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ATTACK OF THE CLONES: ELUCIDATING THE ROLE OF CLONALITY IN THE INVASION SUCCESS OF CARPOBROTUS EDULIS

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A Thesis

Presented to the

Faculty of

California State University,

San Bernardino

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in

Biology

by

Eduardo Luis Cruz

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Approved by:

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ABSTRACT

Transcriptomics is a modern technique in genomics that utilizes RNA sequences to get a snapshot of genetic expression. This is a powerful tool in non-model species lacking a reference genome. Thus, the application of comparative transcriptomics has the potential to help us elucidate the evolutionary mechanisms that facilitate species invasion. *Carpobrotus edulis* is a prolific and widespread invasive succulent plant belonging to the Aizoaceae family. A native to South Africa, this species has become a pervasive invader of many Mediterranean coastal areas. In this study, we leveraged the use of RNAseq to investigate evolutionary changes among invasive populations. RNA-seq data was gathered from experimental native and invasive populations tested under stress conditions and used to assemble a *de novo* transcriptome and perform a differential expression analysis. We provide the first annotated transcriptome for the plant genus Carpobrotus and the Aizoaceae family. Differential expression analysis identified several differentially expressed transcripts between native and invasive populations. Invasive populations showed no increases in putative genes related to flowering and decreased expression in genes related to protection and defense. Supporting the hypothesis that *C. edulis* has undergone significant genetic modification in the invaded range. These results are expected under the Evolution of Increased Competitive Ability hypothesis and Genetic Accommodation hypothesis, wherein novel

metabolic processes and changes in gene regulatory networks evolve when introduced to ecologies.

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DEDICATION

For my brother Ricardo Cruz.

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CHAPTER ONE

AN INTRODUCTION

Migration, Range Expansion, and Species Invasion

Migration and range expansion can naturally occur when plant species search for more suitable environmental conditions (Woodall et al., 2010). The expansion of a species into a new habitat can have ecological implications for the local ecosystem, though the risk of disruption is minimal compared to the disruption of human-mediated translocated species (Simberloff et al., 2012). Global trade and international transport have increased the incidence of anthropogenic disturbance via translocated species, bringing an increased awareness of the ecological impact that invasive species can exert (Richardson, 2010; Simberloff et al., 2013). When introduced into a new environment, invasive species have the potential to outcompete native species (Loo, 2009) and change the function and structure of an ecosystem (D'Antonio & Vitousek 1992; Lovett et al., 2006; Talley et al., 2001; Vitousek & Walker 1989). Thus, understanding the evolution of invasion strategies is crucial for future conservation efforts.

Although the focus of invasion biology has been the detection and mitigation of the invasion's impact, introduced species have been demonstrated to provide one of the best model systems to study contemporary evolution in real-time (Sax et al., 2007; Stockwell et al., 2003). Thus, providing the unique opportunity to elucidate the characteristics and evolutionary mechanisms that facilitate establishment and expansion under variable environmental conditions. These

insights provide: a) higher accuracy when predicting how species might respond to environmental changes, ii) the ability to identify species that have a higher risk of successfully expanding from their ancestral range distribution, finally, iii) a better chance to control/mitigate the potential harm of non-native species in threatened and fragile ecosystems.

It is well documented that species introduced into a new environment often have small founding populations and subsequently reduced genetic diversity, increasing the risk of founder effects such as inbreeding depression and genetic drift (Angeloni et al., 2011; Richards, 2000; Sakai et al., 2001; Valtueña et al., 2021; Vilas et al., 2006). However, comparative studies of invasive species to their native counterparts have demonstrated the potential for increased genetic differentiation within invasive subpopulations despite a significant loss of genetic diversity (Dlugosch & Parker, 2008 a & b). Life history traits such as vegetative growth, beneficial pre-adaptations, novel loci co-adaptation, and multiple reintroductions have been shown to mitigate the effects of substantial bottlenecks among founding populations (Genton et al., 2005; Urquia et al., 2019), providing a means of rapid adaptive evolution in the absence of diverse genetic selection (Dlugosch & Parker, 2008a). This is more evident in species with high phenotypic plasticity where local environmentally induced epigenetic changes, alterations made by silencing or expressing DNA, may act as a substitute for genetic selection (Dodd & Douhovnikoff, 2016; Mounger et al., 2021). Additionally increased utilization of clonal/vegetative growth can minimize the effects of small numbers of individuals

common to founding events, allowing for the development of adaptive traits (Dodd & Douhovnikoff, 2016; Douhovnikoff et al., 2004; Douhovnikoff & Dodd, 2014).

Carpobrotus edulis as a Model Species

A textbook example of such an invasive species is *Carpobrotus edulis* (L.) N.E. Br (*C. edulis*). Commonly known as the highway ice plant, it is a stoloniferous succulent belonging to the Aizoaceae family, and native to the Cape Region of South Africa. However, *C. edulis is* invasive in many coastal Mediterranean climates around the world (i.e., the Iberian Peninsula, US Pacific coast, Coastal South America, Australia, and New Zealand) (Campoy et al., 2018; Carpobrotus edulis [L.] N.E.Br. in GBIF Secretariat, 2021) (Fig 1). *C. edulis* morphology is characterized by extensive plagiotropic (horizontal stems) and radial growth, forming dense mats of physiologically integrated ramets (connected clones) (Roiloa et al., 2010). In the invaded ranges, integrated ramets can extend over large distances of up to 40m (personal communication Dr. Sergio Roiloa) with multiple individuals emerging from the same parent. Over time the extensive radial growth forms dense mats that limit both water and soil availability, effectively suffocating native species in the invaded ranges (Vieites-Blanco & González-Prieto, 2018). As *C. edulis* grows the accumulation of plant necromass under the vegetative mats modifies soil salinity, pH, and Nitrogen Cycling increasing the mortality of co-occurring species (Conser & Conner, 2009; Corbin & D'Antonio, 2004; Vieites-Blanco & González-Prieto, 2018). Additionally, utilization of increased vegetative growth, decreased inbreeding depression, agamospermy

(viable unfertilized seeds), and self-compatibility (via self-pollination) (Suehs et al., 2004), lessen the effects of inbreeding depression from small founding populations. Taken together *C. edulis* exerts a strong negative impact on both the short- and long-term persistence of the native flora in the invaded ranges (de la Peña et al., 2010; Elgersma et al., 2011).

The utilization of RNA Sequencing (RNA-Seq) has become a powerful tool in understanding the role of gene expression and regulation in the evolution of non-model organisms (Adhikari et al., 2022; Duan et al., 2020; Secco et al., 2014). Providing a plethora of excellent tools for the identification of changes in regulation and expression such as *de novo* transcriptome assembly, differential expression, and enrichment analysis (Kukurba & Montgomery, 2015; Spies & Ciaudo, 2015; Wang et al., 2011). Lopez, et al. (2019) used *de novo* transcriptome assembly and differential expression analysis of the parasitic plant Purple Witchweed (*Striga hermonthica* (Delile) Benth.) to identify host-specific parasitic transcripts targeting genes related to nutrient transport, defense mechanisms, cell wall modification, and hormone response during host-parasite interactions. Similarly, Sun et al. (2018) performed an analysis of specific gene loss involved in the evolution of parasitism in the plant genus Cuscuta, by combining *de novo* transcriptome assembly and HiSeq Genome surveys. Thus RNA-Seq has the potential to elucidate specific changes in regulatory expression that can lead to increased invasion potential in species without reference genomes.

Because the increased use of division of labor, reliance on vegetative growth, and higher plasticity are distinct traits among invasive populations of *C.*

edulis, it is reasonable to predict those evolutionary changes can be explained by understanding the genetic basis of those traits. Transcriptomic analysis has the potential to understand changes in regulation and expression that aid in invasive potential. Of specific interest are the differences in gene expression and regulation as they relate to responses to stressful environmental conditions. In the context of using *C. edulis* as a model species for increased invasive potential, this involves i) *de novo* construction of an accurate and complete transcriptome, ii) the differential expression analysis between native and invasive populations grown in controlled experimental conditions, and iii) a direct comparison of their shared but differentially expressed transcriptomic features.

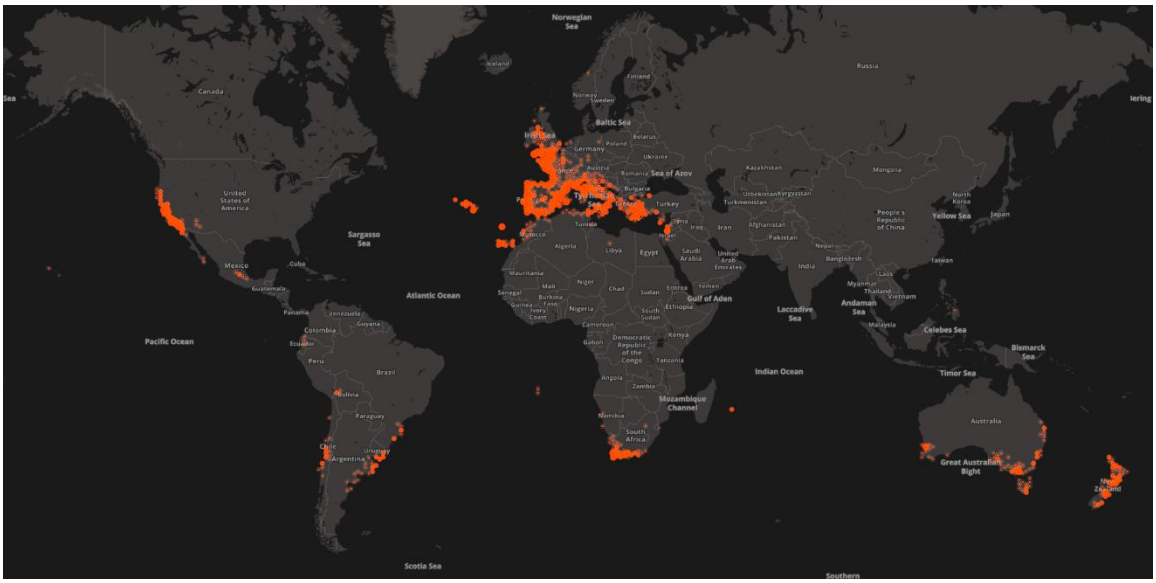

Figure 1. GBIF Distribution.
The following figure illustrates the amount of translocation *C. edulis* has undergone as of Nov 28, 2022. The presence of *C. edulis* is represented as orange points on the map. The information for this figure was compiled from 23,979 georeferenced records accessed through Global Biodiversity Information Facility.

CHAPTER TWO

METHODOLOGY OF STUDY

Study Site and Sampling

In 2015, we collected six coastal populations of *C. edulis*. We sampled three populations in the Iberian Peninsula (invaded range) and three in South Africa (native range) (Fig. 2). To obtain a wide representation of the population's genetic variability, we selected 36 samples per population, per location, with a minimum distance of 25m between vegetative mats. Because *C. edulis* forms compact vegetative mats along the terrain, it is reasonable to assume that each separated vegetative mat represents a different genotype, though no genotyping was performed. From those sample locations, plant fragments from 15 individuals per location were transplanted into a common garden.

Treatments and Tissue Sampling

C. edulis can spread clonally from disconnected fragments, developing into a new individual. All fragments (from now on referred to as individuals) per population were placed in a common garden and were regularly watered (every two days, 100ml per plant). After three months all individuals were well-established and with well-developed roots and thus prepared for common garden sampling. This allowed for the sampling of fully developed plants and the minimization of maternal environmental effects that might affect the outcome of the experiment.

After those three months, we cut the most apical un-rooted ramet of each fully developed individual and planted it in a single 0.5L plastic pot filled with washed sand. By using the apical ramet we ensured that all the experimental plant materials were in the same developmental stage. These individuals were then placed in a new common garden where they were regularly watered (every two days, 100ml per plant). After two months the plants were well established and with well-developed roots and we proceeded to apply the treatments. We randomly assigned two plants per sample location, per individual treatment (7 total treatments, see Table 1 for a detailed description of each treatment). Plants from each of the six populations sampled in the field were equally represented in the treatments, for a total of 96 individuals. The salicylic acid concentration used in the experiment was determined by choosing the highest that did not produce surface damage among the 3 tested solutions (1-, 5-, 10-mM). Plants under treatment were randomly harvested by reversing the shortest possible time to avoid differences in expression from the first to the last plant processed (approx. 1.5 hours). Besides the plants under treatment, we randomly selected plants from each sampled population that were grown under standard conditions. From each plant, two samples were harvested (i.e., leaf and roots). Tweezers and scissors were flame sterilized and disposable blades were used. The cutting board was sterilized with a 2% bleach solution and rinsed with H2Od. The first well-developed leaf from the apex was selected. The roots were washed in distilled water and dried with disposable KimTech Kimwipes (Kimberly Clark, USA). When temperature treatments were applied, distilled water was brought to the temperature of the

treatment. Samples were sliced (pieces of 0.5cm of side) and immediately submerged in RNAlater (Qiagen, The Netherlands) and kept overnight at 4ºC. RNAlater was removed, and samples were finally stored at –20ºC until RNA extraction.

RNA Extraction, Library Preparation, and Sequencing

RNA from both leaves and roots of *C. edulis* were extracted with the Maxwell 16 LEV Plant RNA kit (Promega, USA) following manufacturer instructions. Sample tissue was first dried with a KimTech Kimwipe to absorb traces of RNAlater in which samples were preserved and immediately flash-frozen by submersion in liquid nitrogen. Up to one-hundredth mg of frozen tissue was ground to powder with the Qiagen TissueLyser LT (Qiagen, The Netherlands) bead mill. Grinding was performed in 2 ml round bottom microcentrifuge tubes containing 2 stainless steel beads of 7 mm (about 0.28 in) and 2% w/v of Polyethylene Glycol of high molecular weight (HWM-PEG). The TissueLyser adapter with the prepared tubes was previously incubated for 30 min in dry ice and then maintained in it while proceeding. RNA integrity and quantity were assessed with an Agilent 2100 Bioanalyzer (Agilent, USA) and a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, USA) from the Scientific Research Support Services of the Universidade da Coruña (Spain). A third concentration measurement was taken using Qubit (Thermo Fisher, USA).

After extraction, seven RNA pools were made for library preparation. Six of the pools were made by combining sets of RNA samples in equimolar amounts

and they represented the sampled populations (i.e., PT Iberian Peninsula and SA South Africa). These pools were used for downstream differential expression analysis. An additional pool was made by combining all the RNA samples in equimolar amounts representing the entirety of both populations. This pool was used for the *de novo* transcriptome assembly and annotation. The seven RNA pools were processed using the TruSeq RNA Sample Prep Kit v2 (Illumina, USA), strictly following the manufacturer's instructions. Libraries were single-indexed to allow for demultiplexing. After library construction, all libraries were quantified and quality-checked using an Agilent 2100 Bioanalyzer. According to the Agilent 2100 Bioanalyzer results, all libraries were pooled together. This pool was divided into 2 aliquots and each aliquot was sequenced in 1 HiSeq 4000 PE100 lane (Illumina, USA). The libraries used for downstream differential analyses (PT & SA) were transcripts sequenced at the same depth (Table 2). Meanwhile, the library used for *de novo* transcriptome assembly (Total Control) was sequenced in higher depth to obtain higher coverage of the transcriptome for its annotation (Table 2).

Data Preprocessing

Transcripts containing all the RNA samples of both populations were assessed for quality using FastQC v.0.11.9 (Andrews, 2010). FastQC provides an accurate and simple method for assessing the quality of raw sequence data with an abundance of metrics to assess the quality of high throughput sequences (Leggett et al., 2017). Using the OmicsBox v.1.21.1, transcripts were trimmed for quality with the integrated Trimmomatic v.0.38 (Bolger et al., 2014) feature.

Adapter sequences were removed with Trimmomatic default parameters for Illumina/TruSeq3 adapters. Trimmomatic trimming options were set with mismatches set to a maximum of three, simple clip threshold to 15, and palindrome clip threshold to 30. Trimming included a sliding window of 4 bases with the required quality of 15, quality trimming removed leading bases from the 5' end with a trimming threshold of 20, and length trimming was used to trim from the 5' end with a trim threshold of 10. Finally, we discarded any reads remaining that were shorter than 75 bases long and quality scores below 30. Filtered reads were then reinspected using FastQC v0.11.9 to confirm that they met our quality standards.

de novo Assembly and Transcriptome Annotation

The de novo assembly and annotation of the transcriptome of *C. edulis* was done using OmicsBox v.1.21.1 and its RNA-Seq de-novo assembly pipeline (Götz et al., 2008). The OmicsBox pipeline utilizes software packages such as Trinity v2.13.2 (Grabherr et al., 2011), an efficient and robust method for the *de novo* reconstruction of transcriptomes from short-read sequences using a *de* Bruijn graph algorithm. Total Control Libraries (TCL) were used for the *de novo* assembly of the *C. edulis* transcriptome. We conducted the *de novo* assembly and normalization using Trinity's recommended default parameters. To assess the quality of the *de novo* assembled transcriptome we evaluated the completeness of the assembly in terms of conserved ortholog content using BUSCO v1.2 (Simão et al., 2015) with Embryophyta as a threshold for completeness. After assembly, additional curation was performed to reduce redundancy and improve accuracy.

Sequence clustering was performed in OmicsBox utilizing CD-HIT v.4.8.1 (Li & Godzik, 2006; Fu et al., 2012), with a global sequence identity of 0.95 and the remaining parameters set to recommended settings. Putative coding regions were identified using TransDecoder v.5.5.0, with HMMER v.3.3.2, via OmicsBox Predict Coding Regions pipeline, all with recommended settings (Haas et al., 2013; Hass & Papanicolaou, 2019).

Annotation was performed using Blast2GO v4.1.8 (Conesa et al., 2005; Conesa and Götz, 2008) via the OmicsBox annotation pipeline. To annotate the maximum number of contigs based on sequence and functional similarity, we run all transcripts against multiple databases. OmicsBox enabled the use of a single-step pipeline running the Blastx algorithm to search for homologous sequences (e-value cut-off 1.0E-5) against the non-redundant database from the National Center for Biotechnology Information (NCBI) (Pruitt et al., 2007) while parallel searches in the InterPro from the European Bioinformatics Institute (Finn et al., 2017) to identify conserved protein motifs/domains. Gene Ontology (GO) Mapping was performed using default annotation settings filtering GO IDs by Embryophyta (Taxonomic Filter) and an E-Value Hit-Filter of 1.0E -3 was used to account for the lack of genomic resources for the Aizoaceae family.

Differential Expression Analysis

Differential expression analysis was also performed using OmicsBox. Read alignment was done with our *de novo* assembled transcriptome via integrated Bowtie v2.4.4 (Langmead & Salzburg 2012), and transcripts quantification was

calculated with RSEM v1.3.3 (Li & Dewey 2011). This allowed us to assess the representation of reads to our de-novo transcriptome. Expression patterns were characterized using EdgeR v.3.28.0 (Robinson et al., 2010). EdgeR identifies differentially expressed transcripts based on the assumption that the number of reads produced by each transcript is proportional to its abundance. In our analyses, populations of *C. edulis* collected from the same region (invaded vs native) were considered biological replicates. That is three replicates for native (SA2-4) and three for invasive (PT2-4). Transcripts with very low expression values were filtered using a cut-off of 1 CPM (counts per million) in at least 3 samples (number of biological replicates per group) and we used the Trimmed Mean of M-values method (TMM (Trimmed Mean of M values)) for normalizing the counts. Pairwise Differential Expression (PDE) analysis using EdgeR with a Multifactorial design using both Treatment (Native/Invasive) and Population (South Africa/Portugal) as factors, with a Generalized Linear Model (Likelihood Ratio, GLM) statistical test set with Robust factors set.

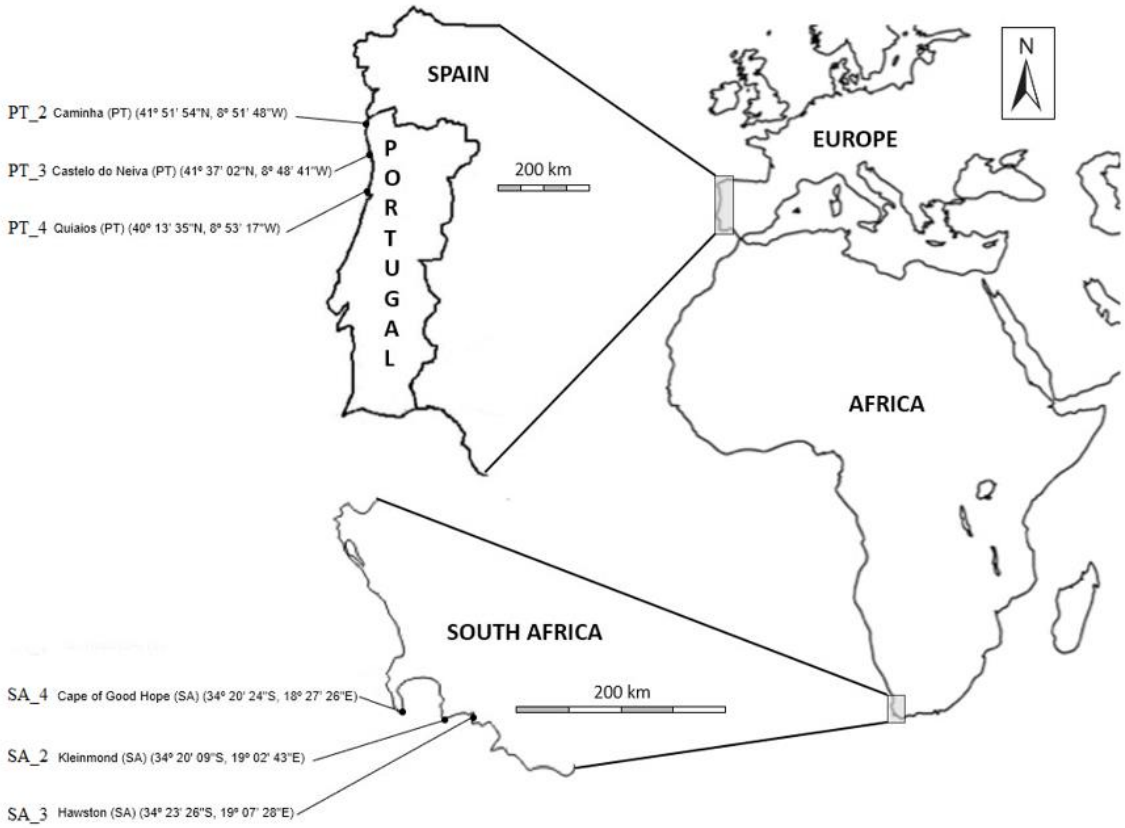

Figure 2. *Carpobrotus edulis* Sample Locations.
Map showing the populations of the *Carpobrotus edulis* sampled for this study from both the native (Cape Region in South Africa) and the invaded (Iberian Peninsula) distribution range. (SA, South Africa; PT, Portugal).

Table 1. Treatments

Treatment	Description	Duration
Dark stress	Culture chamber in darkness at 21ºC	48h
High temperature	Culture chamber at 40ºC	24h
Low temperature	Culture chamber at 4ºC	24h
Nutrients	Watering with fertilizer every 2 days outdoor	Two weeks
Drought stress	No water and covered to prevent rain outdoor	10 days until wilting
Jasmonic acid	10 ml 1mM per plant pulverized outdoor	24 hours
Salicylic acid	10 ml 10 mM per plant pulverized outdoor	24 hours

Description and duration of the treatments applied to native and invasive populations of *C. edulis* in common gardens at the Universidade da Coruña, Spain. Treatments were aimed at simulating various environmental stresses.

Table 2. Library Information

Sample	Coordinates (Lat/Long)	Fraction of a Lane	Raw reads	Trimmed reads
SA_2	34º 20' 09"S 19º 02' 43"E	2/15	74,485,050	63,697,185
SA_3	34º 23' 26"S 19º 07' 28"E	2/15	48,448,959	42,828,974
SA_4	34º 20' 24"S 18º 27' 26"E	2/15	67,796,683	61,608,237
PT_2	41º 51' 54"N 8º 51' 48"W	2/15	56,683,664	50,214,152
PT_3	41º 37' 02"N 8º 48' 41"W	2/15	57,673,167	53,023,361
PT_4	40º 13' 35"N 8º 53' 17"W	2/15	67,925,791	61,163,972
Control_Total	NA	2/3	168,864,835	145,831,704

Library information of *Carpobrotus edulis*. Column headers as follows: Sample, the name assigned to the populations; Coordinates, latitude, and longitude of the sampled populations; Fraction of lane, the fraction of the lane used for sequencing that specific library; Raw reads, number of reads obtained from sequencing each specific library and Trimmed reads, number of high-quality reads retained after trimming.

CHAPTER THREE

RESULTS

Data Preprocessing and Trimming

The total number of raw reads produced across the SA and PT libraries of *C. edulis* ranged from a maximum of 74,485,050 (SA-2) to a minimum of 48,448,959 (SA-3), and TLC libraries reached a total of 168,864,835 reads (Table 2). After trimming, the range of high-quality clean reads remained SA-2 and SA-3 (63,697,185 and 42,828,974 respectively) (Table 2). Following quality filtering individual sample reads retained an average of 89.2% of reading pairs. Deeper sequencing for the Control_T meant the number of raw reads obtained for use in the *de novo* assembly greatly exceeded those of the other libraries. Retaining a total of 145,831,704 reads, a total reduction of 13.6% (Table 2).

de novo Assembly and Transcriptome Annotation

After the quality trimming the high-quality reads were assembled in the OmicsBox using Trinity v2.13.2 (Grabherr et al., 2011). The *de novo* assembly of *C. edulis* resulted in a total of 181,212 transcripts, further refined to 139,080 clusters after curation (Table 3). BUSCO group analysis showed 93.2% complete gene representation conserved across 50 species within Embryophyta (Fig 3). Based on the representation of reads and ortholog completeness, we established that our *de novo* reference transcriptome is of high quality.

Homology searches were done using the most comprehensive, publicly available databases. Transcripts that did not match any sequence could represent novel transcripts, untranslated regions, non-coding RNA, or short sequences not containing a protein domain. However, of those transcripts with homology hits none was phylogenetically close to *C. edulis*, implying the lack of BLAST hits may be the result of underrepresentation of genomic data for more closely related species. Annotation distribution showed that 71,707 (51.6%) of the sequences had a significant hit in the homology search and we were able to ID GO terms for 50,457 sequences (36.28%) (Fig 4). For transcripts with significant hits, we recovered the GO categorizations from the homology search Biological Process (BP GO:0008150), Molecular Function (MF GO:0003674), and Cellular Component (CC GO:0005575) (Fig. 5). The highest number of sequences within the BPs were in the categories of cellular and metabolic processes, within MF the highest distributions were in catalytic activity and binding, and within CCs nearly the entirety of sequences was found in cellular anatomical entities and protein-containing complexes (Fig. 5). Additionally, distribution of sequences, when categorized by enzyme class, was highest among translocases and hydrolases (Fig. 6). Taken together our transcriptome assembly is the first genetic resource for the species *C. edulis*, and the Aizoaceae family.

Differential Expression Analysis

Differential expression analysis was performed using our *de novo* transcriptome to assess transcriptional differences between native and invasive

populations. Each population was represented by group libraries, 3 from South Africa (SA 2-4) and 3 from the Iberian Peninsula (PT 2-4). Using the OmicsBox Count Table function (which utilizes RSEM v1.3.3 & Bowtie v2.1.1), quantization was assessed, with transcripts mapping back with the range of 84.2% (SA4) to 86.8% (PT4), mean mapping of 85.7% (Table 4). Additionally, we sought to ensure that each population could be treated as a set of biological replicates. Principle Component Analysis (PCA) showed distinct clustering of gene expression between invasive and native populations (Fig. 7). Following DE analysis of the initial 139,080 transcripts, 39,146 were preserved after filtering. Additionally, a heat map of differentially expressed genes indicated distinct patterns of expression between Invasive and Native populations across sample groups (Fig 8). The number of DE transcripts with default settings (FDR < 0.05 and an of logFC > 1) totaled 378 DE transcripts, 254 upregulated in the Native (SA) group, and 135 in the Invasive (PT) group. To ensure the highest distinction between expressed transcripts, up and down tags for DE transcripts were filtered conservatively (FDR < 0.001 and logFC > 4). This reduced the total number of transcripts to 148, with 111 upregulated among the native group and 37 transcripts upregulated among the invasive populations.

GO terms were assessed for the upregulated transcripts of both populations. Among the Native population 60 of 111 (54.1%) upregulated transcripts had annotated GO terms, totaling 228 GOs (Fig. 9). Level two GO distribution ranged from 1-42 highest distribution among GO terms associated with cellular and metabolic processes (BP), binding and catalytic activity (MF), and

Cellular anatomical entities and protein-containing complexes (CC) (Fig. 10). Direct GO counts for BP totaled 81 GOs with defense response and translation having the highest counts (Fig. 11). Within the remaining categories, MF totaled 90 GOs with metal ion binding and ATP binding having the highest counts (Data not shown) and CC totaled 57 GOs with membrane and cytosol GOs having the highest counts (Data not shown).

Among the invasive population 21 of 37 (56.8%) upregulated transcripts had associated GO terms, totaling 76 GOs (Fig 12). Level two GO distribution ranged from 1-15 highest among terms relating to metabolic and cellular processes (BP), followed by binding and catalytic activities (MF), and cellular anatomical entity and protein-containing complexes (CC) (Fig 13). Direct GO counts for upregulated invasive DEGs totaled 23 GOs with protein phosphorylation and positive regulation of ubiquitin-protein transferase activity having the highest counts (Fig 14). The remaining direct counts were MF with a total of 29 GOs ATP binding as the highest (Data not shown), and CC totaling 24 GOs with membrane and U2-type prespliceosome GOs having the highest counts (Data not shown).

Table 3. Transcriptome Assembly Metrics

Assembly metrics	
Transcripts > 200 bp	181,212
Total contig length	199,385,844
Mean contig size (bp)	1,100.29
Number of contigs > 1000 bp	89,262
N50	1,977
Longest contig size (bp)	19,652
GC %	41.49
Assembly read support	
% Reads mapped as proper pairs	65.69
% Reads mapped as improper pairs	22.45
% Right reads mapped alone	5.97
% Left reads mapped alone	5.88
BUSCO – Completeness assessment of *de novo* assembly	
Complete, single-copy BUSCOs	488
Complete, duplicated BUSCOs	1016
Fragmented BUSCOs	52
Missing BUSCOs	58
% Complete BUSCOs	93.18

Assembly metrics and quality assembly data for the *Carpobrotus edulis* transcriptome, measuring various aspects of the assembly quality as it pertains to qualitative measures. Assembly read support for the representation of total alignment between RNA-Seq data and the *de novo* assembly. BUSCO metrics for measuring *de novo* assembly completeness by assessing the total number of conserved orthologs among 50 species within Embryophyta (land plants) capturing the most common genes necessary for basic function.

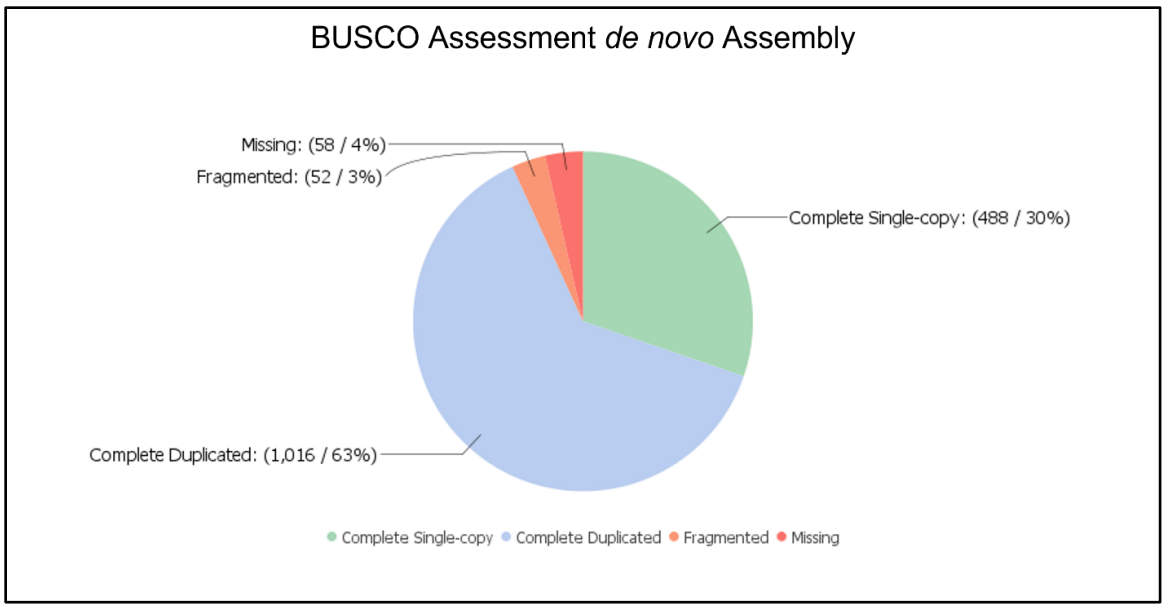

Figure 3. BUSCO Completeness Assessment.
BUSCO measures genomic data quality using the principle of evolutionarily conserved orthologs. In the context of this work, it measures the completeness of a *de novo* assembly searching for homology (strong genetic similarity) for the genes needed for basic functionality as these are phylogenomically conserved (genetically preserved) across members of Embryophyta (land plants). In green 30% are present as single copies, blue represents 63% as duplicates, orange represents 3% fragmented (partially present), and red 4% missing. Results for this assembly indicate a completeness of 93%, indicating a high-quality *de novo* assembly.

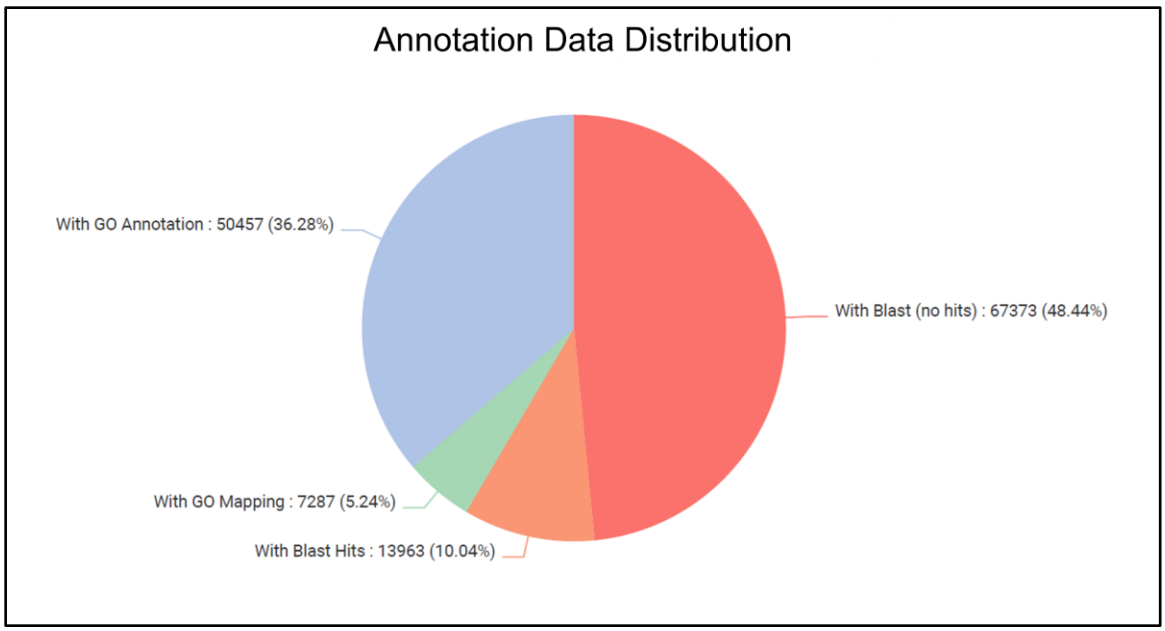

Figure 4. Annotation Distribution

Annotation distribution results for BLAST2GO homology search for the 139,181 sequences identified within the *Carpobrotus edulis de novo* assembly (Transcriptome). Blue represents 50,457 sequences (36.28%) that had full Gene Ontology (GO) annotation, assigning reliable functionality labels to those sequences. Green represents 7,287 sequences (5.24%) with potential GOs based on links to orthologs with functional annotations. Orange represents 13,963 sequences (10.04%) with significant hits in the homology search but no associated GO terms. Red represents the remaining 67,373 sequences (48.44%) with no blast hits in the homology search.

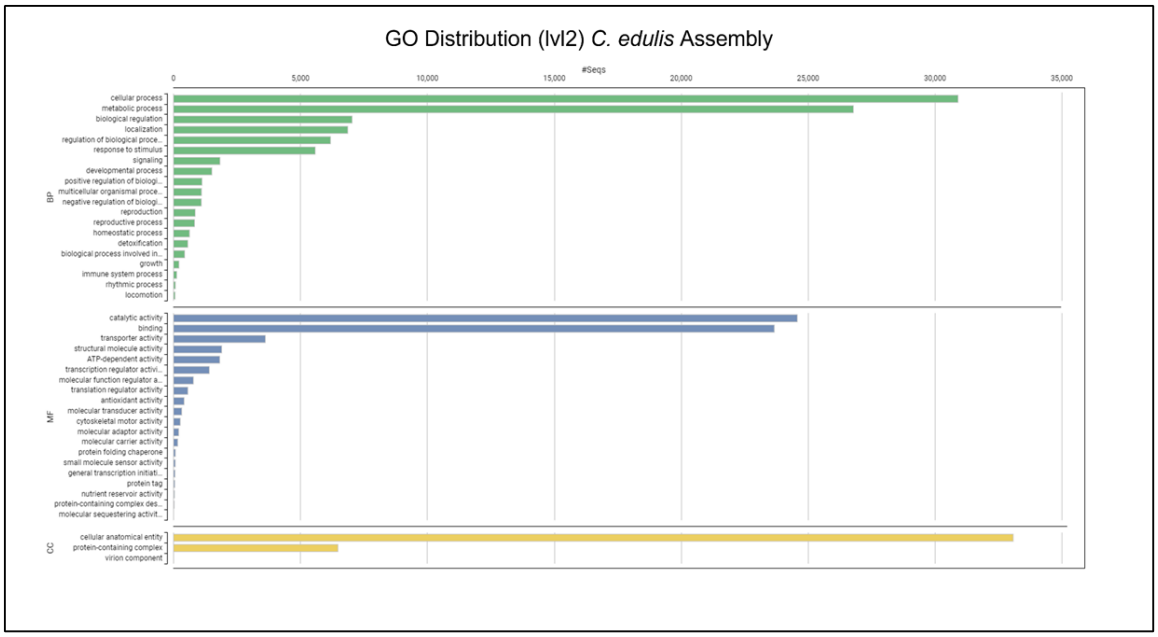

Figure 5. Level 2 Gene Ontology for Assembly.
Gene Ontology (GO) terms distribution for *Carpobrotus edulis* Assembly generated by OmicsBox v.2.2.4. Distribution is categorized by major GO categories. Biological Process (BP GO:0008150) is shown in green, Molecular Function (MF GO:0003674) is shown in blue, and Cellular Component (CC GO:0005575) is shown in yellow, at level 2 (Broadest GO Term association).

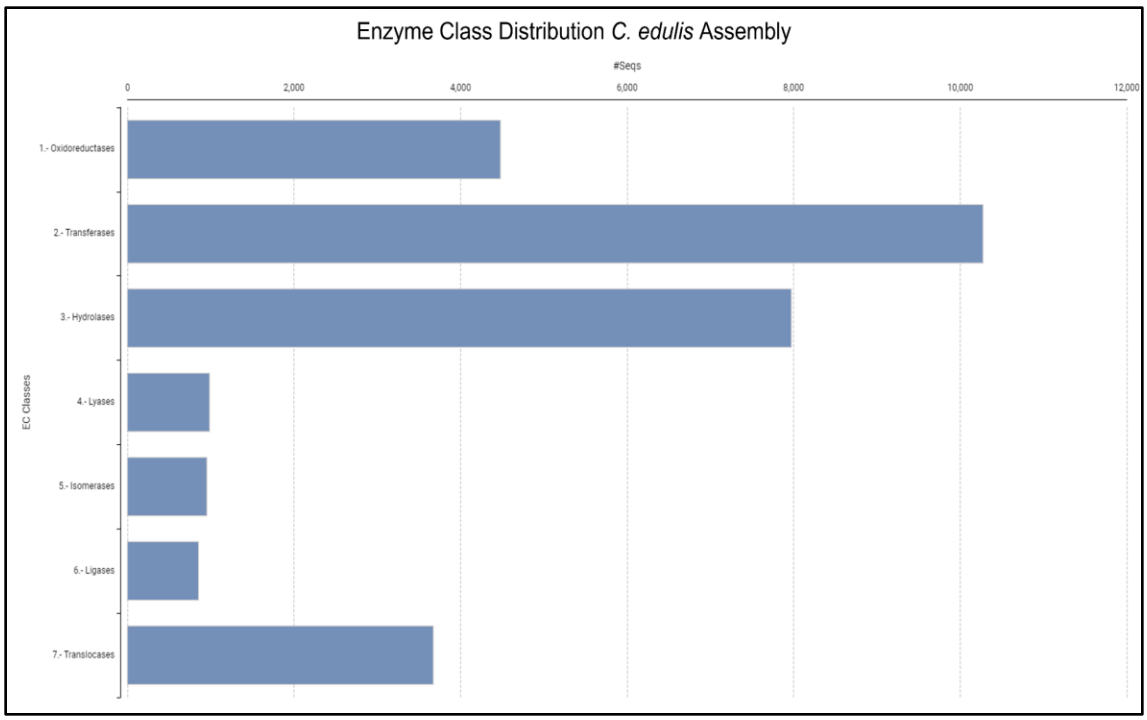

Figure 6. Enzyme Class Distribution.
Distribution of sequences from *Carpobrotus edulis* Assembly characterized by enzyme class generated by OmicsBox v.2.2.4 using Blast2GO integrated software. Shows the breakdown for the various enzyme classes within the identified putative coding regions.

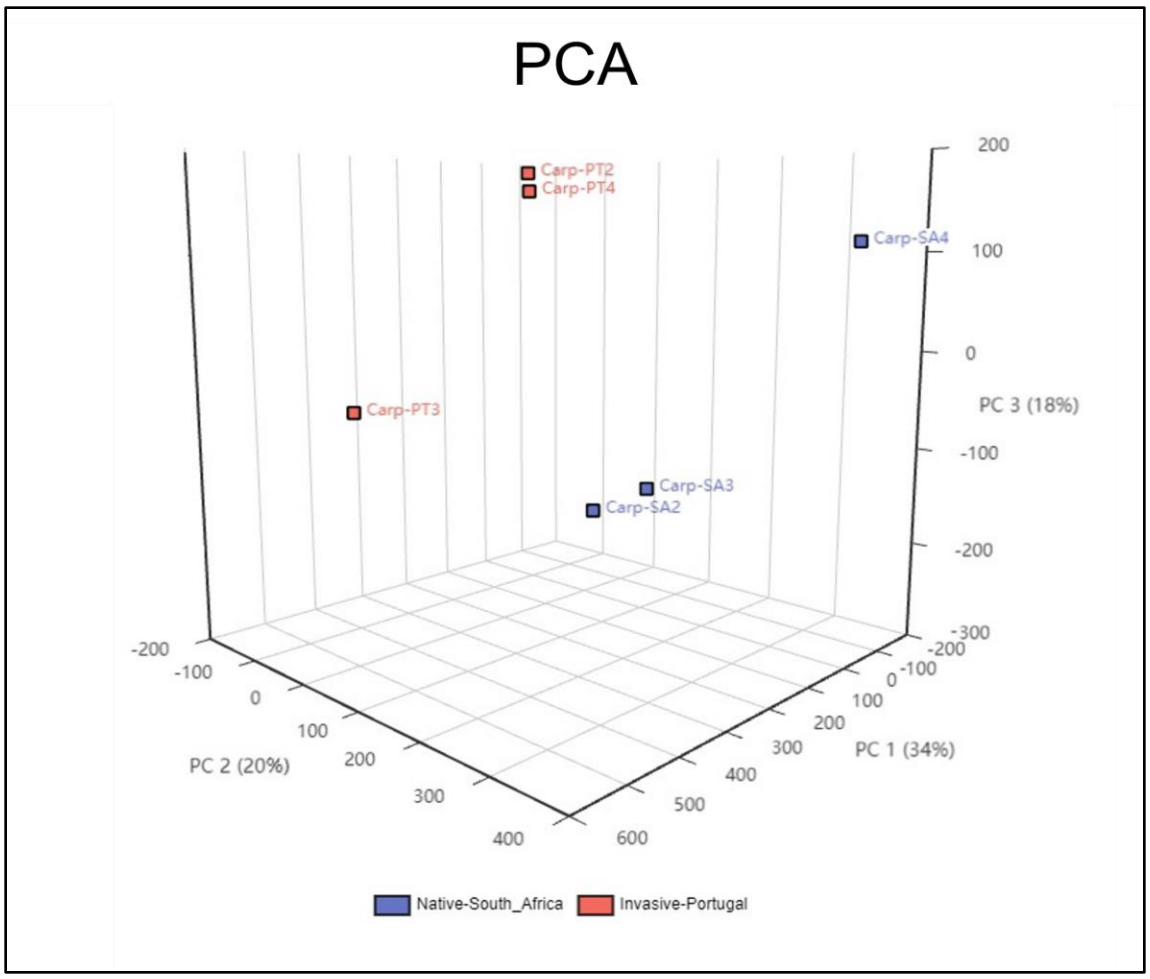

Figure 7. Principle Component Analysis.
The following figure is the PCA of 6 libraries used for the differential expression analysis generated by OmicsBox v.2.2.4 Countable function. PCA represents the comparisons of gene expression between sample groups of the native (SA 2-4 in Blue) and invasive (SA 2-4 Red) as compared in a dimensionally reduced method. Genetic comparisons show a clear delineation between native and invasive populations, indicating RNA-Seq data shows a clear separation between native and invasive populations.

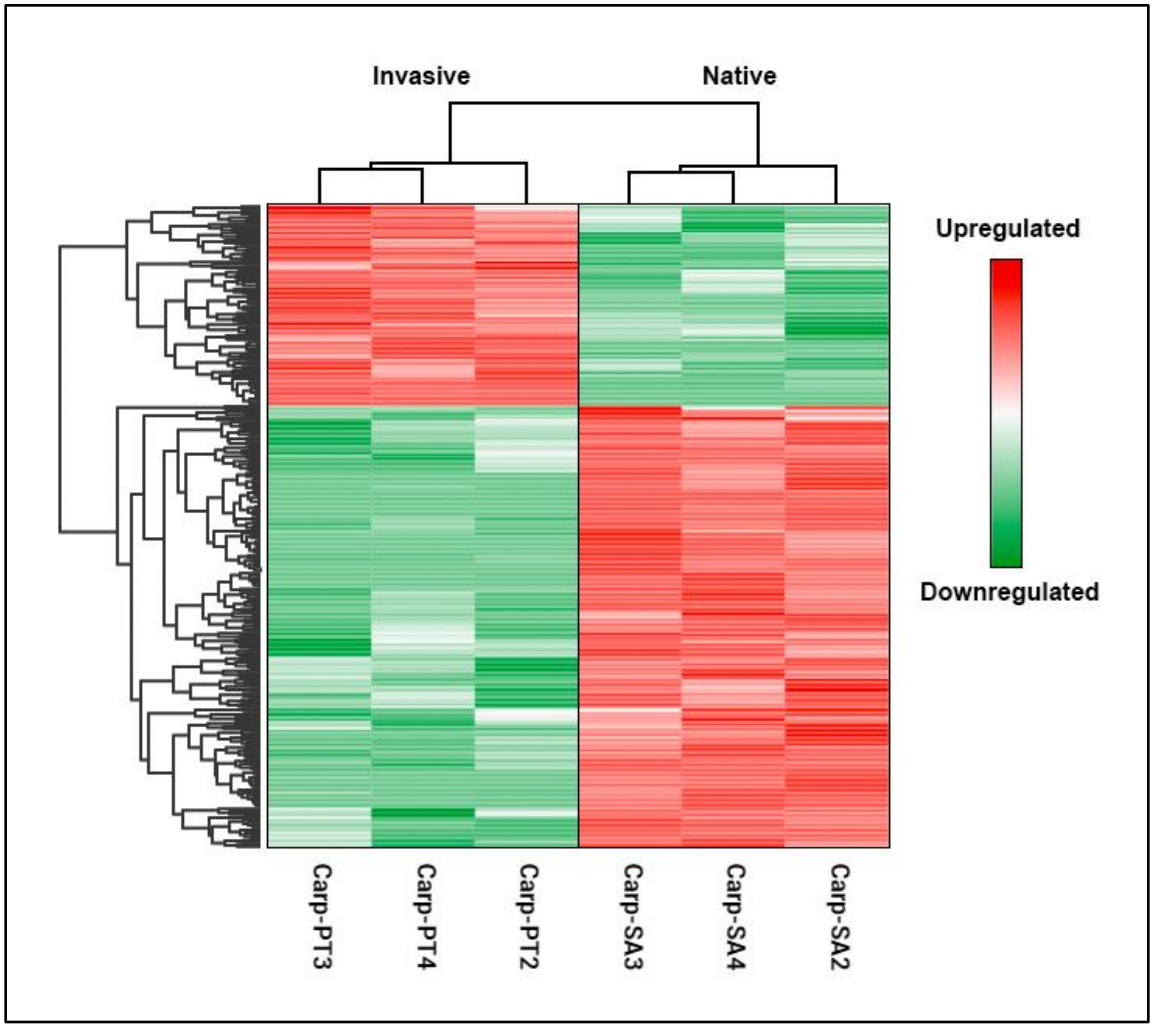

Figure 8. Differential Expression Heatmap.

Heatmap showing the differential expression pattern and clustering of the 148 differentially expressed transcripts (putative genes) between native and invasive populations of *Carpobrotus edulis,* with a False Discovery Rate (FDR) < 0.001 and Logarithmic Fold Change (logFC) > 4, (based on Counts Per Million). The columnar dendrogram across the top of the figure shows the cluster variables by population, Native (Left, SA2-4), and Invasive (Right PT2-4). The horizontal dendrogram illustrates the clustering of transcripts based on the calculation of cluster similarity for individual transcripts. Colors correspond to the regulation of expression, red indicates upregulation, and green indicates downregulation. The figure shows a clear pattern of native and invasive clusters as having distinct patterns of expression for the 148 differentially expressed genes.

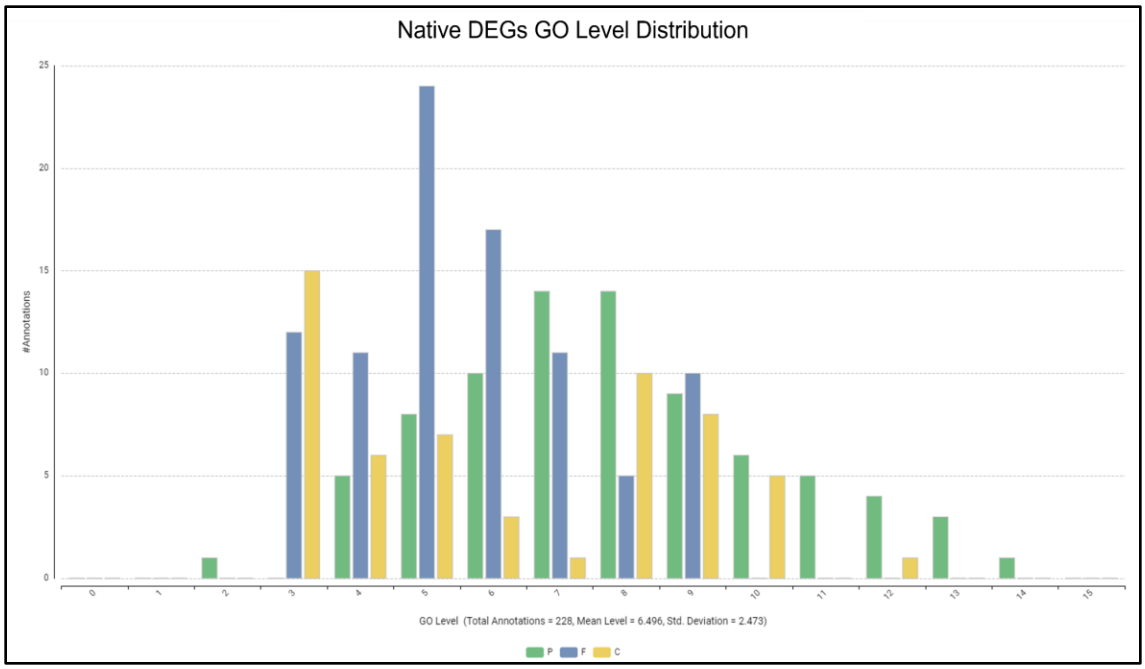

Figure 9. Distribution of Annotations by Gene Ontology Level

(GO) terms associated with the 61 upregulated transcripts from produced by the differential expression (DE) analysis. The figure illustrates the total number of annotations per GO level, with GO categories arranged by color. Green is Biological Process (P), Blue is Molecular Function (F), and Yellow is Cellular Component (C). GO categories are refined along the x-axis according to the increasing specification of GO terms.

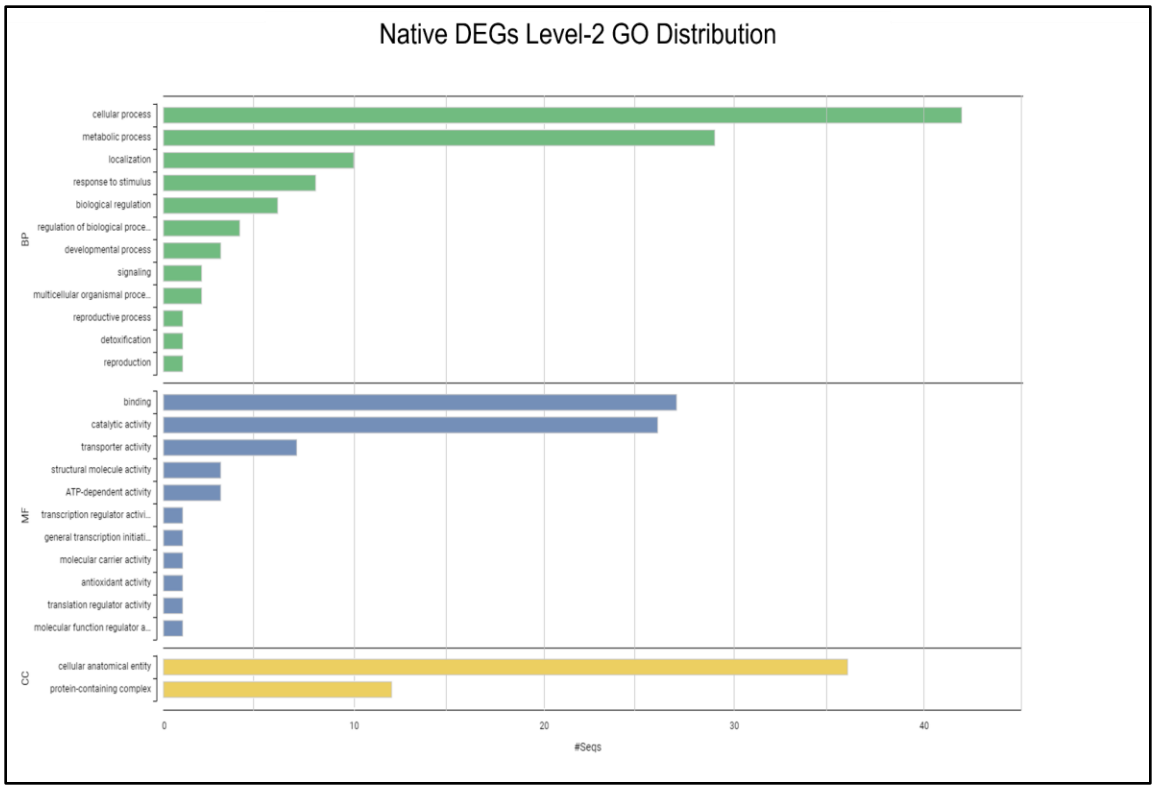

Figure 10. Distribution of Sequences by Level 2 Gene Ontology (GO) terms for the 61 upregulated transcripts from the differential expression (DE) analysis. The figure illustrates the total number of sequences with GOs about a specific GO functional category. GO categories are arranged by color. Green is Biological Process (PP), Blue is Molecular Function (MF), and Yellow is Cellular Component (CC).

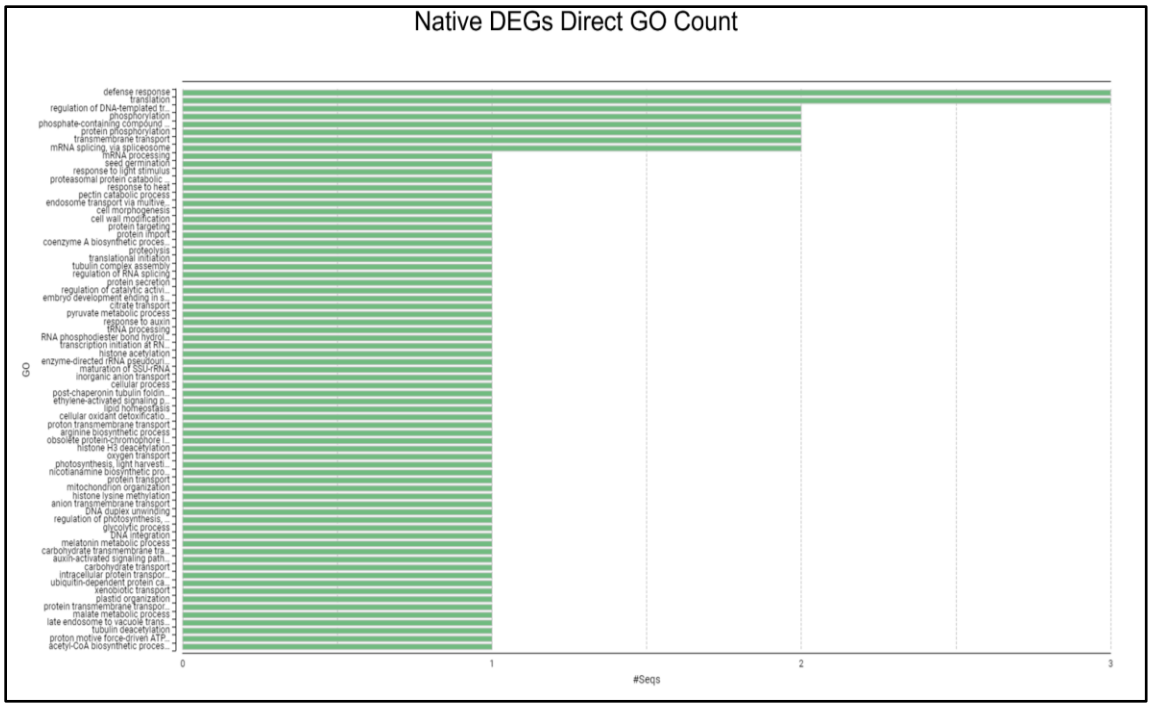

Figure 11. Native GO Direct Count Biological Process.
Distribution of Gene Ontology (GO) terms associated with the 61 upregulated transcripts from the differential expression (DE) analysis. The figure illustrates the total count of sequences categorized by associated GOs within biological processes.

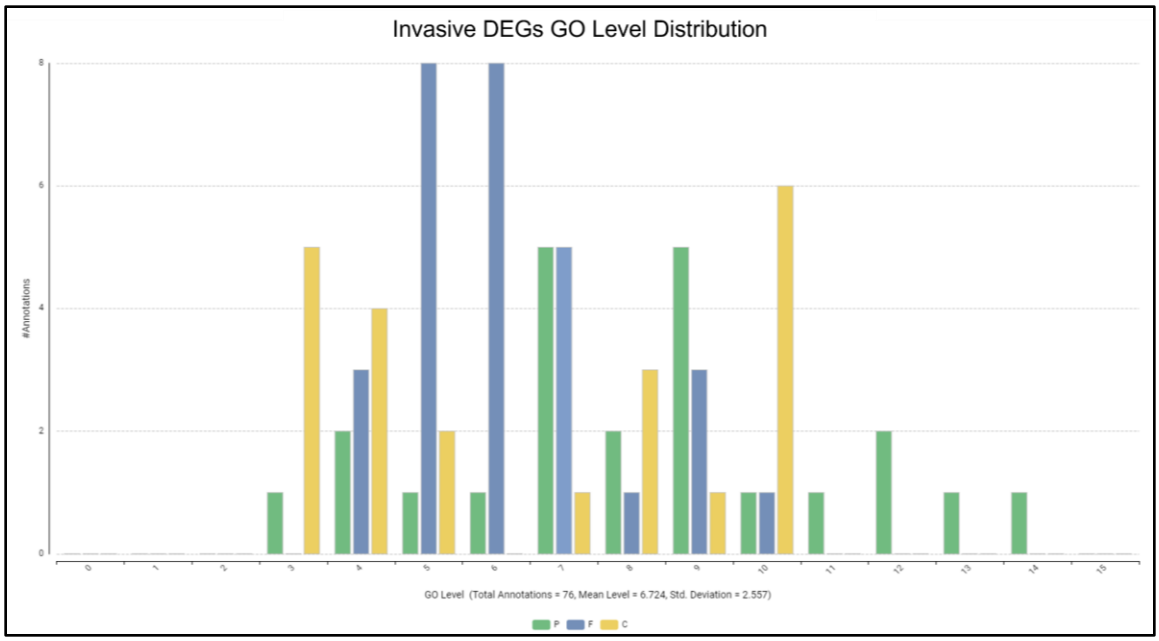

Figure 12. Invasive GO Distribution.
Distribution of Gene Ontology (GO) terms associated with the 21 upregulated transcripts from the differential expression (DE) analysis for the invasive populations. This figure illustrates the total number of annotations per GO level, with GO categories arranged by color. Green is Biological Process (BP), Blue is Molecular Function (MF), and Yellow is Cellular Component (CC).

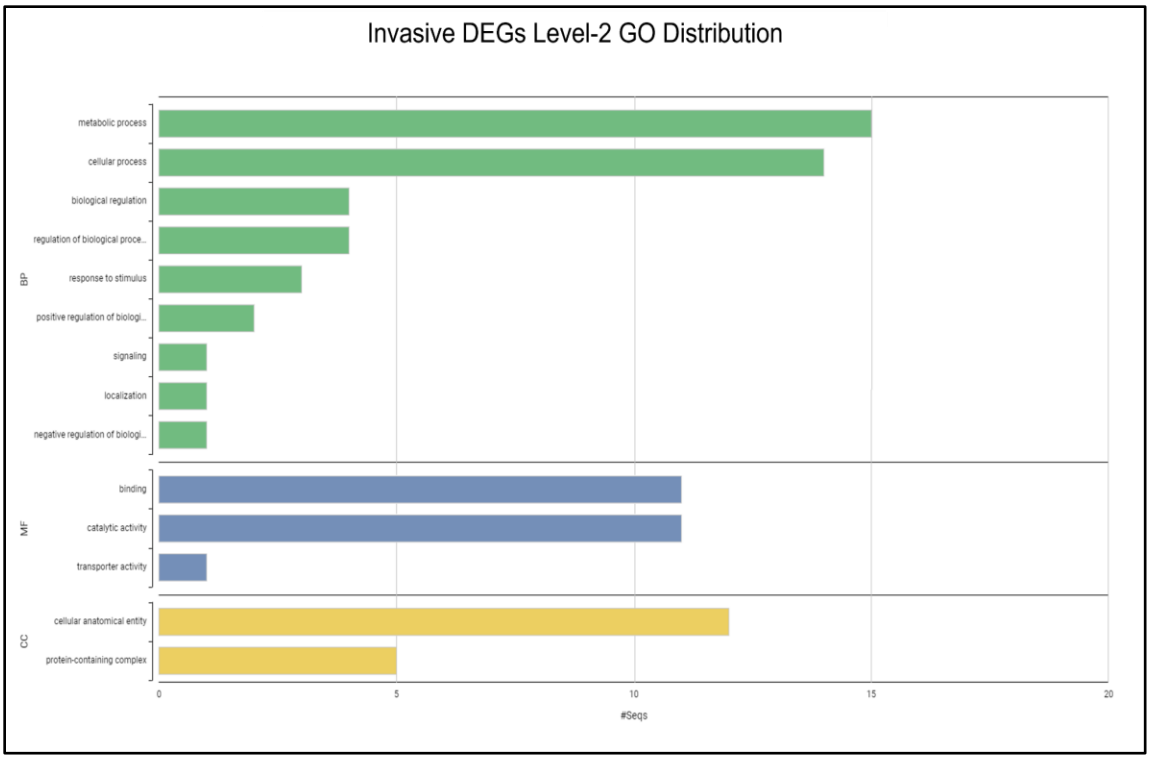

Figure 13. Invasive Level 2 GO Distribution.
Distribution of sequences by Level 2 Gene Ontology (GO) terms for the 21 upregulated transcripts from the differential expression (DE) analysis. The figure illustrates the total number of sequences with GOs pertaining to a specific GO functional category. GO categories are arranged by color. Green is Biological Process (BP), Blue is Molecular Function (MF), and Yellow is Cellular Component (CC).

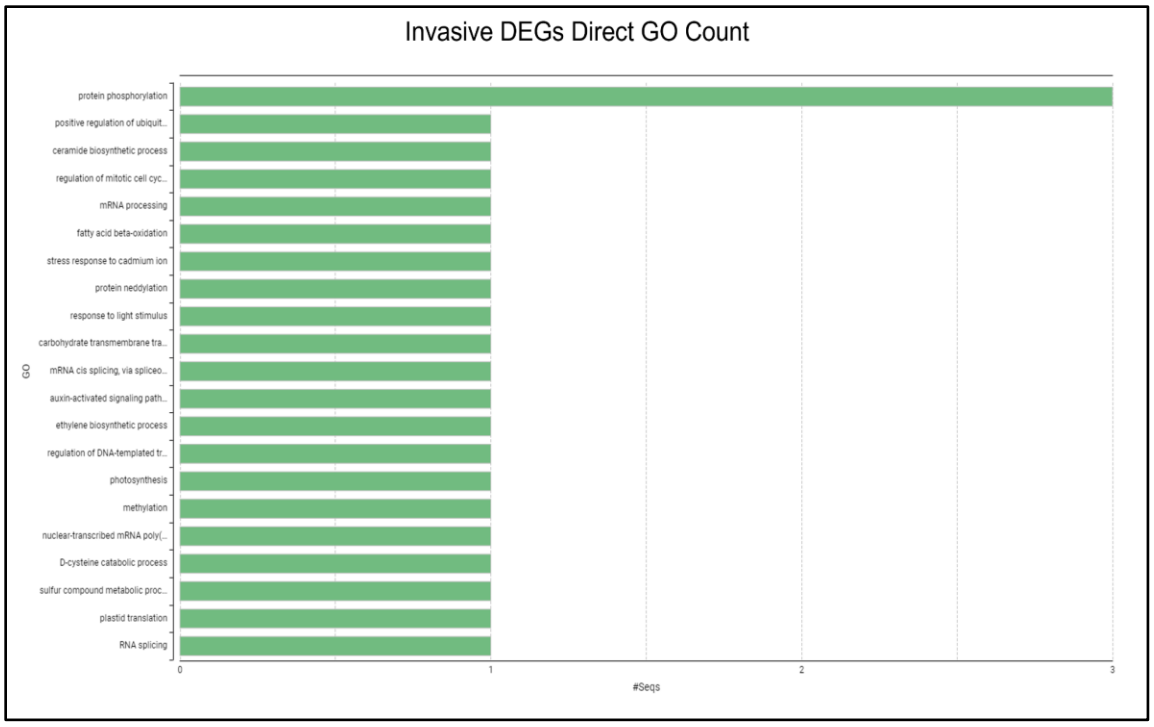

Figure 14. Invasive GO Direct Count.
Distribution of Gene Ontology (GO) terms associated with the 21 upregulated transcripts from the differential expression (DE) analysis. The figure illustrates the total count of sequences categorized by associated GOs within biological processes.

Table 4. Read Alignment

Input Reads		Aligned Reads (%)		Total Alignment (%)	
Sample	**Total Records**	**Once**	**Multiple**	**Overall**	**Not Aligned**
PT-2	50,214,152	33.4	53.4	86.8	13.2
PT-3	53,023,361	30.8	54.3	85.1	14.9
PT-4	61,164,342	32.7	54.1	86.8	13.2
SA-2	63,697,185	34.2	52.3	86.5	13.5
SA-3	42,828,974	31.9	53.1	85.0	15.0
SA-4	61,606,393	31.2	53.1	84.2	15.8

Read Alignment assessment using OmicsBox Count Table Function. An additional metric for assessing the quality of a transcriptome is to check how well your sample RNA data (PT2-4 & SA2-4 libraries) map back to your transcriptome. This ensures that your transcriptome can adequately represent the sample groups. overall alignment reached a maximum of 86.8%, and a minimum of 84.2%, with a mean alignment of 85.7%.

Table 5. The Number of Differentially Expressed Transcripts

Population	Initial	(FDR/logFC)	Post Filter	(FDR/logFC)
Native	254	< 0.05, 1	111	< 0.001, 4
Invasive	135		37	
Total	378		148	

Table of differential expressed transcripts between populations of native *Carpobrotus edulis* from South Africa and invasive *Carpobrotus edulis* from the Iberian Peninsula. Initial values were from results at default settings (FDR < 0.05, logFC > 1) for EdgeR-integrated software within the OmicsBox. Transcripts were subsequently filtered to a more stringent FDR < 0.001 and a logFC > 4. This was done to ensure maximum power in differentiation from DE.

CHAPTER FOUR

DISCUSSIONS ON EICA AND GA

In understanding the invasion potential of a translocated species, traits of interest should be those that are more likely to be impactful on the introduced range, as these often facilitate the transition from an established or naturalized population to an invasive one (Kempel et al., 2013). These traits often are associated with physiological adaptations that enable the negation of competitive exclusion, and ultimately reduce the species richness of its niche competitor (Milanović et al., 2020; Vilà et al., 2007). Increased propagule efficiency, reproductive rates, germination times, and disturbance have been identified as traits facilitating establishment (Kempel et al., 2013; Leishman, 2002). However, it is traits such as higher rates of photosynthesis, increased shoot-root ratio, larger root-shoot biomass allocation, and vegetative growth have been identified as common traits among invasive plants (Dodd & Douhovnikoff, 2016; Suehs et al., 2004; van Kluenen et al., 2010). In fact, not only have these traits been identified as belonging to invasive populations of *C. edulis*, but they are also either lacking or diminished among native populations of *C. edulis* (Roiloa et al., 2014; Roiloa et al., 2016; Portella et al., 2018).

In the context of plant species invasion, many hypotheses have been put forward to explain the possible mechanisms to have facilitated increased invasiveness. Hypotheses such as Genetic Accommodation (GA) propose that in

species with high plasticity, selection for traits once inducible by an environmental stimulus can become genetically determined under directional selection in new environments, in effect streamlining genetic responses as plastic traits under selection maintain the beneficial regulatory networks that produce those responses (Ghalambor et al., 2007; Levis et al., 2017; Moczek et al., 2011; Pfennig et al., 2010; Suzuki et al., 2006). Additionally, hypotheses such as the Evolution of Increased Competitive Ability (EICA) have been proposed to explain the increased invasiveness in plants under the framework that escapes from specialized herbivores and pathogens allows for the relocation of metabolic resources into competitive ability (Blossey & Notzold, 1995; Cornet et al., 2016; Zhang et al., 2019). The following analysis highlights changes that are congruent with both hypotheses.

Patterns within Differential Expression

In setting up this analysis the populations needed to be treated as biological replicates. PCA was used to ensure that the genetic expression of each population could be clustered distinctly between native and invasive populations before the DE (Fig 4). Following DE a heat map of the DE transcripts was produced verifying that in addition to the clustering of the samples as populations, there were distinct clusters in terms of up/downregulation of genetic expression (Fig 5). The heatmap illustrates that invasive populations have observable differences that cluster among groups from the invaded range and have a genetic expression that is

distinct compared to the ancestral native range. This is indicative that rapid adaptive evolution in the invaded range has reshaped patterns of expression across the invaded range (See Fig 2 for the geographic distribution of sample sites).

To ensure maximum confidence in the results we chose to be highly conservative in the selection of DE transcripts, selecting those with an FDR less than 0.001 and logFC over 4. Immediately when looking at the results of the DE, the disproportionate number of DE transcripts between both populations is stark. Total upregulated transcripts in the invasive population were a third of those upregulated among the native populations, and by extension, the number of downregulated transcripts among both populations is inversely proportional. The power of this analysis is that it allows us to filter through high/low expression patterns that are shared by both populations and look specifically at patterns that emerge from the differences. Invasive populations of *C. edulis* are showing altered patterns of expression to respond to the same stressful environmental conditions. This can be interpreted as GA in the invasive populations, as selective pressures in the invaded range may have stabilized genes and subsequent gene regulatory networks that differ from those in the ancestral range. GA, and similarly, EICA propose that such unused genes/gene networks can then be discarded or disused when selection for plastic responses differs from ancestral pressures, as they are no longer under stabilizing selection (Schneider & Meyer, 2017).

An example of possible alterations to gene regulatory networks in the invasive populations of *C. edulis* is the limited responses to salicylic (SA) and jasmonic (JA) acids, both are important hormone signals pertaining to predation and biotic defense responses (Pieterse et al., 2009; Volt et al., 2009). While both were treated SA and JA invasive populations showed different genetic responses to the simulated hormonal stress. Among native populations evidence of a strong reaction can be observed based on associated GO terms, as well as a direct examination of specific DE transcripts (here on DE transcripts will be referred to as, differentially expressed genes, DEGs). Looking at the number of direct GOs, the largest of the native group was that of defense response (Fig. 11). Additionally, the descriptions of corresponding upregulated genes associated with the defense GO term (GO:0006952), were MLP-like protein 31, protein SRC2-like, and MLP-like protein 34, (FDR < 0.001, logFC = 10.59, 7.66, 5.80 respectively) were observed among the native populations. No such defense responses were noted in either GOs or upregulated transcripts among the invasive populations. The results of which demonstrate distinct differences in genetic response to the same hormonal signals. If we look at the response of the invasive populations under the lens of EICA, the lack of response to both jasmonic and salicylic acid may be indicative of a reallocation of genetic resources, or under Genetic Accommodation (GA) the loss of and/or silencing of the gene regulatory networks that would have induced responses to those hormones (more on EICA and GA further in the discussion). While both are speculative without deeper analysis, they follow trends

observed in similar analyses. Transcriptomic analysis of native and invasive populations of *Cirsium arvense* (Canada Thistle) grown under experimental conditions found genetic changes in specific regulatory pathways that altered phenotypic response between populations (Guggisberg et al., 2013). Similarly, transcriptomic differences in genetic expression in response to low-temperature exposure have been identified among newly expanded northern populations of *Alternanthera philoxeroides* (Alligator Weed) (Luo et al., 2020).

Patterns within Gene Ontology

When assessing the overall themes observed in GO analysis, it is important to understand general processes as they related to the subsequent DE. An important distinction was observed between the native and invasive populations, in the overall response to the treatment within common garden treatments. Initial patterns within DE showed support for both EICA and GA, with important themes being reduced metabolic investment in biotic defense hormone response and a reduction in the overall amount of genetic expression (streamlining). Evidence of these changes can be inferred from the total GO count, as the invasive population presented a total of 76 GOs compared to the native 228 terms. While not directly analogous to the genetic response (as a single gene can have many GOs), we can infer this based on the pattern of associated GO terms among the DEGs (Table 5).

Because we are interested in the specific differences in the biological responses between these populations we will limit the scope of the GO analysis to the area of BP, as these are more likely to be responsible for the general physiological changes observed. Among natives, Level 2 distribution showed trends in terms associated with developmental processes, detoxification, reproductive processes, and reproduction (Fig 10). The native populations are responding to increased environmental pressures, with a general theme of preparedness and reproduction. Evidence of this can be seen in responses to cues from the treatments, such as treatment with SA and JA acid, drought stress, and increased temperature. This is an important finding as plants will often accelerate reproduction as a means of escape from increasing environmental pressures including heat and drought stress (Riboni et al., 2014; Shavrukov et al., 2017). A response that has been conserved across many taxonomic groups (Takeno 2016). Predictably, however, invasive populations showed a distinct difference. Level 2 distribution of GO terms associated with upregulated transcripts in the invasive group lacked the aforementioned responses, rather the only GOs present among invasives and absent among natives are positive and negative regulation of biological processes (Fig 13). Moreover, we see that these processes are only a third of those observed among native populations. This is important as this does support possible changes in regulatory gene networks, specifically genetic streamlining.

Additionally, we looked at the direct count of GOs under the category of BP, as this further refines our picture by looking at direct associations to physiological behavior. At this resolution, the native population displayed numerous GOs with a direct count of 81, in contrast, the invasives populations displayed only 23. Additionally, the highest categories listed for native populations were those relating to defense response (3) and translation (3), however of interest were also those relating to seed germination (1), response to heat (1), embryo development (1), cellular oxidant detoxification (1), pectic catabolic process (1), and cell wall modification (1), as these strongly correlate with reproductive growth as well as pathogen/predation response. However, these are also notably absent among the direct counts of GOs for the invasive population, where the highest category is related to protein phosphorylation (3), with the remaining 20 similar to those found among the native populations. It is important to point out that the same GOs present in both populations are associated with differentially expressed transcripts. That is to say that of those responses that are similar in terms of GO association, they are the result of different gene/gene regulatory pathways, a strong indication of GA.

Concerning the invasive populations, protein phosphorylation is an important process in response to environmental pressures as plants will rely on this and alternative splicing to quickly regulate metabolic and biological processes to external stimuli (Li & Liu, 2021; Schweighofer & Meskiene, 2015). It is important to note that protein phosphorylation GOs were also identified among native

populations. However, the nature of our comparison highlights why this is so important because we performed a direct comparison between populations, again showing an altered gene/gene regulatory response. This provides two important takeaways, first, that despite simulated pathogen/predation (via SA and JA), invasive populations did not respond in a manner we could identify. Additionally, in response to conditions such as increased temperature and drought, invasive populations did not shift into reproductive growth as native populations did. While we cannot rule out that the conditions were insufficient to induce flowering or defense response among invasive populations, the altered regulatory response to the same conditions is evidence of changes in gene expression that are consistent with both EICA and GA.

DE Transcripts of Interest Among Native Populations

Refining our analysis even further when assessing the 10 most differentially expressed genes, we looked for themes that could be drawn from the most identifiable sequences (Table 6). This was done using a combination of NCBI Blast results for a specific description of ortholog function as well as GO for generalized function. Stress and reproduction were again observed as patterns in the DEGs. GO terms provided very generalized functionality, however assessment of ortholog function revealed a sharper image of the possible environmental responses. The highest up-regulated transcript was identified as being an ortholog of trans-cinnamate 4-monooxygenase (TRINITY_DN10168_c0_g1_i5, FDR < 0.001,

logFC = 14.98), a catalytic enzyme involved the production of various bioactive secondary metabolites (Betz et al., 2001; Hrazdina et al., 1985; Jaing et al., 2017). The production of secondary metabolites is an important defense response among plants to both predation and pathogen infection, indicating a response to the SA and JA treatments. The second highest up-regulated transcript was described as Non-symbiotic hemoglobin 1-like (CeNon-Hb1) (TRINITY_DN12781_c0_g1_i3, FDR < 0.001, logFC = 13.09) a key enzyme involved in the scavenging of Nitrous Oxides (NO) during hypoxia or low-temperature stress (Kuruthukulangarakoola et al., 2017; Shimoda et al., 2005; Thiel et al., 2011). Transcriptomic analysis of transgenic *Arabidopsis thaliana* indicated that over-expression of AtHb1 (CeNon-Hb1 ortholog) induced several stress-related hormones involved in signaling regulatory factors and genes related to abscisic, salicylic, and jasmonic acids, and production or latex proteins (Thiel et al., 2011). This upregulated response in *C. edulis* native populations could be indicative of one or all corresponding treatments (low-temperature, SA, and JA treatments). Additionally, this also means Non-Hb1 was significantly downregulated among the invasive population. This finding is consistent with those of Fenollosa et al, (2018) who found variations in ABA, SA, and JA, responses to cold stress. Additionally, comparisons between native and invasive populations of *C. edulis* indicated protective responses to cold stress, and a differential chilling sensitivity was identified, with individuals from the native range displaying increased sensitivity to chilling with corresponding differences in

physiological response (Fenollosa & Munné-Bosch, 2019). This finding is consistent with expectations of GA, among invasive populations.

DEGs related to environmental stress response were upregulated transcripts described as Serine-rich-protein-like (TRINITY_DN129_c0_g1_i3, FDR < 0.001, logFC = 13.06) and Serine/arginine-rich splicing factor SR34A-like (TRINITY_DN20524_c0_g1_i6, FDR < 0.001, logFC = 12.28). Expression patterns of Serine-rich-proteins (SrPs) are largely linked to a wide array of abiotic stresses (Kishor et al., 2020; Mahalakshmi et al., 2006). Additionally, some SrPs have been identified as being integral to specific tissue development, with Serine/arginine-rich splicing factors integral for the regulation of SrP-isoforms relating to flower development and root development (Chen et al., 2019) or as alternatively spliced regulatory factors (Kalyna et al., 2012). Additionally, SrPs are used as fast-response signaling factors, because of their utility as signal transductors (Chen et al, 2019). Because these are correspondingly downregulated among invasive populations, these indicate an altered means of responding to the same environmental conditions.

With respect to reproductive growth, the remaining two of interest were those relating to aspects of reproduction. Respiratory burst oxidase homolog protein B-like and protein translocase subunit SECA2, chloroplastic isoform X1 (TRINITY_DN1232_c0_g1_i12, FDR <0.001, logFC = 11.97859; TRINITY_DN2263_c0_g1_i2 FDR < 0.001, logFC = 6.98 respectively). Both have GO terms relating specifically to seeds, particularly seed germination, and embryo

development. Flowering time, and by extension seed production, is known to be affected by various abiotic stresses, including drought (Riboni et al., 2014) and nutrient deficiency (Shimakawa et al., 2012). These factors are adaptations that evolved as part of complex regulatory networks aimed at escaping certain abiotic environmental conditions via seed dispersal (Ionescu et al., 2017). Such regulatory networks have been shown to include upregulation in pathways involving chlorophyll transport such as SEC2A during the transition from vegetative to flowering growth (Liu et al., 2022). Additionally in *Arabidopsis thaliana* SEC2A mutants were shown to be lethal to embryos at globular stages (Skalitzky et al., 2011). Indicating a strong relationship between SEC2A and embryogenesis. With respect to *Ce-RbohB*, respiratory burst oxidases (Rboh) serve varied functions in plants, with RbohB orthologs relating to biotic defense (Torres & Dangl, 2005; Hawamda et al., 2020) and heat response (Larkindale et al., 2005). However, of interest are functions related to seed dormancy and germination. The RbohB ortholog in *Arabidopsis thaliana* plays a vital role in the preparation of seed ripening, additionally in the potential for alternative splicing post translation as a mechanism for seeds to react to their environments (Müller et al. 2009). Upregulation of these transcripts supports the hypothesis that native populations may be undergoing or preparing for stress-induced flowering. These transcripts are also significantly downregulated among the invasive populations, indicating either a lack of or delayed response (compared to native). This is consistent with the physiological traits displayed in the invaded range, and alterations in regulatory

networks expected under GA. Together, these provide evidence for a strong genetic response among native populations of *C. edulis* to simulated predation, reaction to environmental stress, and preparation for alteration of growth patterns. Additionally, these genetic responses were absent among invasive populations under the same controlled conditions.

DE Transcripts of Interest Among Invasive Populations

Among the invasive populations, a similar analysis was performed on the most identifiable differential expressed and related transcripts of interest (Table 7). The highest upregulated transcript among invasive was described as DCN1-like protein 4 isoform X1 (CeDCN1) (TRINITY_DN4963_c0_g2_i6, FDR < 0.001, logFC = 13.69), cyclin-dependent kinase G-2 (CDKG2) - like isoform X1 (TRINITY_DN5089_c0_g1_i3, FDR < 0.001, logFC = 10.82), and E3 ubiquitin-protein ligase (ATL23) (TRINITY_DN2884_c2_g1_i11, FDR < 0.001, logFC = 4.98). Choi et al. (2014) have linked DCN1 and E3 ubiquitin ligases to the highly conserved neddylation/NEDD8 pathway, a strong means of responding to environmental conditions. The ubiquitin-proteasome (NEDD8) is responsible for the regulation of growth and development in plants (Santner & Estelle, 2010). Additionally, an ortholog of CeDCN1 in *Arabidopsis thaliana* (aar3) acts as a regulator for 2,4-dichlorophenoxyacetic acid (Biswas et al., 2007). 2,4-dichlorophenoxyacetic acid, also known as synthetic auxin, acts as a selective herbicide inducing uncontrolled growth (Wilson et al., 2010). Moreover, in *A.*

thaliana, CDKG2 has been identified as relating to salt mediated flowering, where in constitutively (always on) mutants showed delayed flowering time (Ma et al., 2015). This is supported by Nibau et al. (2020) that showed CDKG2 is involved in the regulation of flowering time by balancing flowering transcription factors, and that loss of CDKG2 results in *A. thaliana* mutants results in an early flowering phenotype. Together these indicate that invasive populations of *C. edulis* may be regulating strong responses to growth hormones in responses to environmental cues.

Interestingly, the second highest was described as protein early responsive to dehydration 15 (CeERD15) (TRINITY_DN5035_c0_g1_i11, FDR < 0.001, logFC = 11.65) which acts as a regulator of both abscisic and salicylic acids to mitigate stress responses in *Arabidopsis thaliana* decreased drought resistance while increasing freezing tolerance when overexpressed (Kariola et al., 2006). However, function diversity among this family is large, in soybeans, the ERD15 homolog connects endoplasmic reticulum with osmotic stress-induced death (Alves et al., 2011). While it may seem counterintuitive for invasive populations to overexpress a gene that decreases their drought tolerance, this was not reflected in the physiology of the invasive plants under drought treatment (Personal Observation Dr. Lopez). What is telling however is that if the putative CeERD15 works as it does in A. thaliana, this may explain the increased cold tolerance (Fenollosa & Munné-Bosch, 2019), as well as the altered hormonal responses to cold, observed among invasive populations (Fenollosa et al., 2018). Overexpression in the

invasive group may have evolved as a mechanism to ensure cold tolerance along the coasts of Europe. As such, it would be appropriate for the native populations to utilize putative CeERD15 for drought response, as there is little need for cold tolerance in South Africa with mean temperature ranges from 11ºC - 17.5ºC (Climate Change Knowledge Portal, 2022). This finding is supported by work performed by Fenollosa & Munné-Bosch (2019) which showed invasive populations outperforming natives' cold tolerance at various fridged temperatures, except at 12ºC, where performance was similar. This is an interesting temperature, as that is the historically low mean temperature observed in the ancestral range. Indicating the presence of ancestral regulatory networks beneficial to cold tolerance has been preserved among invasive populations, a finding congruent with GA. The elevated level of expression among these putative orthologs indicate invasive populations are increasing growth and regulation of growth pathways, while delaying flowering. This is consistent with what we have observed in the contrasting gene expression among native populations; reduced growth response, transition to reproductive growth and increased flowering.

The last group of upregulated invasive transcripts is associated with drought response, but more specifically with regulation of growth in response to drought conditions. The upregulated transcript descriptions were cyclin-dependent kinase G-2 - like isoform X1 (CeCDKG2) (TRINITY_DN5089_c0_g1_i3, FDR < 0.001, logFC = 10.82), Mitogen-activated protein kinase (CeMAPKKK) (TRINITY_DN2255_c1_g2_i12, FDR < 0.001, logFC = 8.44), and

Serine/threonine-protein kinase AFC2 (CeAFC2) (TRINITY_DN4384_c0_g1_i89, FDR < 0.001, logFC = 10.75). CeCDKG2 had a high percent identity (93%) to protein kinase MK5 in *Mesembryanthemum crystallinnum* (McKM5), also a member of Aizoaceae. McKM5 has been identified as belonging to a complex AFC2-based drought response network (Bidabadi et al., 2015). Similarly, Alimohammadi et al., (2013) identified orthologs of all three of the AFC kinases, as having functions related to water-deficit resistance, regulating growth via sugar-mediated signaling, and histone modification. Further, MAPKKKs are a highly conserved signal in plant development and drought stress response, with various MAPKKK orthologs related to regulatory cascades involved in drought tolerance (Li & Liu, 2021). Together, these indicated a strong response among invasive populations to the drought treatment, indicating a strong selection for water deficit management in the invasive populations. Conversely, these are downregulated among the natives, which is counterintuitive when considering drought treatments. However, this may be indicative of differences in drought response depending on whether the plant is responding during reproductive growth (Native) and vegetative growth (Invasive).

Closing Remarks

Among one the most looming concerns are the impacts of climate change, as many researchers have identified synergistic effects of plant invasion and climate alteration (Giejsztowt et al., 2020). In addition to the stresses introduced

by climate change, invasive species have shown to be capable of increased physiological efficiency at increased temperatures and drought stress (Vilà et al., 2007), increasingly with high plasticity species such as *C. edulis* (Campoy et al., 2021). Evidence of these sorts of rapid evolutionary changes has been observed in other invasive species. Based on this analysis we can conclude that there are clear genetic changes that have been selected for in the invaded range of *C. edulis*. These changes have partially explained aspects of the increased invasiveness observed between native and invasive populations. A finding that supports the notion of selection that results in EICA and GA. Future work could be done in identifying regulation changes among transcription factors, to identify if there has been a selection that led to rapid adaptive evolution in the form of differential modifications to specific gene regulatory networks.

Table 6. Native Differentially Expressed Genes of Interest

Sequence Name	Description	FDR	logFC
TRINITY_DN10168_c0_g1_i5	trans-cinnamate 4-monooxygenase	< 0.001	14.99
TRINITY_DN12781_c0_g1_i3	non-symbiotic hemoglobin 1-like	< 0.001	13.09
TRINITY_DN129_c0_g1_i3	serine-rich protein-like protein	< 0.001	13.06
TRINITY_DN20524_c0_g1_i6	serine/arginine-rich splicing factor SR34A-like	< 0.001	12.52
TRINITY_DN1232_c0_g1_i12	respiratory burst oxidase homolog protein B-like	< 0.001	11.98
TRINITY_DN2263_c0_g1_i2	protein translocase subunit SECA2 *	< 0.001	6.98
TRINITY_DN177_c1_g1_i1	Probable pectinesterase 53	< 0.001	9.83
TRINITY_DN1324_c0_g1_i8	protein SRC2-like	< 0.001	7.66
TRINITY_DN14061_c0_g1_i10	MLP-like protein 31	< 0.001	5.79
TRINITY_DN1561_c0_g1_i27	MLP-like protein 34	< 0.001	10.59

Table of differentially expressed genes significantly upregulated among native populations, their relevant metrics such as *de novo* Trinity designation, False Discovery Rate (FDR), and Log Fold Change (logFC).
*Abbreviation of *protein translocase subunit SECA2 chloroplastic isoform X1*.

Table 7. Invasive Differentially Expressed Genes of Interest

Sequence Name	Description	FDR	logFC
TY_DN4963_c0_g2_i6	DCN1-like protein 4 isoform X1	< 0.001	13.69
TY_DN5089_c0_g1_i3	cyclin-dependent kinase G-2 - like isoform X1	< 0.001	10.82
TY_DN2884_c2_g1_i11	E3 ubiquitin-protein ligase ATL23	< 0.001	4.98
TY_DN5035_c0_g1_i11	protein ERD15-like**	< 0.001	11.65
TY_DN2187_c0_g1_i3	Sugar transporter ERD6-like 5	< 0.001	7.74
TY_DN4384_c0_g1_i89	Serine/threonine-protein kinase AFC2	< 0.001	10.75
TY_DN2255_c1_g2_i12	Mitogen-activated protein kinase kinase kinase	< 0.001	8.44

Table of differentially expressed genes significantly upregulated among native populations, their relevant metrics such as *de novo* Trinity designation, False Discovery Rate (FDR), and Log Fold Change (logFC).
** Abbreviation of protein EARLY RESPONSIVE TO DEHYDRATION 15- like.

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