplast genome of *Zostera marina* (Olsen et al. 2016) will become a valuable resource for understanding light responses. Given the realistic threat of meadow decline in relation to low-light, low-light related senescence needs examined in detail. In recent work (Grandellis et al. 2016) molecular profiling has identified several mechanisms, which play a role in the process of senescence during light starvation in the potato crop. The roles of brassinosteroids in low light response are of interest, as brassinosteroids have been shown to promote resistance to low light stress in tomato (Cui et al. 2016). These hormones are indeed conserved in seagrasses (Olsen et al. 2016).

The application of gene expression profiling technology, such as RT-qPCR has also played an important role in shaping the molecular research of seagrasses, Pernice et al. (2015) have recently utilized a molecular tool kit to detect dredging-associated stress (light-starvation through increased turbidity) in *Z. muelleri* in the port of Gladstone in Queensland, Australia. It is possible that tool kits like this one can provide a model for the implementation of further molecular-based monitoring efforts. These approaches should; however, be designed carefully and treated with caution as gene expression has been found to be highly variable between genotypes of *Z. marina* in shading and recovery experiments (Salo et al. 2015). As a result we therefore suggest that ecological consultancy and marine scientists use a combination of chlorophyll fluorometry, physiology and molecular techniques until a further understanding of molecular light responses and the genetic variation within regional meadows is acquired.

**3.2 – Carbon fixation in seagrasses – challenging old beliefs with new technology.**

It has long been accepted that seagrasses contain a carbon concentrating mechanism (CCM) to support carbon sequestration. A detailed conceptual diagram of the suggested seagrass CCM is clearly explained and illustrated by Larkum et al. (2006). CCM’s are a common adaptation in many autotrophic organisms (Badger and Price 2003; Raven et al. 2008) with CO2-limited environments often observed as the driving force behind such selectivity (Raven et al. 2008). Despite comprehensive reviews on seagrass carbon fixation and metabolism (Touchette and Burkholder 2000; Beer et al. 2002; Larkum et al. 2006), our knowledge of carbon fixation in seagrasses at the molecular level is still poor. Given the emergence of omics, interest seems revived, now that we possess higher resolution capability. In respect to photosynthetic systematics; to classify seagrasses as C3 or C4 photosynthetic autotrophs remains a challenge in its own right; past studies have observed C3 and C4 carbon signatures present across a range of seagrasses (Andrews and Abel, 1979; Benedict and Scott 1979; Beer et al. 1980). Of course such conflicting reports are perplexing given that we know seagrasses lack true Kranz anatomy and bundle sheath cells. A recent analysis of an EST-derived dataset may of course provide subtle clues of evolutionary based pressure occurring within photosynthetic and carbon metabolism pathways in *P. oceanica* and *Z. marina* (Wissler et al. 2011); however, given the size of the dataset, more effort is needed to validate such findings.

*Z. marina* carbonic anhydrase and boron HCO3 transporter genes have also been identified, perhaps providing evidence of the CCM operation (Olsen et al. 2016). RubisCO sub-units have also been shown to be negatively regulated within *P. oceanica* in response to lower levels of light (Mazzuca et al. 2009; Datollo et al. 2014) while methylation activity of phosphoenolpyruvate carboxylase (PEPC) is altered during changes in irradiance level (Greco et al. 2013). The previous theory of C3-C4 intermediate photosynthesis existing in seagrass species (Touchette and Burkholder 2000) remains plausible; however, C4 related enzymes are also known to play roles in anaplerotic reactions within plants (Doubnerová and Ryšlavá 2011). It is possible C4-type photosynthesis within seagrasses, could operate independently of true Kranz anatomy; however, this is supported by a theory of single-cell C4 photosynthesis (Edwards et al. 2004), which has been shown to operate in the aquatic plant, *Hydrilla verticillata*, a close relative of the *Halophila* genus of seagrass (Bowes et al. 2002; Bowes et al. 2011). We therefore suggest that a range of carbon fixation pathways may exist across the seagrass group until further work elucidates the exact carbon fixation pathways. We believe that omics alone will not unlock the carbon fixation pathway of seagrasses, but perhaps an integrated approach involving omics, microscopy and immuno-localization techniques is necessary. Such work will allow us to accurately determine seagrass response to predicted CO2 fluctuations in the future.

**3.3 –** **Are stress and environmental response signatures between land and marine plants different?**

An obvious difference between land plants and seagrasses is that seagrasses are fully submerged. Our understanding of how plants respond to their environment is growing steadily, but the differences between land plants and seagrasses remain largely elusive. In seagrasses, oxidative stress protective genes have commonly been identified and associated with light response (Datallo et al. 2013; Salo et al. 2015), immune modulation (Brakel et al. 2014), thermal stress (Franssen et al. 2011; Winters et al. 2011; Franssen et al. 2014), extreme environments (Lauritano et al. 2015). Whilst typical eukaryote reactive oxygen species (ROS) and antioxidant activity has been observed in seagrasses, the catalase gene has been reduced to a single copy in *Z. marina* (possibly due to reduced xylem characteristics of submerged plants), whilst all 3 types of superoxide dismutase remain (Olsen et al. 2016). Another interesting observation is that increased ROS activity has been observed in *P. oceanica* meadows (Datollo et al. 2013) under low light. Typically, increased activity is characteristic of high irradiance exposure in land plants; however, Datollo et al. (2013) suggested that seagrasses under low light may be more vulnerable to the extrinsic environment, given that immune functioning is an energy costly process.

Heat Shock Proteins, a group of molecular chaperones involved in stabilizing proteins (Rocheta et al. 2014) have also been identified (Reusch et al., 2008; Bergmann et al., 2010; Franssen et al 2011; Franssen et al 2014; Lauritano et al, 2015; Massa et al., 2011; Piro et al., 2015a; Salo et al. 2015), whilst molecular chaperones, detoxifying cytochromes and metallothionein-type molecules have been found to play important roles in seagrass adaptability and resilience (Bergmann et al., 2010; Kong et al., 2013; Kong et al., 2014; Lauritano et al., 2015). One metallothionein in particular, *MT2L* is found to be highly expressed in *Z. marina* (Olsen et al. 2016). Molecular chaperones aid in promoting correct protein folding, stabilization and preventing proteins from aggregating during stressful conditions (Hartl et al., 2011), whilst cytochromes and metallothioneins aid in scavenging and detoxification processes (Gautam et al. 2012). Glutathione-related transcripts have been isolated and profiled in *P. oceanica, Z. muelleri* and *Z. marina* (Lauritano et al., 2015; Massa et al., 2011; Pernice et al., 2015; Olsen et al. 2016). Glutathione is an essential molecule used for signalling, detoxification of ROS and Reactive Oxygen Intermediates (ROIs) as well as normal development in plants (Noctor et al. 2011). Transcription factors (TFs) which bind to cis-regulatory elements in the genome and which help regulate transcriptional processes are known to play a wide role in plant development, growth and stress response within plants, in seagrasses transcription factors are present (Kong et al. 2013; Kong et al. 2014; Golicz et al. 2015; Olsen et al. 2016). Additionally Olsen et al. (2016) have identified and characterized many miRNA families present in *Z. marina,* including those miRNA families that appear to be lost through evolution.

Molecular studies (Datollo et al. 2013; Kong et al. 2014; Golicz et al. 2015; Olsen et al. 2016) have provided novel insight into the role of hormones and associated transcription regulators in seagrasses. Of particular interest, it has been suggested that the ethylene-signalling pathway has been partially or wholly lost in Z*. muelleri* (Golicz et al. 2015). This study suggested that the loss of the ethylene pathway may be an adaptation to a fully submerged lifestyle, and in additional analysis the authors also failed to detect similar transcripts in EST databases of *Z. noltii* and *Z. marina* species of the Northern Hemisphere, indicating a general phenomenon across seagrass species. Given that ethylene is a volatile gaseous hormone and that seagrasses lack stomata, the partial loss of this hormone pathway in seagrasses is likely to reflect adaptation to a fully submerged life in the marine environment. The *Z. marina* genome has indeed (as of this year) backed such hypothesis and findings, showing that the ethylene pathway is largely reduced in *Zosteraceae* species(Olsen et al. 2016). If silencing of the ethylene pathway has occurred, seagrasses must therefore possess alternative signalling pathways, which are involved in germination, root hair growth and senescence that work independently of ethylene. As a result there is a need to clarify such findings with further experimentation.

In respect to other hormones, Z*. marina* has lost the ability to produce volatiles. Key terpenoid pathways involved in producing volatiles are absent, whilst terpenoid pathways involved in primary metabolism remain (Olsen et al., 2016). The presence of non-volatile phytohormones, the cytokinins, abscisic acid and gibberillic acid pathways have also been identified (Olsen et al. 2016). Suggestively, future work should utilize the power of deep sequencing technology in combination with analytical chemistry, e.g. metabolomics and HPLC/UPLC to verify the presence and function of phyto-hormones within seagrasses. Studies on higher plants could of course be used as sound examples of how we may accomplish such feats (Jia et al. 2016; Cui et al. 2016). Whilst the cross-talk between metabolic pathways including ROS homeostasis, hormone signalling, and downstream signalling cascades remains largely elusive in model plants, it will arguably be a long time before such complexity is understood in seagrasses.

**3.4 – Osmoregulation at the molecular level**

Perhaps the most intriguing characteristic of seagrasses is their superior ability to live in highly saline environments compared to terrestrial plants. Whilst in depth reviews on seagrass osmo-regulation and salt tolerance are available (Touchette 2007), our focus lies with the molecular realm of seagrasses. To our knowledge, the first EST dataset came from Kong et al. (2013). In this study the authors identified 163 genes, which were suggested to play a role in hyper-saline response; photosynthesis, metabolism and energy pathways. Metallothionein, metallothionein-like, transporter proteins, stress proteins, ROS scavengers and carbohydrates played significant roles, supporting earlier beliefs that carbohydrates act as osmolites and regulate cellular osmolality (Touchette 2007). Furthermore, it has recently been confirmed the cell wall composure of *Z. marina* has been found to contain many celluloses, pectins and algal-like polysaccharides, which help to adjust osmolarity during low tides. Even more interestingly, seagrasses have regained their ability to produce sulphated polysaccharides, further promoting the success of osmoregulation in these marine plants (Olsen et al. 2016).

Characterization of the *de novo* *Z. marina* transcriptome (Kong et al. 2014) has identified K+ channel and transporter transcripts, as well as *SOS*, *NHX*, *CLC* and Na+/H+ pump associated transcripts which all play a role in osmoregulation. Olsen et al. (2016) have made the discovery of an H+-ATPase gene, which is highly expressed in vegetative tissue of *Z. marina* and encodes for a salt tolerant H+-ATPase. *AHA* genes, which are unique to the alismatids, have also been identified by Olsen et al. (2016). Such discoveries have indicated compartmentalization and detoxification of sodium and chloride ions are important molecular mechanisms operating within seagrasses to counteract high external salinities, which would otherwise be toxic to vascular plants (Kong et al. 2014); as Na+ is one of the most disruptive ions to cellular processes. Whilst seagrasses are extremely salt tolerant compared to most other plants, a very recent combination of semi-quantitative proteomics and physiology methodologies have been used to study the response of the seagrass *Cymodocea nodosa* to hyper-salinity (43 psu) over 30 days. From protein expression, they found the metabolism of the leaf changes through over-expression of cytochrome b559 subunits and the down-regulation of structural PSII, PSI proteins and RuBisCo, with a shift in carbon metabolism (Piro et al. 2015a). Cytochrome b559 is considered a prerequisite for PSII assembly, suggesting assembly and repair of photosystems are enhanced during hyper-saline stress. Interestingly, Beer et al. (1980) found an increase in the carbon fixing enzyme PEPC in *C. nodosa* in response to hyper-salinity, possibly signifying that the decrease in RubisCO is compensated for by PEPC, to maintain the rate of carbon fixation (Piro et al. 2015a). PEPC may also be playing an anaplerotic role in providing intermediates for the TCA cycle or serving a role in stress response (Doubnerová and Ryšlavá 2011). Piro et al. (2015a) also found an increase in cellular respiration, hardening of cell walls and evidence of sodium compartmentalization in the vacuole of *C. nodosa*. It can be stated though, that such findings by Piro et al. (2015a) are congruent with previous studies (Muramatsu et al. 2002; Kong et al. 2014) indicating that seagrasses exhibit a decrease in photosynthetic activity under hyper-salinity, but an increase in detoxification and osmoregulation mechanisms.

Attention should also be directed towards hypo-salinity. No current studies in the omics frontier have investigated hypo-salinity at this time; however, Collier et al. (2014) have identified a signature stress response in seagrasses whereby significant shoot proliferation occurs before mortality in response to hypo-salinity. Whilst the study was rigorous, we do need to study such responses at the molecular level as increased rainfall has recently been attributed to seagrass decline in parts of the world, including Cairns, Northern Australia (McKenna et al. 2015). On an evolutionary note, Tyerman et al’s (1984) theory of new leaf growth in *Posidonia australis* (via gradual osmotic exposure) should be re-investigated. Such a phenomenon may hold vital clues of how seagrasses evolved and populated the marine environment. Future research efforts on seagrass osmoregulation are important for numerous reasons, i) Such research allows us to understand the complexity of osmoregulation in seagrasses, ii) it helps to explain why some seagrass species are more tolerant to brackish / hyper-saline waters than others, iii) it allows us to determine how seagrasses will survive in future climates where it is expected that the frequency and extent of rainfall events will change, and iv) such research may also play a role in enhancing salt tolerance in agricultural crops. To date, omics has provided us with much knowledge on the mechanisms of salt tolerance in seagrasses, but it can be said that this area of study holds further promise for the future and has the potential to expand significantly.

**3.5 – Tolerance to anoxia and phytotoxic sediment**

Seagrasses are regularly exposed to anoxia/hypoxia in the marine environment (Borum et al. 2006; Brodersen et al. 2015; Hasler-Sheetal et al. 2015). Possibly the most ingenious adaptation of the seagrasses is the presence of a well-developed aerenchyma (lacunae) system, which provides (i) an efficient sulphide detoxification system, (ii) channels oxygen to the below ground biomass to form protective oxic microshields and (iii) the ability to self-induce efficient anaerobic respiration in the below-ground biomass without damaging tissues caused by excess acidosis and ethanol toxicity during anaerobiosis (Hasler-Sheetal. 2015). It must be stated that terrestrial plants do not normally possess aerenchyma as terrestrial soils are abundant with oxygen; however, like most biological phenomena, there are exceptions such as rice and sorghum which are regularly subjected to water-logging. Aquaphytes also possess aerenchyma, therefore it is assumed that such anatomy is likely to have been an early evolutionary adaptation in aquatic plants.

Although recent work has helped to establish the interactions of sediment sulphides with *Z. muelleri* (Broderson et al. 2015) and *Z. marina* (Hasler-Sheetal & Holmer, 2015), a more recent non-targeted metabolomics approach utilizing GC-MS and metabolite enrichment analysis has uncovered critical metabolic systems which are responsible for anoxia tolerance in seagrasses (Hasler-Shetal et al. 2015). *Z. marina* has been found to possess metabolic measures which prevent harmful lactate and pyruvate from accumulating in the tissues via the alanine, GABA and 2-oxoglutarate shunts (Hasler-Sheetal et al. 2015). It has been hypothesized that a regulatory ‘low oxygen-sensing’ pathway exists, which induces fermentative respiration in the belowground biomass (Hasler-Sheetal et al. 2015). In land plants such a pathway has already been identified in *Arabidopsis* (Gibbs et al. 2011). Given that several seagrass die-off events have been attributed to anoxia and sulphide intrusion in the past (Zieman et al. 1999; Koch et al. 2007), it is critical that we uncover the genetic networks involved, in the hope of implementing suitable monitoring efforts.

**3.6 – Other areas needing investigation and development**

Reviews on seagrass sexual reproduction and seed dispersal exist (den Hartog 1970; Ackerman 2006, Kendrick et al. 2012); however, only two studies (Golicz et al. 2015; Olsen et al. 2016) have provided molecular evidence that *Z. muelleri* and *Z. marina* have lost functional genes associated with pollen development and inflorescence morphology; MADS-box transcription factors associated with floral development have been shown to be reduced in *Z. marina* (Olsen et al. 2016). Given that sexual reproduction has been shown to significantly increase in response to disturbance events (Cabaço and Santos 2012), it is vital that we understand what triggers sexual reproduction and how it is regulated at the molecular level. Ultimately the ability to out-cross ensures the maintenance of genetic diversity (Ackerman 2006) and meadow resilience.

It terms of invasive seagrass species, it is surprising that no omics work has yet been initiated on *Halophila stipulacea*. This species that is native to the western Indian Ocean and Red Sea has shown rapid geographical dispersion since the 1800’s, spreading to the Mediterranean and the Caribbean (Willette et al. 2014). It rapidly colonizes, has the ability to survive in a wide range of environmental conditions and possesses great resilience to disturbance events. The photo-physiology of this species alone suggests a high degree of plasticity in response to extreme light environments (Sharon et al. 2009; Sharon et al. 2011). As such its tolerance and resilience make it an excellent candidate species to examine at the molecular level, allowing us to understand what makes this species more tolerant and resilient to environmental change than the majority of seagrasses. Furthermore, such work conducted using artificial mesocosm environments will also allow us to predict its geographical boundary limits.

Further investigation of the rhizosphere is also necessary. The only rhizome and root tissue specific omics research to date is metabolomics by Hasler-Sheetal et al. (2015) investigating anoxia in *Z. marina*. We therefore encourage the targeting of such plant tissue as previously conducted in crop species (Zhang et al. 2014; DuanMu et al. 2015). Additionally, the use of meta-genomics in seagrass bilogy is an area, which can undergo expansion. Understandably, the utilization of metagenomics is time-consuming and often difficult; however, with the observed improvement in resources and computational processing (Land et al. 2015), biologists should be expanding their research interests towards the rhizosphere. This will allow us to learn more about seagrass-sediment interaction, seagrass-microbe interaction and the fundamentals of growth and development, which seem to be under-represented in the previous literature (Wetzel and Penhale. 1979; Hansen et al. 2000). Given that pathogenicity has caused seagrass meadow loss in the past (Brakel et al. 2014), we must understand and learn more about the concepts of microbes and seagrass interactions in the rhizosphere, and also in the canopy.

In terms of omics and experimental protocols there is a lot to consider with aquatic plants. For plants, it can be challenging to conduct accurate sequencing based experiments in general; however, given that seagrasses grow in an aqueous environment, a further level of difficulty presents itself; uniformity in non-investigative parameters is critical for a successful experiment. Initial considerations should focus on replicate number, depth of sequencing, and the time frame in which gene expression occurs between treatments; as such, running pilot experiments can help. Gene expression is known to be stochastistic; such variation may arise from differences due to environmental factors, multi-genotype presence and the influence of genetic mosaics within experimental populations (Becheler et al. 2010; Reusch & Boström. 2011; Sherman et al. 2016). Moreover, given that seagrasses are polyploid, polyploidy can also promote transcriptome and proteomic downstream re-arrangement within a time frame of as little as one or two generations (Leitch & Leitch. 2008); as a result it is important to work with plants of minimal genetic variation in order to maximize the resolution of detecting accurate changes in gene expression. Genotyping before conducting such experiments may therefore be of benefit to the researcher to minimize genic variation. Batch affects arising from the use of a multiple aquaria set-up, non-uniform RNA processing, different tissues, and sequencing cell lane bias must also be accounted for. In relation to genic variation, no transcriptomic studies on seagrasses to date have essentially provided records of variation between biological replicates of treatments when conducting differential expression analysis. We therefore encourage that future efforts do so; to give researchers an idea of how uniform gene expression is between samples.

As for the majority of non-model organisms, the bioinformatic resources for seagrasses are limited. The necessity of online database portals specific to seagrass research is arguably one area, which can undergo further development. To date, the only publically available database, to our knowledge is the EST database, *Dr. Zompo* (Wissler et al. 2009) that contains ESTs for *P. oceanica* and *Z. marina*. Whilst the sequence reads of many experiments in Table 2 are available in sequence archives, there is a need for user-friendly and interactive web portals, whereby the user can perform annotation, mine for candidate genes of interest and genetic markers. As such sequencing of many more seagrass species is necessary. Higher plant specific databases such as TAIR (Swarbreck et al. 2008), PLAZA DB (Proost et al. 2009) and the Mangrove Transcriptome Database (MTDB) (Dassanayake et al. 2010) are just some examples of what can be achieved for seagrass-specific bioinformatics portals.

**4 – Genetic marker utilization in seagrass biology**

The advent of molecular technologies over the past 20 years has undoubtedly paved the way to a better understanding of seagrass genetics and their adaptability to environmental stress and climate change (Reusch et al. 2001; Procaccini et al 2007). For the first time, these studies have provided (i) insight into the genetic diversity within and between meadows (ii) clearer understanding of seagrass phylogenetics, (iii) improved systematic nomenclature) and (iv) observation of meadow resilience in the wake of disturbance events (Procaccini et al. 1999; Reusch et al. 2002; Waycott et al. 2006). These studies of course have relied on the availability of validated molecular markers. The need for such markers is a requisite given the amounting pressures which seagrass meadows are faced with.

**Section 4.1 - Development and advancement of molecular markers**

Molecular markers were introduced into seagrass biology in the 1980’s; initial markers included simplistic phylogenetic evolutionary markers and allozymes (Les 1988; Triest 1991a; Waycott et al. 2006). With the increasing popularity of PCR (Polymerase Chain Reaction) protocols and development of molecular biology techniques, molecular markers for genotyping became established. Development of RFLP (Random Fragment Length Polymorphism) and RAPD (Random Amplified Length Polymorphic DNA) markers occurred, alongside the development of AFLP (Amplified Fragment Length Polymorphism) markers. RAPD markers quickly gained the reputation for being problematic as they possessed low-resolution capacity (failing to detect levels of polymorphism) and were associated with poor reproducibility (Reusch 2001; Procaccini et al. 2007). Earlier work utilizing these methods revealed little to no polymorphism in seagrasses as such systems were based on dominant markers rather than co-dominant markers. With improvements in procedures in the mid 90’s (Alberte et al. 1994; Procaccini and Mozella. 1996), higher levels of polymorphism were detected in seagrass populations (Reusch 2001; Waycott et al. 2006). It wasn’t until the introduction of high-resolution SSR markers (Simple Sequence Repeats; Litt and Luty. 1989) that detection rate of variability and polymorphism increased significantly within seagrass species (Procaccini and Mazella 1998; Reusch 1999b; Reusch 1999c). Additionally, nuclear and plastid encoded markers such as *ITS*, *rbcL*, and *matK* have had impact in population genetics work, especially in determining taxonomy of seagrass species (Lucas et al. 2012; Coyer et al. 2013).

**4.2 – SSR markers: High-resolution popularity**

SSR markers occur at frequent intervals, are easily identifiable, and have co-dominant characteristics. As a result SSRs are the current choice of high-resolution genetic marker in seagrass population genetics due to their ability to identify polymoprhisms (Table 1). *Thalassia hemprechii* and *Zostera marina* are perhaps the most widely exploited seagrasses for SSRs to date (Table 1). Until recently SSR marker validation and usage in tropical species was somewhat lagging behind. Only in the past 5 years have SSR markers been validated for select *Cymodocea, Enhalus, Syringodium* and *Thalassia* species (Gao et al. 2012; Nakajima et al. 2012; Matsuki et al. 2012; Matsuki et al. 2013; Wainwright et al. 2013a, 2013b; Arriesgado et al. 2014, 2015). It could also be argued that the majority of SSR work has arisen from the Americas, Europe and Asia to date (Table 1). Recent efforts in Australia have; however, identified SSRs for Australian seagrass species *Z.* *muelleri, Z. nigricalis, Posidonia australis* and *Posidonia sinuosa* (Sinclair et al. 2009; Sherman et al. 2012; Smith et al. 2013). More work needs conducted in the Indo-Pacific, a hot spot for seagrass diversity. Additionally the numbers of polymorphic loci remain limited for *Halophila*. In seagrasses, validated SSRs have shown the capacity to cross-amplify in other closely related species of seagrass (Reusch 2000; Sinclair et al. 2009; Smith et al. 2013); however, cross-amplification of markers has not been a major focus in seagrass biology as compared to crop plants.More recently, novel methods have been developed to detect genic-SSRs in seagrasses. Genic-SSRs have so far been identified in the seagrasses *Zostera marina* and *Posidonia oceanica* (Oatjen and Reusch. 2007; Reusch et al. 2008; Massa et al. 2011). Such methodologies reduce time and cost by scanning assembled transcriptomes or EST libraries for SSR’s using software. The main benefit of genic-SSRs is that they can be associated with functionally annotated transcripts and functional protein domains, thus serving as FDM-SSRs (Functional Domain Marker-SSRs); however, compared to genomic SSRs, the rate of polymorphism discovery can be lower.

**4.3 - Is there a need for further advances in the field of genetic markers?**

Whilst single nucleotide polymorphisms (SNPs) are the most widely abundant polymorphism in organisms (Appleby et al. 2009), their use in seagrasses has been limited. SNP’s have desired characteristics including low error rates, high frequencies of occurrence and a simple mutation mechanism; however, they are often bi-allelic (Appleby et al. 2009) and as a result are not as desirable as SSRs. Thirty-seven SNPs have been identified in *Zostera marina* (Ferber et al. 2008) and utilized in population genetics (Oetjen et al. 2010). Whilst the availability of sequenced genomes allows for SNPs to be detected easily, novel and cost effective methods such as ddRAD (double digest Restriction site Associated DNA sequencing) and GBS (Genotyping By Sequencing) can make use of reduced genome complexity and therefore offer cheaper alternatives than whole genome sequencing (Elshire et al. 2011; Peterson et al. 2012). Such methods have yet to be implemented in seagrass studies. GBS and ddRAD can detect SNPs, microsatellites, deletions and insertions. The approach provides a higher resolution than microsatellite screening. Additionally it can provide information on QTL (quantitive trait loci) and associate mapping, giving us the ability to identify regions of the genome that may offer greater levels of phenotypic fitness, as previously conducted in higher plants (Zeng et al. 2006; Båga et al. 2007). Fine scale genetic diversity, mosaics and somatic mutations are also worth further investigation in seagrasses (Reusch and Boström. 2011; Sherman et al. 2016), in order to determine how their presence can affect meadow resilience and the success of using SNPs whilst minimalizing error rates. The final challenge (although not discussed much in this review) is the ability to discriminate between taxa. It is our belief that many seagrass taxonomists still encounter trouble whilst determining the phylogenetics of seagrass species. As such, advancements in molecular markers and high-throughout genomic sequencing can surely aid in such research and provide clearer relationships between species.

**Concluding remarks**

Seagrass biologists have undoubtedly made up for lost time by uncovering a wealth of molecular knowledge over the past decade. Perhaps the most significant contribution has been the genome sequencing of *Z. marina* and various transcriptome studies. Such knowledge has allowed us (i) to obtain a clearer idea of evolutionary traits in seagrasses and (ii) to elucidate the role that molecular processes play in the systematic regulation of seagrasses in response to their environments. We envision that omics and profiling techniques will become much more common in the seagrass biology field, as observed in the higher plant field. As such seagrass biologists should seek out higher plant studies as suitable models for deciphering the molecular biology of seagrasses. The development of future technology, sequencing of more omic datasets and implementation of epigenetic research will no doubt aid in increasing the accuracy and knowledge gained from such research. Whilst SSR markers remain the preferred choice of marker, the expansion and utilization of ddRAD and GBS approaches in the future can help overcome the limitations in that approximately only one third of seagrass species have suitable genetic markers. On a final note, omics and molecular profiling approaches should be considered as complimentary to physiological and ecological approaches and not as the sole answer to all biologically relevant questions. As such integrated studies will help to play a more influential role in our understanding of seagrasses, especially in regard to future climate change and disturbance events.

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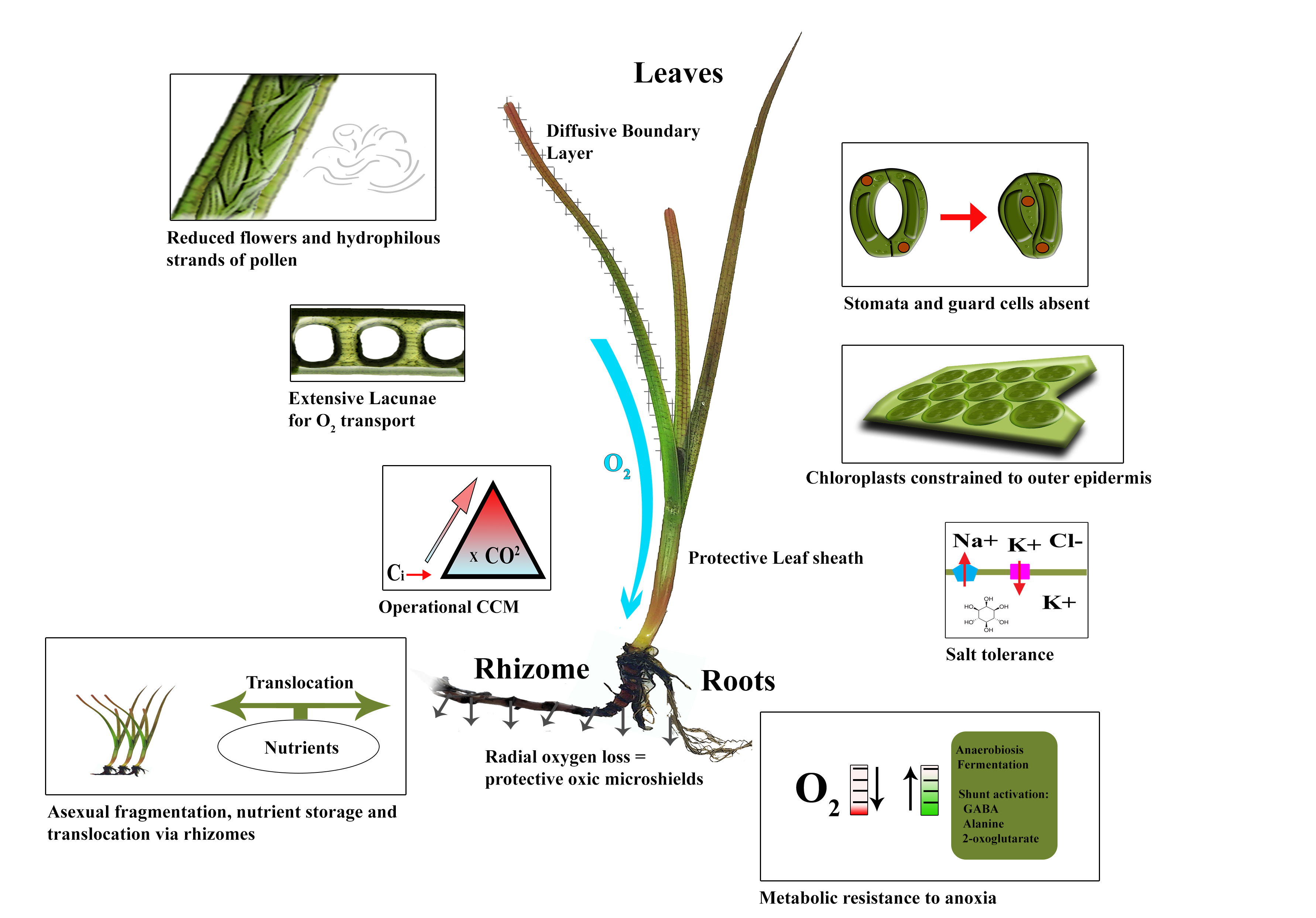
|  |  |  |
| --- | --- | --- |
| **Species** | **Source(s) of information** | **# Polymorphic SSR Loci** |
| *Thalassia hemprichii* | Matsuki et al. 2012; Wainwright et al. 2013b; Van Dijk et al. 2014 | 49 |
| *Zostera marina* | Reusch et al. 1999b; Reusch 2000; Oetjen and Reusch. 2007; Peng et al. 2012; | 45 |
| *Zostera japonica* | Coyer et al. 2004; Jiang et al. 2011a; Zhang et al., 2015 | 34 |
| *Cymodocea rotundata* | Arriesgado et al. 2014 | 29 |
| *Syringodium isoetifolium* | Matsuki et al. 2013; Wainwright et al. 2013a | 27 |
| *Cymodocea nodosa* | Alberto et al. 2003b; Ruggiero et al. 2004 | 22 |
| *Zostera muelleri* | Sherman et al. 2012 | 20 |
| *Syringodium filiforme* | Bijak et al. 2014 | 17 |
| *Enhalus acoroides* | Gao et al. 2012; Nakajima et al. 2012 | 16 |
| *Cymodocea serrulata* | Arriesgado et al. 2015 | 15 |
| *Posidonia oceanica* | Procaccini and Waycott. 1998; Alberto et al. 2003a | 14 |
| *Thalassia testudinum* | Van Dijk et al. 2007 | 14 |
| *Zostera nigricaulis* | Sherman et al., 2012; Smith et al. 2013 | 14 |
| *Posidonia australis* | Sinclair et al. 2009 | 10 |
| *Halophila ovalis* | Xu et al. 2010 | 10 |
| *Ruppia cirrhosa* | Martinez-Garrido et al. 2014 | 10 |
| *Ruppia maritima* | Yu et al. 2009 | 10 |
| *Zostera noltii* | Coyer et al. 2004 | 9 |
| *Halodule wrightii* | Larkin et al, 2012 | 8 |
| *Posidonia sinuosa* | Sinclair et al. 2009 | 6 |
| *Halophila Beccarii* | Jiang et al. 2011b | 6 |
| *Halophila minor* | Xu et al. 2010 | 6 |
| *Zostera caespitosa* | Peng et al. 2012 | 2 |
| **Species** | **Source(s) of information** | **# Polymorphic SNP loci** |
| *Zostera marina* | Ferber et al. 2008 | 37 |

**Table 1:** Known to us, the number of polymorphic SSR loci and SNP loci validated in seagrass species to date. Organised by number of polymorphic loci per species. Information collated in this table comes from the citations under ‘Source(s) of information’ column. A comprehensive literature search was conducted using ‘Web of Science’ with the search terms ‘microsatellite loci seagrass’, ‘microsatellite markers seagrass’, ‘polymorphic microsatellite seagrass’ and ‘polymorphic loci seagrass’.

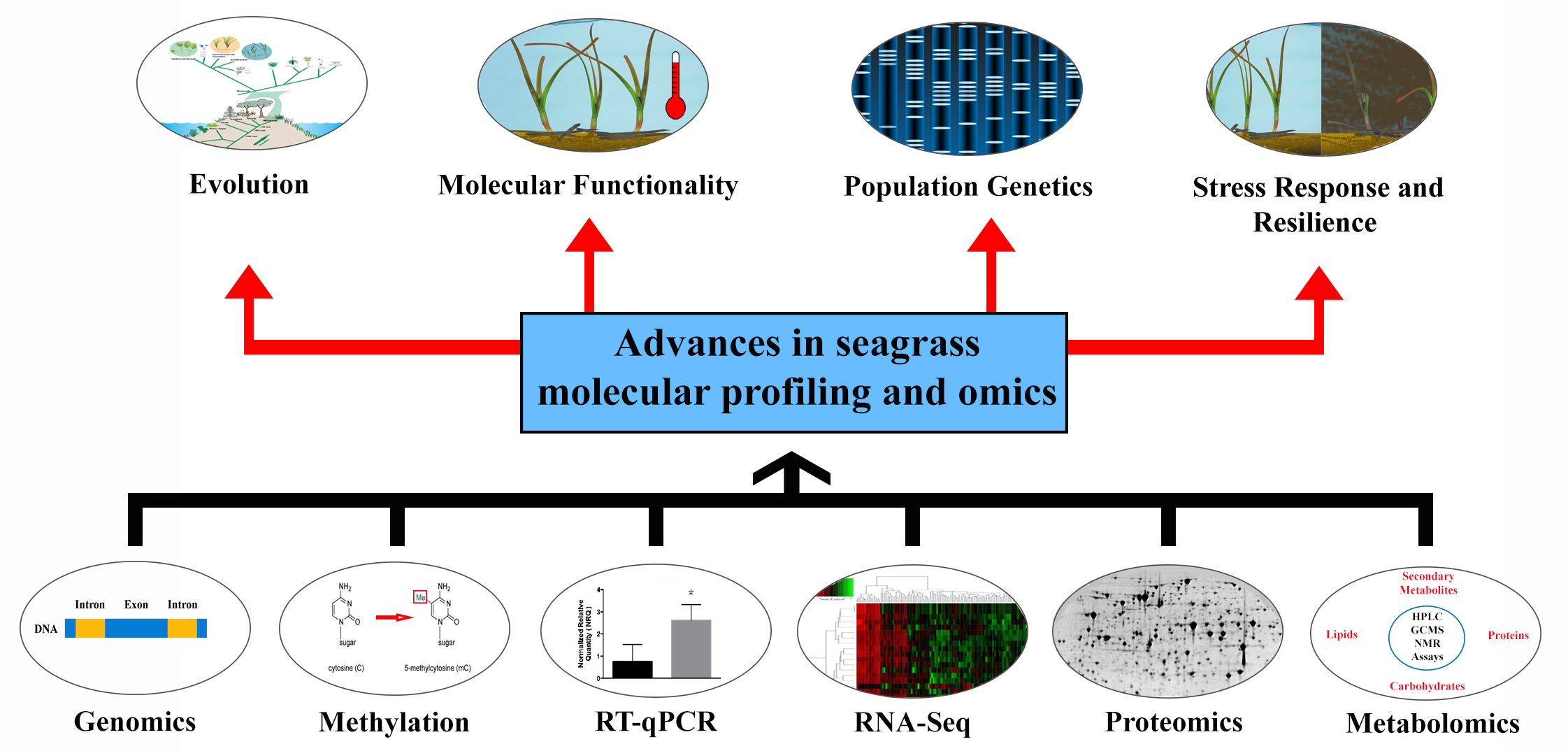
|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Species** | **Study/ Stress Type** | **Molecular / NGS Application** | **Author** | **Year** |
| *Z. marina* | Thermal | RT-qPCR | Ransbotyn & Reusch. | 2006 |
| *Z. marina* | Thermal | EST | Reusch et al. | 2008 |
| *P. oceanica* | Light | Proteomics | Mazzuca et al. | 2009 |
| *P. oceanica, Z. marina* | Online database repository | Online repository creation | Wissler et al. | 2009 |
| *Z. marina* | Thermal | Genotyping and RT-qPCR | Bergmann et al. | 2010 |
| *Z. marina* | Detection of *L. zosterae* pathogen | qPCR | Bergmann et al. | 2011 |
| *Z. marina* | Thermal | EST, *de novo* assembly, RT-qPCR | Franssen et al. | 2011 |
| *Z. noltii* | Thermal | EST | Massa et al. | 2011 |
| *Z. marina* | Thermal | RT-qPCR | Winters et al. | 2011 |
| *P. oceanica, Z. marina* | Evolution | EST | Wissler et al. | 2011 |
| *P. oceanica* | Cadmium toxicity | DNA methylation | Greco et al. | 2012 |
| *P. oceanica* | Thermal | Transcriptomics and metabolomics | Gu et al. | 2012 |
| *P. oceanica* | Reference genes | RT-qPCR | Serra et al. | 2012 |
| *P. oceanica* | Depth gradient / Light | SSH-EST and Proteomics | Datollo et al. | 2013 |
| *P. oceanica* | Light | DNA Methylation | Greco et al. | 2013 |
| *Z. marina* | Salinity | EST | Kong et al. | 2013 |
| *Z. marina* | Defence gene modulation | RT-qPCR | Brakel et al. | 2014 |
| *P. oceanica* | Depth gradient / Light | Micro-array and RT-qPCR | Datollo et al. | 2014 |
| *Z. marina, Z. noltii* | Thermal | Genome-wide transcriptome analysis | Franssen et al. | 2014 |
| *Z. marina* | Thermal / Light / Salinity | RNA-Seq *de novo* assembly | Kong et al. | 2014 |
| *P. oceanica* | Analysis of repetitive genome | Genomics approach | Barghini et al. | 2015 |
| *Z. muelleri* | Genome wide characterization | Comparative genomics approach | Golicz et al. | 2015 |
| *Z. marina* | Anoxia | Metabolomics | Hasler-Sheetal et al. | 2015 |
| *P. oceanica* | Reference genes / Volcanic vents | RT-qPCR | Lauritano et al. | 2015 |
| *Z. muelleri* | Dredging stress | RT-qPCR | Pernice et al. | 2015 |
| *C. nodosa* | Salinity | Proteomics | Piro et al. | 2015a |
| *P. oceanica* | Chloroplast proteomic methods | Proteomics | Piro et al. | 2015b |
| *Z. marina* | Light | Genotyping and RT-qPCR | Salo et al. | 2015 |
| *Z. muelleri* | Reference genes | RT-qPCR | Schliep et al. | 2015 |
| *Z. marina* | Characterization of *LHC* genes | EST-library and RT-qPCR | Kong et al. | 2016 |
| *Z. marina* | Whole genome characterization | Genome assembly | Olsen et al. | 2016 |

**Table 2:** Known to us, themolecular profiling and omic studies published since the establishment of the seagrass NGS era in 2006. A comprehensive search was conducted on ‘Web of science’ for the topics ‘seagrass sequencing’, ‘seagrass gene expression’, ‘seagrass proteomics’, ‘seagrass metabolomics’. Additional studies based on personal knowledge are also provided.

**Figure 1**



**Figure 2**

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