Catarina Constantino Siopa

The role of genome duplication in the genesis of diversity:

The role of selfing in the establishment of neotetraploid lineages of *Jasione maritima*

Tese de mestrado em Ecologia, orientada pela Doutora Sílvia Raquel Cardoso Castro Loureiro e pela Doutora Maria Celeste Pereira Dias (Universidade de Coimbra) e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Junho 2018

· U C ·

UNIVERSIDADE DE COIMBRA

Agradecimentos

À Sílvia Castro, Maria Celeste Dias e João Loureiro. Obrigado por aceitarem orientar a minha tese. Obrigada por toda a ajuda, conselhos, orientação e motivação para ser e fazer melhor. Aprendi e evolui muito graças a vocês.

Ao Departamento de Ciências da Vida e ao Centro de Ecologia Funcional pela disponibilização dos laboratórios que permitiram a realização das experiências laboratoriais, e ao Jardim Botânico da Universidade de Coimbra, que disponibilizou também espaço para a realização da experiência em estufa.

A todo o FLOWer group, obrigado por me acolherem tão bem! Especial obrigado à Mariana Castro por todos os conselhos e ajuda ao longo de todo o processo, foste muitas vezes a minha salvação. Todas as vossas palavras de ânimo e motivação foram essenciais para sobreviver a estes dois anos.

Aos meus primos de Mestrado, Daniela e Mikael, obrigado por tudo. Estes anos não seriam os mesmos sem vocês. Foi um gosto partilhar os melhores dois anos da minha vida convosco. É um prazer ver-vos fechar mais um capítulo, e nada melhor que fazê-lo juntos.

Ao Ricardo, obrigado por estares sempre presente (mesmo longe) e pronto a acudir e ouvir todas as minhas lamúrias, por me motivares e acreditares sempre em mim. Obrigado pela ajuda e conselhos, pela companhia nas noites longas de trabalho. Obrigado por me arrastares para fora de casa para parar de trabalhar e viver um pouco para além da tese. Esta tese é também um pouquinho tua.

Por último, obrigada aos meus pais e ao Paulinho por todo o apoio e preocupação. Obrigado, mãe, por seres uma guerreira e moveres mundos e fundos para que pudesse ter a oportunidade de estudar e seguir esta área. Obrigado por me ensinares como lutar e trabalhar para alcançar os meus objectivos. Esta tese é dedicada especialmente a ti.

Obrigada a todos que me acompanharam e apoiaram de alguma forma nesta aventura.

Table of contents

Abbreviations

2*n* – diploid number of chromosomes

2*x* – diploid

ABA – abscisic acid

Chl $_a$ – Chlorophyll *a*

Chl $_b$ – Chlorophyll *b*

CV – coefficient of variation

DNA - deoxyribonucleic acid

DW – Dry weight

Φ_{PSII} – Effective quantum yield of photosystem II

et al. – (L. *et alia*) and others

FCM – Flow cytometry

FL – fluorescence pulse integral

F_m – Maximum fluorescence at dark

F_m' – Maximal fluorescence at light

F_o – Minimum fluorescence

F_s – steady-state fluorescence

FS – forward light scatter

F_v / F_m – Maximum quantum yield of photosystem II

FW – Fresh weight

GLM – Generalized Linear Models

GLMM – Generalized Linear Mixed Models

e.g. – (L. *exempli gratia*) for example

i.e. – (L. *id est*) that is

ICN – Portuguese Nature Conservation Institute

IUCN – International Union for Conservation of Nature

LSmeans – least square means

n – number of

neo4*x* – neotetraploid

out – outcrossing

P – probability

pi – proportion

pg – picograms

RNase – ribonuclease

REL – Relative Electrolyte Leakage

RWC – Relative Water Content

SCI – Self-compatibility index

SD – Standard deviation

self – selfing

SE – Standard error

SS – side light scatter

sp. – (L. *species*) species

TSS – Total Soluble Sugars

TW – Turgid Weight

UV – ultraviolet

vs. – versus

Note: all the units used follow the SI (Système International d'Unités)

List of Figures

List of Tables

List of Appendices

Abstract

Polyploidization is considered a major force in the evolutionary history of Angiosperms. This phenomenon can modify various aspects of a plant, including its morphology and physiology. Similarly, the biology of reproduction may also be changed and will have strong consequences for the polyploid in its early stages after emergence. All these possible changes can be crucial for the establishment allowing the neopolyploid to overcome the minority cytotype exclusion. So far, polyploid studies have been mostly focused in genetics and epigenetics, with studies in the field of ecology and reproduction biology being very scarce. These areas, however, are equally important for understanding the mechanisms that lead to the emergence, establishment, and maintenance of polyploids in nature.

Jasione maritima is a Campanulaceae growing in dune systems and a polyploid complex bearing diploids and tetraploids, in a currently allopatric distribution. The mechanisms behind the establishment of the tetraploid are, however, unknown. Here, we studied the role of selfing in the establishment of neotetraploids of *J. maritima*, with the use of synthesized neotetraploids in order to evaluate the real consequences of polyploidization. Reproductive success was measured after controlled pollinations to understand the effects of polyploidization in the selfing ability. In addition, a set of fitness parameters including physiological parameters were measured to assess the performance of the offspring obtained after selfing and outcrossing.

The results of this study revealed that diploids and neotetraploids of *Jasione maritima* are self-incompatible. Thus, polyploidization does not seem to impact the ability to self-fertilize in the neotetraploids. The reproductive success was negatively affected by polyploidization and by the use of selfing. When analysing the neotetraploids, the overall fitness of the offspring was lower than diploids. However, in neotetraploids, outcrossing and selfing treatment of the offspring had similar plant performances. Therefore, in the early generations, selfing may act as a reproductive assurance for neotetraploids, enabling them to produce offspring with similar fitness when compared with neotetraploids of the outcrossing treatment. However, selfing by itself is not sufficient to explain the successful establishment of the neotetraploids and is most probably part of a combination of mechanisms that provide a fitness advantage to neotetraploids in the early stages of emergence enabling them to overcome the minority cytotype exclusion.

Keywords

Jasione maritima, neotetraploids, plant performance, polyploidization, selfing.

Resumo

A poliploidização é considerada uma força importante na história evolutiva das Angiospérmicas. Este processo pode modificar vários aspectos de uma planta, incluindo a sua morfologia e fisiologia. De igual forma, a biologia da reprodução pode também ser alterada gerando consequências significativas para o poliplóide nas fases iniciais após a sua emergência. Todas estas possíveis alterações podem ser cruciais para o estabelecimento, permitindo ao neopoliplóide ultrapassar a exclusão do citótipo minoritário. Até à data, os estudos sobre poliplóides focam-se sobretudo na genética e epigenética, sendo os estudos na área da ecologia e biologia da reprodução muito escassos. Estas áreas são, no entanto, igualmente importantes para a compreensão dos mecanismos que levam à emergência, estabelecimento e manutenção dos poliplóides na natureza.

Jasione maritima é uma Campanulacea de sistemas dunares e um complexo poliplóide constituído por plantas diplóides e tetraplóides, numa distribuição actualmente alopátrica. Os mecanismos envolvidos no estabelecimento do tetraplóide são, contudo, desconhecidos. Neste trabalho, estudou-se o papel da autofertilização no estabelecimento de neotetraplóides de *J. maritima*, recorrendo ao uso de neotetraplóides sintetizados de forma a avaliar as consequências reais da poliploidização. O sucesso reprodutivo foi medido após polinizações controladas para compreender os efeitos da poliploidização e a capacidade de autofertilização. Adicionalmente, mediram-se um conjunto de parâmetros de fitness, incluindo parâmetros fisiológicos, para avaliar a performance da descendência obtida através da auto-polinização e da polinização cruzada.

Os resultados deste trabalho revelam que os diplóides e neotetraplóides de *J. maritima* são auto-incompatíveis. Estas observações sugerem que a poliploidização não parece ter tido impacto na capacidade de autofertilização nos neotetraplóides. O sucesso reprodutivo foi negativamente afectado pela poliploidização e pela auto-fertilização. A performance das plantas descendentes foi menor para os neotetraplóides em relação aos diplóides. Contudo, dentro dos neotetraplóides, observou-se que o fitness geral da descendência não foi afectado pelo uso de auto-polinização. Assim, nas primeiras gerações, a auto-fertilização pode actuar como uma garantia reprodutiva para os neotetraplóides nos primeiros momentos após a sua emergência, permitindo a produção de descendência de fitness semelhante aos neotetraplóides de polinização cruzada. No entanto, a autofertilização por si só será insuficiente para explicar o estabelecimento dos neotetraplóides, sendo provavelmente parte de uma combinação

de mecanismos que providenciam uma vantagem competitiva aos neotetraplóides nas primeiras fases de emergência e que lhe permitem superar a exclusão como citótipo minoritário.

Palavras-chave

Autofertilização, *Jasione maritima*, neotetraplóides, performance da planta poliploidização.

CHAPTER 1 – Introduction

1. Introduction

Polyploidization corresponds to the process in which a cell has more than two pairs of chromosomes. In Angiosperms, polyploidization is a common process that is usually associated with events of species diversification (Otto and Whitton, 2000; Blanc and Wolfe, 2004; Soltis and Soltis, 2009; Madlung, 2013). So far, several articles addressed the frequency of polyploidization events on Angiosperms. Stebbins (1971) estimated that 30 to 35% of angiosperm species underwent polyploidization events in their history. In the Artic flora, 51.1% of taxa are exclusively polyploids and 9.6% are diploid/polyploid complexes (Brochmann *et al.*, 2004). In the Mediterranean region, a polyploid incidence of 36.5% was detected, with higher values being detected for the Iberian Peninsula (48.8%; Marques *et al.*, 2018). The process leads to a profound genetic change that can be manifested in biochemical, cytological, morphological and physiological traits of the plant with, lastly, ecological implications that can act as an advantage enabling polyploids establishment (Levin, 2002).

Until now, there are several studies unravelling the questions around polyploids. However, most of them deal with genetic and epigenetic changes. The areas of ecology, reproduction and physiology have received less attention, but are fundamental to understand the mechanisms of establishment and maintenance of polyploids in nature (Soltis *et al.*, 2010; Husband *et al.*, 2013; Segraves, 2017).

1.1 Polyploids formation

In order to understand the dynamics of polyploidy it is essential to understand the mechanisms behind polyploid formation. Unreduced gametes (i.e. 2*n* gametes) formation is considered as a major mechanism for polyploid emergence (Ramsey and Schemske, 1998). The production of polyploids through 2*n* gametes formation may occur by two different pathways: (1) direct formation of a new polyploid entity by the fusion of two *2n* gametes (i.e. bilateral polyploidization) and (2) formation of a triploid-bridge with the fusion of an *2n* gamete and a reduced one (n) (i.e. unilateral polyploidization; Bretagnolle and Thompson, 1995; Ramsey and Schemske, 1998). These pathways may lead to the formation of gametes with different ploidy levels. The 2*n* gametes produced by diploids can cross with gametes of natural polyploids, forming neopolyploid offspring. For example, this was observed on the complex diploid/tetraploid of *Dactylis glomerata*, where cytotypes live in sympatry, with the ongoing formation of 2*n* gametes by diploids allowing the formation of tetraploids offspring through crosses between diploids with unreduced gametes and tetraploids (Bretagnolle and Lumaret, 1995).

In Angiosperms, the incidence of *2n* gametes in nature ranges from 27.52% on hybrids and 0.56 % on nonhybrid species (Ramsey and Schemske, 1998), but these values are highly variable among species, populations, individuals or, even, reproductive structures (Bretagnolle and Thompson, 1995). For example, in *Dactylis glomerata*, the incidence of *2n* eggs produced was 0.49%, but in 2*n* pollen this value reached the 0.98%. The production of unreduced eggs also varied from 0.1% to 26%, while unreduced pollen varied from 0.1% to 14% (De Haan *et al.*, 1992; Maceira *et al.*, 1992). The incidence of *2n* gametes may also be stimulated by stress conditions (see De Storme *et al.*, 2012; Ramsey and Schemske, 1998), which may explain the higher incidence of polyploids on subarctic regions or at higher altitude (Grant, 1971).

Polyploids are often divided into two groups taking into account its mode of origin: auto- and allopolyploids (Kihara and Ono, 1926; Ramsey and Schemske, 1998). Autopolyploids are formed within a population of a single species, through the fusion of two *2n* gametes; while allopolyploids are formed by processes of hybridization followed by polyploidization (Ramsey and Schemske, 2002). By opposition to autopolyploids, allopolyploids have been deeply studied (Soltis *et al.*, 2016), with numerous reports being available in the literature (e.g. *Senecio cambrensis* in Hegarty *et al.,* 2012; *Spartina anglica* in Gray *et al.,* 1991; *Iris versicolor* in Lim *et al.* 2007a, *Nicotiana* family in Lim *et al.,* 2007b). This is mainly due to the fact that autopolyploids are more difficult to detect in nature due to their morphological resemblance with the diploid parental. With the advances on genetic techniques it became possible to more easily detect these entities, being now established that autopolyploids are also common in nature, and thus, in the past, its occurrence may have been underestimated (Soltis *et al.*, 2010; Barker *et al.*, 2016).

1.2 Neopolyploids establishment in nature

After emergence, the first stages of polyploid establishment are usually characterized by small populations of the neopolyploid growing in sympatry with the diploid progenitors (Levin, 2002). Subsequently, the new cytotype may be subjected to the Minority Cytotype Exclusion (as formulated by Levin 1975). Levin (1975) hypothesised that the polyploid, under random mating, is in disadvantage in comparison with the diploid, once it occurs at a lower frequency. Considering that the mating success is frequency-dependent, the new cytotype will struggle to produce offspring since most of the matings of the polyploid cytotype will occur with diploids, which produces odd ploidy offspring and, subsequently, reduce the reproductive success of the neotetraploid**.**

Being reproduction the main factor not only for the establishment, but also for the maintenance of a population, the new cytotype will probably be excluded (Levin, 2002).

Due to the cytotype minority exclusion theory, previous studied defined as very restrictive the likelihood of establishment of neopolyploids (Levin, 1975; Felber, 1991; Rodríguez, 1996). However, it is currently considered that the probability of establishment of polyploids is much higher and dependant on the acquisition of new ecological and reproductive features that may confer the neopolyploid an advantage (Rodríguez, 1996; Rieseberg and Willis, 2007). Neopolyploids may present breeding barriers that mediate assortative mating, including heterogeneity of spatial distribution, temporal or mechanical isolation caused by different flowering phenologies or morphologies, different behaviours or preferences of pollinators, gametic isolation or reduced fertility/fitness of hybrids (reviewed in Castro *et al.*, 2011). Alternatively, if assortative mating is not achieved, neopolyploids may have new traits that increase the capacity to disperse/colonize new niches escaping competition with diploids or that increase fitness and fertility allowing competition with the diploids. Strategies such as higher rates of asexual reproduction or selfing, perenniality, low dispersal, different niche preferences, production of 2*n* gametes or higher competitive ability may all enable the neopolyploids to cope with minority cytotype disadvantage (Rodríguez, 1996; Rausch and Morgan, 2005; Rieseberg and Willis, 2007; Castro and Loureiro, 2014). If all or a portion of these changes occur after polyploidization the probability of establishment of the neopolyploid, much likely, increases.

1.3 Shifts mediated by polyploidization

Numerous studies have tried to identify the shifts mediated by polyploidization and its ecological significance, through comparison between polyploids and diploids counterparts, with differences being often found in morphological, physiological and developmental traits (Bretagnolle and Thompson, 1996; Husband and Schemske, 2000; Buggs and Pannell, 2007; Maherali *et al.*, 2009; Hao *et al.*, 2013; Madlung, 2013).

Morphological consequences are often related with the “gigas effect” (Hetherington and Woodward, 2003; Segraves, 2017) once polyploids usually acquire larger features. For example, tetraploids and hexaploids of *Atriplex canescens* presented larger leaves, less stem thickening and shorter internodes, resulting in a higher leaf to sapwood area ratio (Hao *et al.*, 2013). In *Spartina pectinata*, biomass was 1.5x higher in hexaploids than in tetraploids (Kim *et al.*, 2012).

Physiological differences are also common and often related with morphological differences. Polyploids tend to present larger cells due to the increase of genetic material, including larger stomata or vascular cells. Consequently, larger stomata may increase photosynthetic rates and gas exchanges (Molin *et al.*, 1982). Water relations may also be affected by stomata features. Water relation, plant hormones and secondary metabolism may also be changed after polyploidization (Levin, 2002; secondary metabolism reviewed on Dhawan and Lavania, 1996). For example, in *Arabidopsis thaliana*, polyploids presented larger stomata and increased stomatal closure response to the hormone, abscisic acid (ABA), due to decreased expression of ABA-responsive genes, which conferred higher tolerance to drought (Del Pozo and Ramirez-Parra, 2014). In *Atriplex confertifolia*, photosynthetic rates increased with ploidy level, but this effect was counterbalanced by a decrease on the number of cells (Warner and Edwards, 1989). In *A. canescens*, a higher resistance to xylem cavitation on higher ploidy levels was observed, which may be the physiological basis for higher resistance to extreme drought (Hao *et al.*, 2013).

Changes on development traits have also been observed. Different growth rates are a common result of genome duplication, with consequences on the phenology of the polyploids. The time and duration of flowering are also commonly affected traits on polyploids (Levin, 2002). In *Dactylis glomerata*, tetraploids experienced an earlier flowering time when compared with diploids. Also, tetraploids germinated faster and in higher percentages than their diploid counterparts (Bretagnolle *et al.*, 1995; Bretagnolle and Thompson, 1996). These new features may have a final impact on the ecology of the polyploid. Species interactions, resistance to diseases and pathogens and competitive ability may also suffer changes due to polyploidization (Segraves and Thompson, 1999; Husband and Schemske, 2000; Castro *et al.*, 2011; Thébault *et al.*, 2011).

Of the empirical studies that tried to understand the effects of polyploidization, the majority used established polyploids. Therefore, these studies did not consider the time passed after the polyploid emergence (Husband *et al.*, 2008), and, thus, the observed results will reflect not only the effect of a duplicated genome but also natural selection and other post-polyploidization processes (Bretagnolle and Lumaret, 1995; Ramsey and Schemske, 2002; Ramsey, 2011). Therefore, the use of synthetic or natural neopolyploids is of high value in order to assess the real effect of polyploidization (Husband *et al.*, 2008). Natural neopolyploids can be detected in nature using large scale screening analyses of natural populations using flow cytometric analyses (e.g. Ramsey, 2011). When natural neopolyploids are not detected in nature, synthetic polyploids can

be obtained in the laboratory using c-mitotic agents (e.g. colchici ne; Eigsti, 1938). Despite rarely used, some studies have already used synthetic neopolyploids or natural polyploids of recent origin, enabling to quantify the effects of polyploidization *per se* (Bretagnolle and Lumaret, 1995; Liu *e al.*, 2007; Husband *et al.*, 2008; Trojak-Goluch and Skomra, 2013).

1.4 The importance of the reproductive system

Reproductive traits represent the most common and important changes on neopolyploids (Grant, 1956; Husband *et al.*, 2008). Despite the lack of empirical studies, some patterns have already been observed in the available studies. Polyploids usually acquire larger floral organs, which may impact pollinator's interactions and, consequently, the reproductive fitness of the cytotypes (e.g. Husband and Schemske, 2000). Lower numbers of seeds accompanied by bigger seeds are also common in polyploids and may confer an advantage towards germination and seedling survival (e.g. Bretagnolle *et al.*, 1995). Other common change is the disruption of the self-incompatibility system, allowing the polyploid to self-reproduce in the initial stages of polyploid establishment (Miller and Venable, 2000; Baack, 2005; Rausch and Morgan, 2005). A change of the mating system during these stages is of major significance once selfing can act as a reproductive assurance when the available mates or pollinators are scarce, enabling the polyploid to deal with the minority cytotype exclusion (Levin, 1975).

Indeed, reproductive assurance is a major advantage when self-reproduction is possible; however, selfing also bears some disadvantages. Selfing results on inbreeding depression, i.e., the loss of fitness of the selfed individual in relation to the outcrossed equivalent (Husband and Schemske, 1995, 1997; Rausch and Morgan, 2005; Ozimec and Husband, 2011). This was observed, for example, on *Amsinckia gloriosa* where tetraploids presented higher inbreeding depression than their diploid counterparts, *A. spectabilis* (Johnston and Schoen, 1996). However, some studies have also demonstrated that polyploidization may reduce inbreeding depression caused by high selfing rates (Husband and Schemske, 1997; Miller and Venable, 2000; Mable, 2004). If inbreeding depression is buffered by polyploidization, selfing would be favoured as mating system, at least on the first stages (Rausch and Morgan, 2005). This hypothesis was observed in *Chamerion angustifolium*. Despite the fact that the use of selfing caused lower fitness on all parameters measured, including seed set and seed germination, for both diploids and tetraploids, tetraploids presented 29% less cumulative inbreeding depression that diploids (Husband and Schemske, 1997), revealing a trade-off by

polyploidization with the use of selfing. If neopolyploids combine high rates of auto-fertilization with lower inbreeding depression, polyploidization may act as a short-term solution for offspring production, thus helping neotetraploids overcoming the minority cytotype disadvantage. After its successful establishment, the mating system may change to adjust to newer scenarios.

1.5 Study system – *Jasione maritima*

The genus *Jasione* L. belongs to the Campanulaceae family and is found in a wide range of ecosystems, from dune systems to rocky alpine areas. *Jasione maritima* (Duby) Merino is a perennial plant found in dune systems (Figure 1a) from the French Gironde to the coast of Portugal, being an Iberian endemism (ICN 2006). It is considered as endangered by the International Union of Conservation for Nature (IUCN) on the Red List of Threatened Species, due to habitat fragmentation. The plant size varies from 25 to 50 cm, usually acquiring the shape of flat cushions with 30-40 cm (Figure 1b), or sterile rosettes, in the winter. The inflorescences are blueish, rarely white to pinkish, with a glomerulus that can reach 8 to 15 mm of diameter (Figure 1c and 1d) with their flowering spans between June and August. This species has been described recently as a polyploid complex, bearing diploids ($2n = 2x = 12$ chromosomes) and tetraploids ($2n = 4x = 24$ chromosomes) (Castro, 2018). Similarly to the closely related *J. montana* (Parnell 1987), *J. maritima* was recently described as self-incompatible (Castro, 2018).

Figure 1: *Jasione maritima*: a) Dune habitat; b) Plant habit; c) Inflorescence at field; d) Inflorescence at the greenhouse experiment.

Nowadays, the two cytotypes present an allopatric distribution, with diploids occurring in the northern area of the distribution and tetraploids in southern locations (Figure 2). While tetraploids occupy their potential niche, diploids are restricted to a smaller area, not equivalent to its potential niche (Castro, 2018). As the two cytotypes are very similar in their morphology it is hypothesized that the tetraploid originated from auto-polyploidization event(s). Therefore, this complex represents a good model to understand the mechanisms related with neopolyploid establishment. Additionally, the use of neotetraploids of *J. maritima*, already synthesized in the FLOWer laboratory, allows to develop studies in a contemporary time scale.

1.6 Objectives and hypotheses

The main goal of this MSc Thesis was to understand the role of selfing in the establishment of neotetraploids in the diploid-tetraploid complex of *J. maritima*. To evaluate this, the following questions were addressed: (1) How genome duplications

change the selfing ability in *J. maritima*? (2) How the offspring fitness is affected when neotetraploids use selfing as mating system?

To address the first question, outcross and self-pollinations were performed on diploid (i.e. 2*x*) and synthetic neotetraploid (i.e neo4*x)* individuals, and reproductive success was measured through fruit and seed production and seed germination. We hypothesize that neotetraploids have the ability to self-pollinate, thus enabling the production of offspring in the absence of sexual compatible mates, fundamental in in the first stages after emergence.

To address the second question, the offspring of the diploid and synthetic neotetraploid cytotypes, obtained by outcrossing and selfing, were set to grow in a greenhouse experiment, and plant performance was measured through several fitness parameters, including developmental and physiological traits. We hypothesize that, after selfing, neotetraploids offspring has similar or higher plant performance than diploid offspring. If that is verified in *J. maritima*, selfing can be a mechanism to overcome the minority cytotype exclusion, thus helping neotetraploids establishment in the first stages after its emergence.

1.7 Key innovations

The first and most important key innovation of this MSc Thesis is the use of neopolyploids in a comparative study between newly generated polyploids and their diploids progenitors. This approach enables to assess the effects of polyploidization *per se* on the parameters measured. The parameters measured are by itself new in the study of polyploids, once the areas of ecology and physiology are largely neglected, although they are essential to understand the mechanisms behind the emergence, establishment and maintenance of polyploids in nature. In fact, a group of morphological, physiological and fitness traits are measured in synthetic neotetraploids and compared with the diploid parental for the first. Therefore, this study contributes with new insights on the effects and mechanisms behind polyploidization on polyploid species with potential to be interpreted in a broader context.

CHAPTER 2 – Materials and Methods

2. Materials and Methods

2.1 Plant material

This study, as starting material, used seeds collected in natural populations of diploids of *Jasione maritima*. Seed collection was carried out by the FLOWer group during the fruiting period of July 2013. For this study, three diploid populations were selected: population MS13 from Pedrosa beach, in Mourín, La Coruña, Spain (43.15818, -9.19126); population SC77 from Afora beach, Fisterra, La Coruña, Spain (42.90851, -9.27328); and population SC73 from Lariño, La Coruña, Spain (42.77103, -9.12227; see Figure 2). Population SC73 corresponds to the diploid population present on the contact zone with tetraploid populations (see Figure 2).

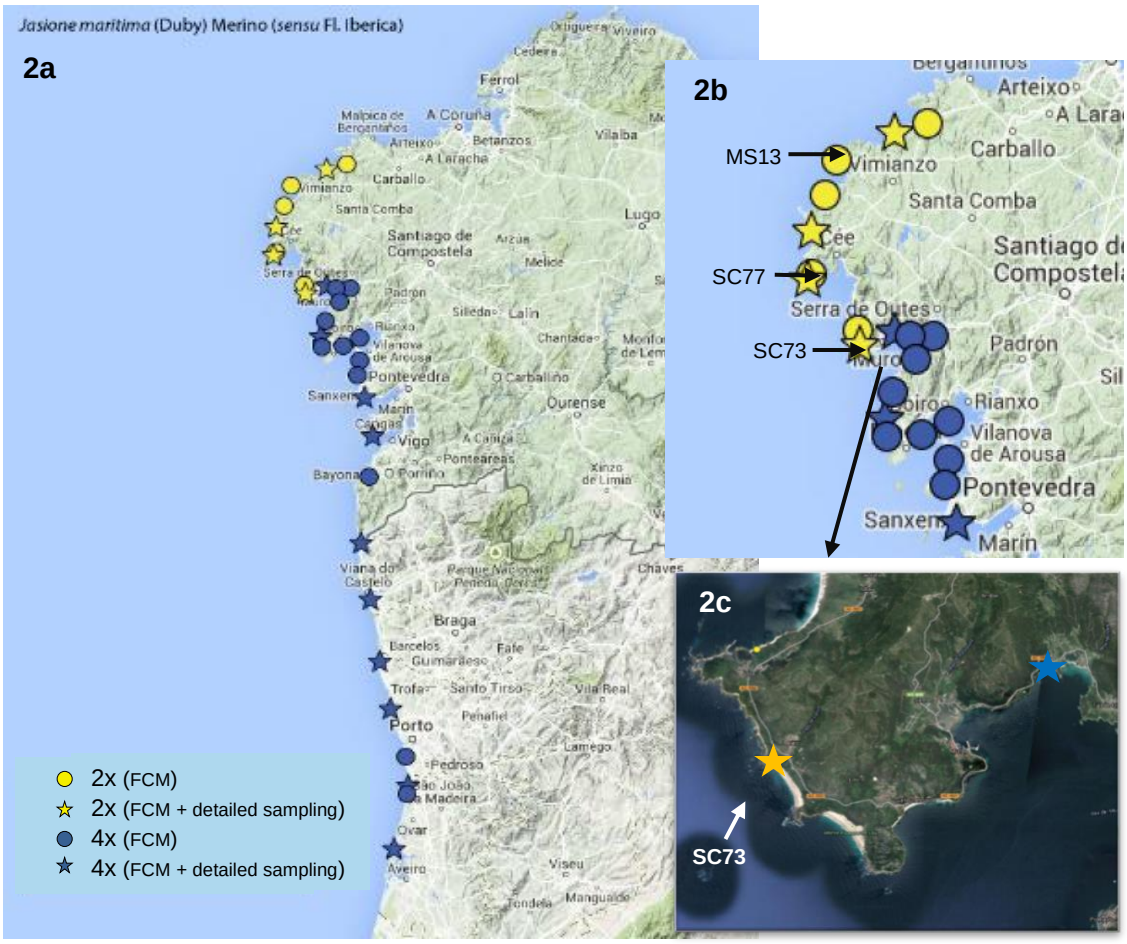

Figure 2: Distribution of *Jasione maritima*: a) Distribution map of *J. maritima* cytotypes; b) Cytotypes and the three diploid populations used for the experiment; c) Contact zone of the two cytotypes (adapted from Castro *et al.* 2015).

The seeds collected in the field were used for two experiments. First, seeds were used to produce synthetic neotetraploids (section 2.2); second, they were used to obtain diploid plants. Then, both synthetic neotetraploids and diploids were used to perform controlled pollinations (section 2.4). The seeds obtained in the controlled pollinations were subsequently used to study the performance of the F1 after outcrossing and selfing (sections 2.6 and 2.7). The ploidy levels of the plants were assessed using flow cytometry (section 2.3) at several stages of the experiment, namely for neotetraploid identification and confirmation of ploidy levels of the F1 generation.

2.2 Neotetraploids production

Synthetized neotetraploids were obtained in the laboratory, through treatment of seeds of *J. maritima* with colchicine following Castro (2018). Briefly, the seeds were maintained in the cold (4 ºC) for one week and transferred to the growth chamber (at 24 ºC with a cycle of 16h/8h light/dark exposure photoperiod) for seed germination. The young seedlings with 3-4 days were then submerged into a solution of 0.5% colchicine for 14 h and subsequently washed several times with ddH_2O. After the colchicine treatment, plants were planted in trays filled with standard soil and maintained in greenhouse conditions. The ploidy level of all the plants that survived was screened using flow cytometry (see section 2.3) and the plants with the double of the genome size were transplanted to 1 L pots (filled with standard soil).These plants were then used for the crossing experiment.

2.3 Ploidy level estimation

DNA-quantification analyses using flow cytometry were performed to verify the ploidy level of all the plants of the experiment. The flow cytometry procedure enabled the isolation of nuclei in a suspension that, after staining using a DNA fluorochrome, was analysed in a flow cytometer to quantify the emitted fluorescence of the nuclei and estimate the nuclear DNA content and ploidy level.

Briefly, two to three fresh leaves were collected per individual. In a Petri dish, 50 mg of sample material along with 50 mg of leaves of an internal reference standard (*Solanum lycopersicum* ‘Stupické’, 2C = 1.96 picograms (pg), Doležel *et al.*, 1992) were chopped in 1 ml of Woody Plant Buffer to obtain a nuclear suspension (Loureiro *et al.*, 2007)

After removing the debris by filtration using a 50 µm nylon filter, a DNA staining fluorochrome (propidium iodide, 50 µg.mg $^{-1}$) and RNAse (50 µg.mol $^{-1}$) were added to the solution to stain the nuclei and to degrade the double-stranded RNA, respectively.

Then, samples were analysed individually in a CyFlow Space flow cytometer. Graphics with the results were given by the software Partec FloMax v2.4d. The graphics obtained were: a histogram with the fluorescence pulse integral in linear scale (FL); forward light scatter (FS) versus (vs.) side light scatter (SS), both in logarithmic scale; FL vs. time; FL vs. SS in log scale. In the graphic of FL vs. SS we define a polygon to remove the debris. At least 1300 nuclei were analysed for both the sample and the reference standard (Suda *et al.*, 2007). As a quality standard, only when the coefficient of variation (CV) obtained was below 5% the analysis was accepted (Dolezel *et al.,* 2007). If CV was higher than this value, a new sample was prepared and analysed.

To determine the genome size, the following equation was used: $me\ size\ of\ J.maritima\ (pg) = \frac{J.maritima\ \mathrm{FL}}{S.\ lycopersicum\ FL} \times S.lycopersicum\ genome\ size$; using the mean values of relative fluorescence of the obtained peaks. Ploidy level was then defined based on the estimates of genome size and previous chromosome counts. Diploids were identified when the genome size was around 2.98 ± 0.07 pg, while neotetraploids presented genome size values of 6.06 ± 0.11 pg (Castro, 2018).

2.4 Reproductive fitness of neotetraploids

Along with neotetraploids germination and growth, diploid seeds were sown in 1 L pots (using standard soil) and placed to grow in the same greenhouse conditions as the neotetraploids. Both diploid and neotetraploid individuals were grown until the adult stage to perform controlled pollinations and obtain a F1 generation after outcrossing and self-pollinations. Controlled pollinations were performed during the flowering period on both cytotypes. For that, the inflorescences were kept isolated from pollinators using mesh bags in order to prevent natural pollination. The bags were kept until fruiting to prevent seed losses as the fruit is a dehiscent capsule. Outcrossing pollinations were made using at least five pollen donors (in order to increase pollen diversity) for several days as the flowers of the inflorescence opened gradually. Self-pollinations were made using pollen from two inflorescences from the same plant. Pollinations were performed by gently rubbing the two inflorescences with each other. After fruit maturation the inflorescences were stored in identified paper bags for subsequent analysis.

In the laboratory, the number of flowers, fruits and viable seeds were quantified for each infructescence under a binocular microscope (Figure 3). The seeds were then used to assess germination rates. For this, 25 seeds from 45 different individuals in each treatment (15 per population) were selected and put to germinate in Petri dishes with moistened filter-paper. The seeds were exposed to a cold treatment for one week and were then moved to a climate chamber (24 ºC of temperature with a photoperiod of 16h/8h of light/dark exposure, respectively). After one month, total germination rate was assessed for each mother plant.

To assess if neotetraploids had the ability to self-pollinate, the following parameters were assessed: fruit production ($Fruit\ set = \frac{nº\ fruits}{total\ nº\ flowers}$), seed production ($Seed\ set = \frac{nº\ seeds}{nº\ fruits}$) and total seed germination ($Total\ seed\ germination\ (\%) = \frac{nº\ germinated\ seed}{nº\ seeds}$). Additionally, the Self-Compatible Index (i.e. SCI) was calculated for diploids and neotetraploids. SCI was calculated using each reproductive variable as, for example, the proportion between the fruit set of selfed inflorescences and the fruit set of outcrossed inflorescences: ($fruit\ set: SCI = \frac{fruit\ set\ selfing}{fruit\ set\ outcrossing}$). Since the mothers of the diploids and neotetraploids were not all the same, for outcrossing we used the mean values of the data obtained.

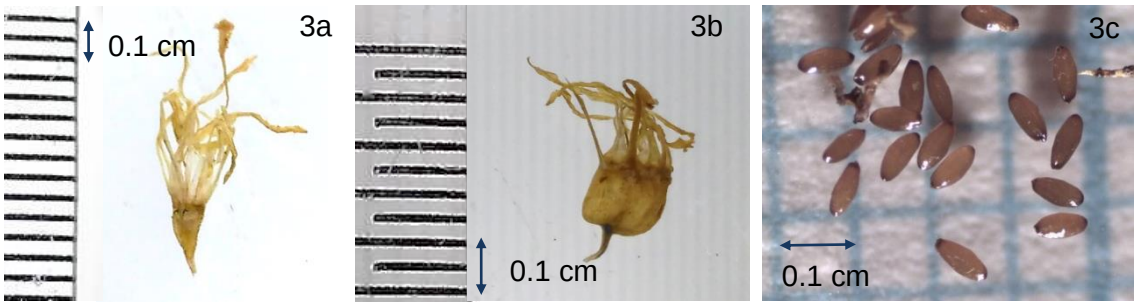

Figure 3: Morphology of *Jasione maritima* reproductive structures: a) Dry flower; b) Dry fruit; c) Morphologically viable seeds.

2.5 Performance of the F1 generation: Development traits

Seeds from 45 different individuals from each treatment (15 per population) were placed to germinate in Petri dishes (one per mother plant) with moistened filter-paper. After germination, we selected one seedling from each mother plant and transplanted it

a 1 L pot in a greenhouse (using standard soil, beach sand and standard sand in 1:1:2 proportions, respectively).

The plants were grown since November of 2016 to September of 2017. Before the end of the greenhouse experiment, the ploidy level of each individual was confirmed using flow cytometry (see section 2.3 for further details).

In the end of the experiment, the height (cm) and number of leaves of each plant was measured. Afterwards, eight plants per treatment were randomly selected to assess physiological traits (section 2.6) and the remaining plants were harvested, separated in belowground and aboveground organs, and collected in identified paper bags. The paper bags were put to dry in an oven at 68 ºC for 48 h. Finally, belowground, above ground and total weight were assessed by weighing the corresponding parts in a precision scale (0.01 mg accuracy).

2.6 Plant performance: Physiological traits

At the end of the greenhouse experiment, eight plants per treatment (including individuals from all the populations) were randomly selected to evaluate the impact of polyploidization in several physiological traits. While some physiological parameters were measured *in vivo* or by collecting leaves (fresh material) from the plant, for other parameters, leaves were harvested, immediately frozen in liquid nitrogen and stored at -80 ºC in individual aluminum foil envelopes. Afterwards, leaves were macerated in frozen conditions (with liquid nitrogen) for later analysis.

Photosynthesis, relative water content, electrolyte leakage and stomatal measurements were performed before harvesting. Photosynthetic parameters were assessed *in vivo* by measuring chlorophyll *a* fluorescence with a fluorometer (FluorPen FP 100-Max). Chlorophyll fluorescence parameters, minimum fluorescence (F_o) and maximum fluorescence (F_m), were measured after leaf dark adaptation for 30 min by applying a weak-intensity modulated light and a high saturation pulse of white light (> 1500 µmol m^{-2} s^{-1}), respectively. Then, leaves were acclimated to ambient light and the steady-state fluorescence (F_s) was averaged over 30 s, followed by exposure to a saturating light (> 7500 µmol m^{-2} s^{-1}) to determine the maximal fluorescence (F_m'). Maximum and effective quantum yield of photosystem II (F_v/F_m and Φ_{PSII}, respectively) were calculated: $F_v/F_m = (F_m-F_o)/F_m$ and $\phi_{PSII} = (F_m'-F_s)/ F_m'$.

For relative water content (i.e. RWC), two leaves of eight individuals were collected, weighed (fresh weight i.e. FW) and placed in 1.5 ml microtubes. Then, the microtubes were filled with water, placed over night in dark at 4 ºC to obtain the turgid weight (TW). After that, leaves were dried for 7 days at 70 ºC to obtain the dry weight (DW). The RWC was calculated as a proportion according to Dias *et al. (*2014) as: $RWC = \frac{(FW-DW)}{(TW-DW)}$.

Membrane permeability was assessed by measuring leakage of UV-absorbing substances (UVAS; Redman, Haraldson, and Gusta, 1986). Two to three leaves were selected from each individual, and were subsequently washed and reserved on microtubes containing deionized water and incubated overnight at 25 ºC. The absorbance was then measured in a spectrophotometer at 280 nm (A_{280nm}). Afterwards, leaves were autoclaved for 20 min (120 ºC) and the absorbance was measure again (A'_{280nm}). Relative electrolyte leakage (i.e. REL) of UVAS was measured as the following ratio: $REL = {A_{280nm}}/{A'_{280nm}}$.

Stomatal density and length were also measured. For that, a leaf from 20 diploid and 20 neotetraploid individuals was collected. After peeling the epidermis of the abaxial face of each leaf, they were put in a microscopic slide and prepared following Weyers and Travis (1981). Stomatal density was measured by counting the number of stomata of five different plans per leaf. The area of the plans was taken in order to determine stomatal density (number of stomata / mm^2). Stomatal length was measured by selecting six stomata from each individual. The measurements were performed using the ImageJ software.

Macerated frozen leaves were used to determine the concentration of photosynthetic pigments and carbohydrates content. For pigments content, leaf samples were homogenized with acetone/50mM Tris buffer and centrifuged. The absorbance of the supernatant was read at 470, 537, 647, 663 nm on a spectrophotometer. The concentration of chlorophyll *a*, chlorophyll *b* and carotenoids was calculated according with Sims and Gamon (2002) using the following formulas:

$$\mathbf{Chl_a} = 0.01373\ A_{663} - 0.000897\ A_{537} - 0.003046\ A_{647}$$

$$\mathbf{Chl_b} = 0.02405\ A_{647} - 0.004305\ A_{537} - 0.005507\ A_{663}$$

$$\mathbf{Carotenoids} = (A_{470} - (17.1 \times (Chl_a + Chl_b) - 9.479 \times \text{Anthocyanins})) / 119.26$$

$$\mathbf{Anthocianyns} = 0.08173\ A_{537} - 0.00697\ A_{647} - 0.002228\ A_{663}$$

For total soluble sugars (TSS), leaf samples were homogenized with 80% ethanol, and placed in a bath at 80 ºC for one hour (Irigoyen *et al.*, 1992). After a 10 min centrifugation at low temperature, the obtained supernatant was incubated with an anthrone solution (40 mg anthrone + 20 ml sulfuric acid + 1 ml H_2O) for 10 min at 100 ºC and the mixture was centrifuged. The supernatant was used to read the absorbance at 625 nm on a spectrophotometer. For the determination of starch content the pellet obtained from the TSS extraction was used (Osaki *et al.*, 1991). Perchloric acid (30%) was added to the pellet and the mixture was incubated for 1h in a bath at 60 ºC, and subsequently centrifuged. The obtained supernatant was incubated with an anthrone solution (40 mg anthrone + 20 ml sulfuric acid + 1 ml H_2O) for 10 min at 100 ºC and the mixture was centrifuged. The supernatant was used to read the absorbance at 625 nm. The content of TSS and starch were calculated using a glucose standard curve, constructed for obtaining the absorbance of solutions of known glucose concentrations.

2.7 Statistical Analyses

Generalized Linear Mixed Models (GLMM) were used to study each parameter. In the analyses, we considered ploidy level (2*x* and neo4*x*) and pollination treatment (outcrossing and selfing) as fixed factors. Population (SC73, SC77 and MS13) was initial included as random factor but, once population was not influencing the data (i.e. when the variance of the residuals was higher), Generalized Linear Models (GLM) were used instead. After applying the models, Least Square Means for multiple comparisons were performed to evaluate differences between the factors. Below are presented the specific questions and the comparison made:

(1) How genome duplications change the selfing ability in *J. maritima*? To evaluate this, differences between neotetraploids and diploids of after the selfing treatment were analyzed. Therefore, differences on reproductive traits were observed, using the following response variables: fruit set, seed set and seed germination. For fruit set and seed germination an arcsine transformation was used. For seed set, once the assumptions of the GLM were not accomplished, Mann-Whitney-Wilcoxon tests were performed to test the differences between ploidy level and crossing treatment and differences between cross treatment within ploidy level and between ploidy level within cross treatment. GLM's were followed by a Least Square Means for multiple comparisons. Differences on self-compatibility index for fruit set, seed set and seed germination were also tested using GLM analysis after arcsine transformation.

(2) How the offspring fitness is affected when neotetraploids use selfing as mating system? To evaluate this, we assessed first how plant performance is affected by the use of selfing on neotetraploids (2.1) by analysing the differences between neotetraploids offspring obtained after outcrossing and selfing treatments; and second how neotetraploids may compete with diploids (2.2) by analyzing differences between neotetraploids (of outcrossing and selfing treatment) and diploids of outcrossing treatment. We analyzed plant performance in a group of developmental, fitness and physiological parameters. The developmental and fitness traits were the number of leaves, plant height and belowground, aboveground and total biomass. For all these parameters, a logarithmic transformation was used to normalize the data. Physiological traits included stomatal density and stomatal length, maximum and effective quantum yield of photosystem II (F_v/F_m and Φ_{PSII}, respectively), chlorophyll *a*, *b* and carotenoids content, total soluble sugars and starch content, relative water content (RWC) and relative electrolyte leakage (REL). For RWC, REL, an arcsine transformation was used. Additionally, a square-root transformation for starch content, F_v/F_m and $\Phi_{PSII,}$ and a logarithmic transformation for stomatal length were used to normalize the data. When a significant interaction between factors was detected, a nested model was used, with ploidy level nested within crosses and crosses nested within ploidy levels. For 2.2 (see above) only comparisons between diploid and neotetraploid offspring obtained after outcrossing were made.

For descriptive analysis, mean and standard error of the mean were calculated for the different parameters studied. Standard deviation of the mean and sample size were also obtained. Outliers were inspected and excluded from the analysis. Analyses and graphics construction were performed using R software version 3.4.3 ("*car"* and "*lme4"* packages for assumption's tests and GLM analysis; "*lsmeans"* package for multiple comparisons tests).

CHAPTER 3 – Results

3. Results

3.1 How genome duplication affected the reproductive fitness of neotetraploids of *Jasione maritima*?

Fruit set. Fruit set was significantly higher in diploids than in neotetraploids, regardless of the crossing treatment (Table 1; Appendix 1). There were no significant differences in fruit set between crosses on both diploids and neotetraploids (Figure 4a). Concerning the self-compatibility index (SCI) calculated based on fruit set, no significant differences were observed between cytotypes (Table 2; Figure 5b); still SCI was slightly higher in neotetraploids, which presented a gain in self-compatibility (i.e. SCI > 1), while in diploids it was lower than one.

Seed set. Neotetraploids presented significantly lower seed set in comparison with the diploids (Table 1; Appendix 1), but no differences were observed between cytotypes for the selfing treatment (Figure 4b). Also, in both cytotypes, the outcrossing treatment presented significantly higher values of seed set in comparison with selfing (Table 1; Appendix 1; Figure 4b). SCI calculated based on seed set did not vary significantly between cytotypes, with both values being low (Table 2; Figure 5b).

Seed germination. Overall, neotetraploids presented significantly lower seed germination than diploids (Table 1; Appendix 1). Also, in both cytotypes, seed germination was significantly lower after selfing (Table 1; Appendix 1; Figure 4c. However, it is interesting to notice that, seed germination after selfing in diploids was very similar to that obtained after outcrossing in neotetraploids. Regarding seed germination, SCI was significantly higher in diploids than in neotetraploids (SCI<1; Table 2; Figure 5c).

Table 1: Effect of ploidy level and crossing treatment on fruit set, seed germination rate, seed set on diploids and neotetraploids of *Jasione maritima*. For fruit set and seed germination, obtained values of *F*-statistic and *P* value of the GLM's are showed. For seed set, obtained values of *W* and *P* value of the Mann-Whitney-Wilcoxon test are given. Significant *P* values are highlighted in bold.

Reproductive traits	Ploidy level	Crossing	Crossing * Ploidy
Fruit set	F = 25.096, **P < 0.001**	F = 0.027, P = 0.869	F =0.765, P = 0.384
Seed germination	F = 34.484, **P < 0.001**	F = 37.062, **P < 0.001**	F = 0.523, P = 0.471
Seed set	W = 2838, **P = 0.003**	W = 3690, **P < 0.001**	-

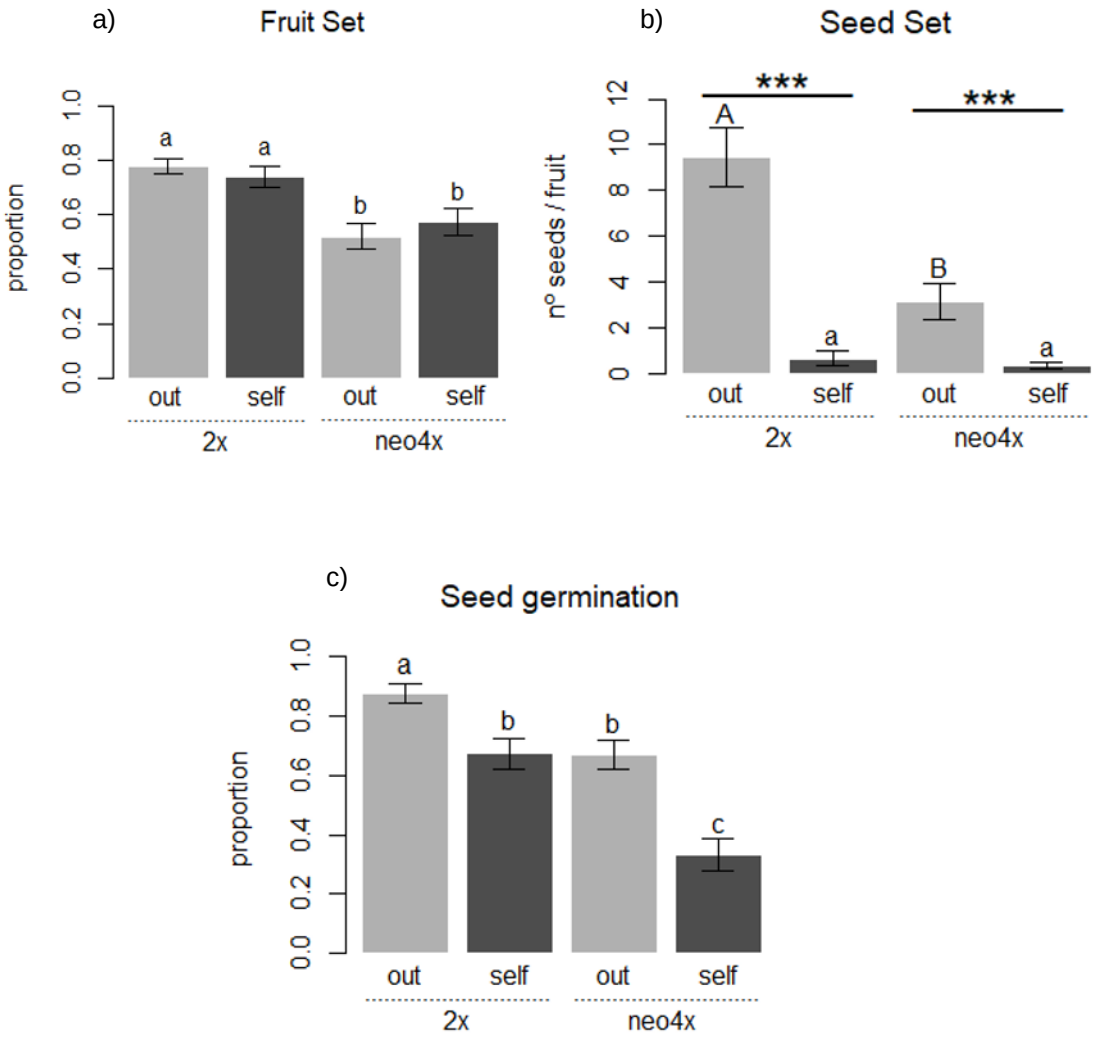

Figure 4: Reproductive fitness of diploids (2*x*) and neotetraploids (neo4*x*) of *Jasione maritima*: (a) Fruit set; (b) Seed set and (c) Seed germination. Values are presented as mean and standard error of the mean. Different letters represent statistical differences at $P < 0.05$ for parameters without interaction between factors and according with the post-hoc analysis. For seed set, once non-parametric test were performed, asterisk (*) indicates statistical differences between crosses within cytotypes; different uppercase letters represent differences between cytotypes on outcrossing treatments and different lowercase letters differences between cytotypes on selfing treatments. * $0.01 < P < 0.05$; ** $0.01 < P < 0.001$ and *** $P < 0.001$.

Table 2: Effect of ploidy level on self-compatibility index (SCI) of fruit set, seed set and seed germination on diploids and neotetraploids of *Jasione maritima*. Obtained values of *F*-statistic and *P* value of the GLM's are showed. Significant *P* values are highlighted in bold.

Reproductive traits	Ploidy level
SCI – Fruit set	$F = 0.869$, $P = 0.360$
SCI – Seed set	$F = 0.603$, $P = 0.440$
SCI – Seed germination	$F = 8.249$, $\mathbf{P = 0.006}$

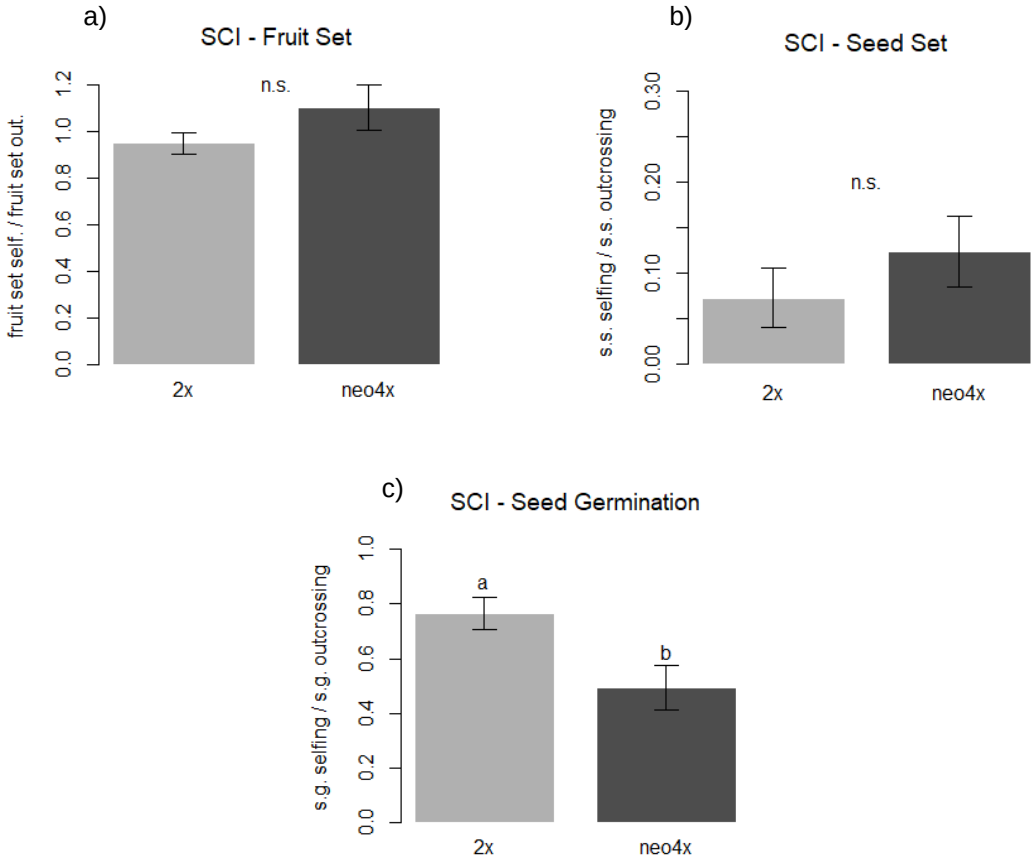

Figure 5: Reproductive fitness of diploids (2*x*) and neotetraploids (neo4*x*) of *Jasione maritima*: (a) Self-compatibility index (SCI) based on fruit set; (b) Self-compatibility index based on seed set and (c) Self-compatibility based on seed germination. Values are presented as mean and standard error of the mean. Different letters represent statistical differences at $P < 0.05$; n.s. denotes no statistical differences between levels at $P > 0.05$. f.s. – fruit set, s.s. – seed set, s.g. – seed germination.

3.2 Plant performance of neotetraploid offspring – Morphological parameters

Number of leaves. Ploidy level and crossing treatment significantly affected the number of leaves. The number of leaves was significantly lower in the neotetraploids (for both outcrossing and selfing treatment) than in the diploid individuals (Table 3; Appendix

2; Figure 6a). In diploids, individuals obtained after the selfing treatment presented a significantly lower number of leaves than those obtained after outcrossing. In neotetraploids, no significant differences were obtained between crossing treatments (Figure 6a).

Plant height. Ploidy level did not significantly affected this trait. Selfing treatment presented significantly lower height than diploids (Table 3; Appendix 2; Figure 6b). No differences were observed between ploidy levels on both outcrossing and selfing treatment (Figure 6b). In diploids, selfing treatment presented lower height than outcrossing, but no significant differences were observed on neotetraploids between crossing treatments (Figure 6b).

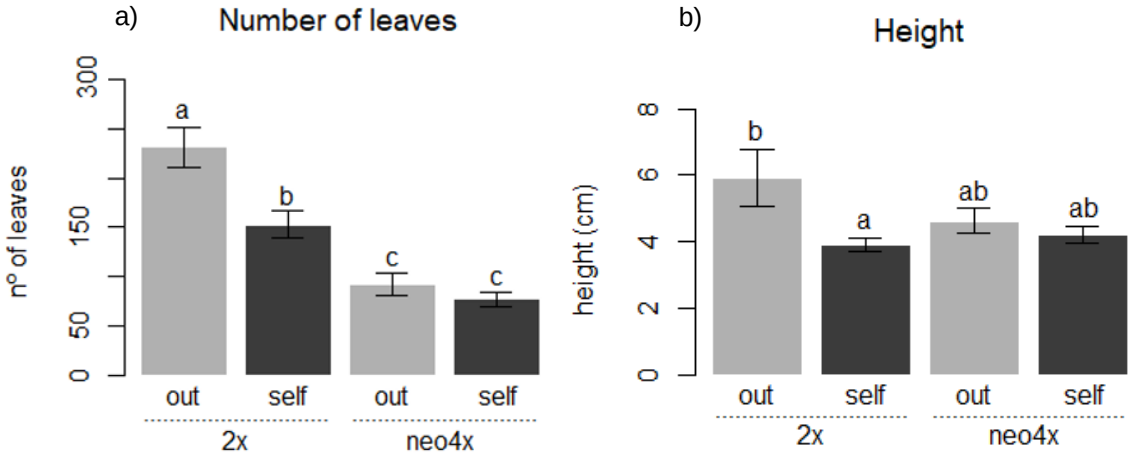

Figure 6: Plant performance of diploids (2*x*) and neotetraploids (neo4*x*) of *Jasione maritima:* (a) Number of leaves and (b) Plant height (in cm). Values are presented as mean and standard error of the mean. Different letters represent statistical differences at $P < 0.05$ for parameters without interaction between factors and according with the post-hoc analysis.

Aboveground, belowground and total biomass. Overall, both the ploidy level and the crossing treatment affected the aboveground biomass, while only ploidy level affected belowground biomass (Table 3; Appendix 2; Figures 7a and 7b). These traits were lower on neotetraploids, despite significant differences were only obtained between diploid individuals that resulted from outcrossing and neotetraploids obtained after selfing. As expected, considering the trends obtained for above- and belowground biomass, in total biomass the same pattern was observed (Table 3; Appendix 2; Figure 7c).

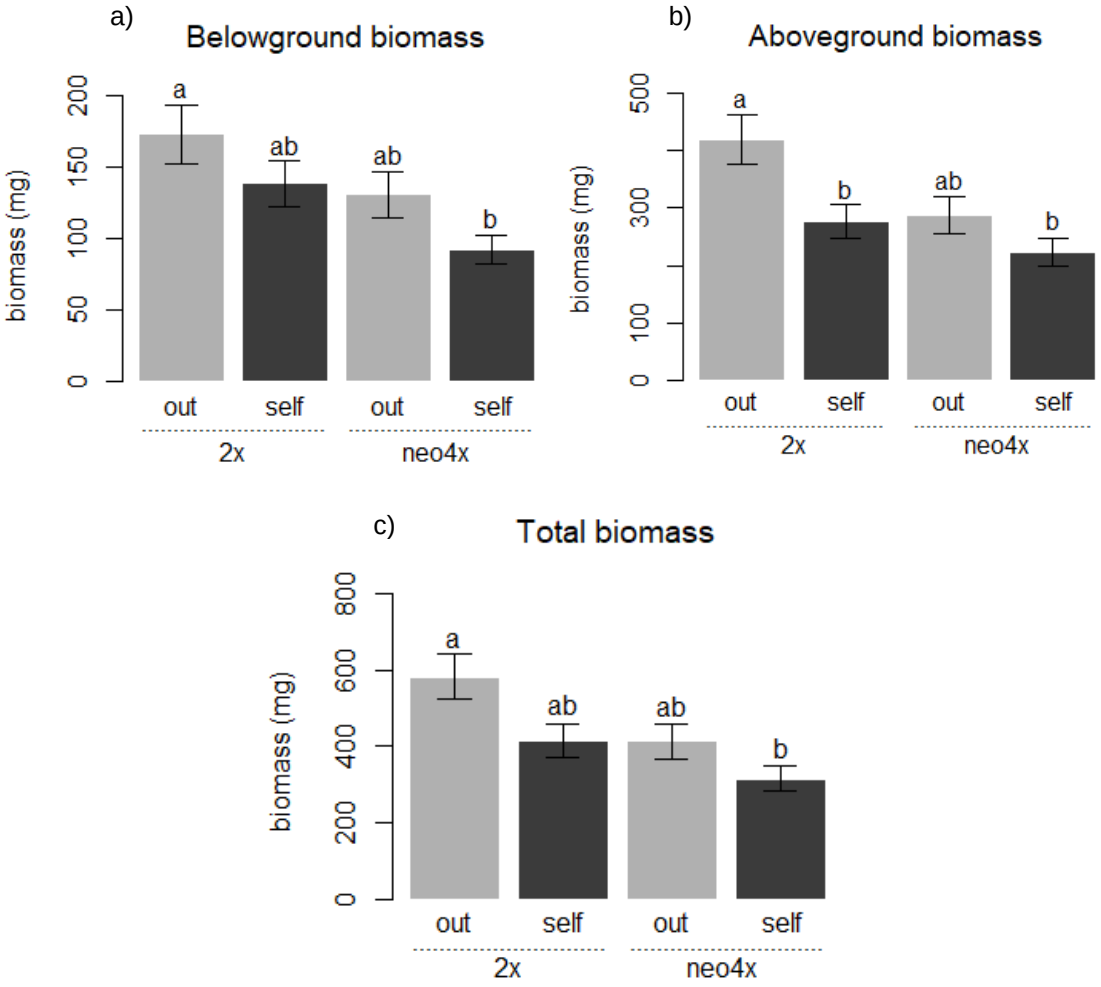

Figure 7: Plant performance of diploids (2*x*) and neotetraploids (neo4*x*) of *Jasione maritima:* (a) Aboveground biomass (in mg); (b) Belowground biomass (in mg) and (c) Total biomass (in mg). Values are presented as mean and standard error of the mean. Different letters represent statistical differences at $P < 0.05$ for parameters without interaction between factors and according with the post-hoc analysis.

Table 3: Effect of ploidy level and crossing treatment on developmental and fitness plant performance of diploids and neotetraploids of *Jasione maritima*. Obtained values of *F*-statistic and *P* value of the GLM's are given. Significant *P* values are highlighted in bold.

Developmental and fitness traits	Ploidy level	Crossing	Crossing * Ploidy
Number of leaves	*F* = 68.619, ***P* < 0.001**	*F* = 6.000, ***P* = 0.015**	*F* = 2.168, *P* = 0.143
Height	*F* = 0.211, *P* = 0.647	*F* = 6.177, ***P* = 0.014**	*F* = 1.956, *P* = 0.164
Belowground biomass	*F* = 7.881, ***P* = 0.006**	*F* = 3.591, *P* = 0.060	*F* = 0.002, *P* = 0.968
Aboveground biomass	*F* = 5.818, ***P* = 0.017**	*F* = 7.174, ***P* = 0.008**	*F* = 0.988, *P* = 0.322
Total biomass	*F* = 6.618, ***P* = 0.011**	*F* = 5.756, ***P* = 0.018**	*F* = 0.463, *P* = 0.497

3.3 Plant performance of neotetraploid offspring – Physiological parameters

Chlorophyll a, b and Carotenoids. Pigment content were not affected by ploidy level and crossing treatment (Table 4; Appendix 3.1), as no significant differences were observed on the comparisons made at all levels (Figures 8a-c).

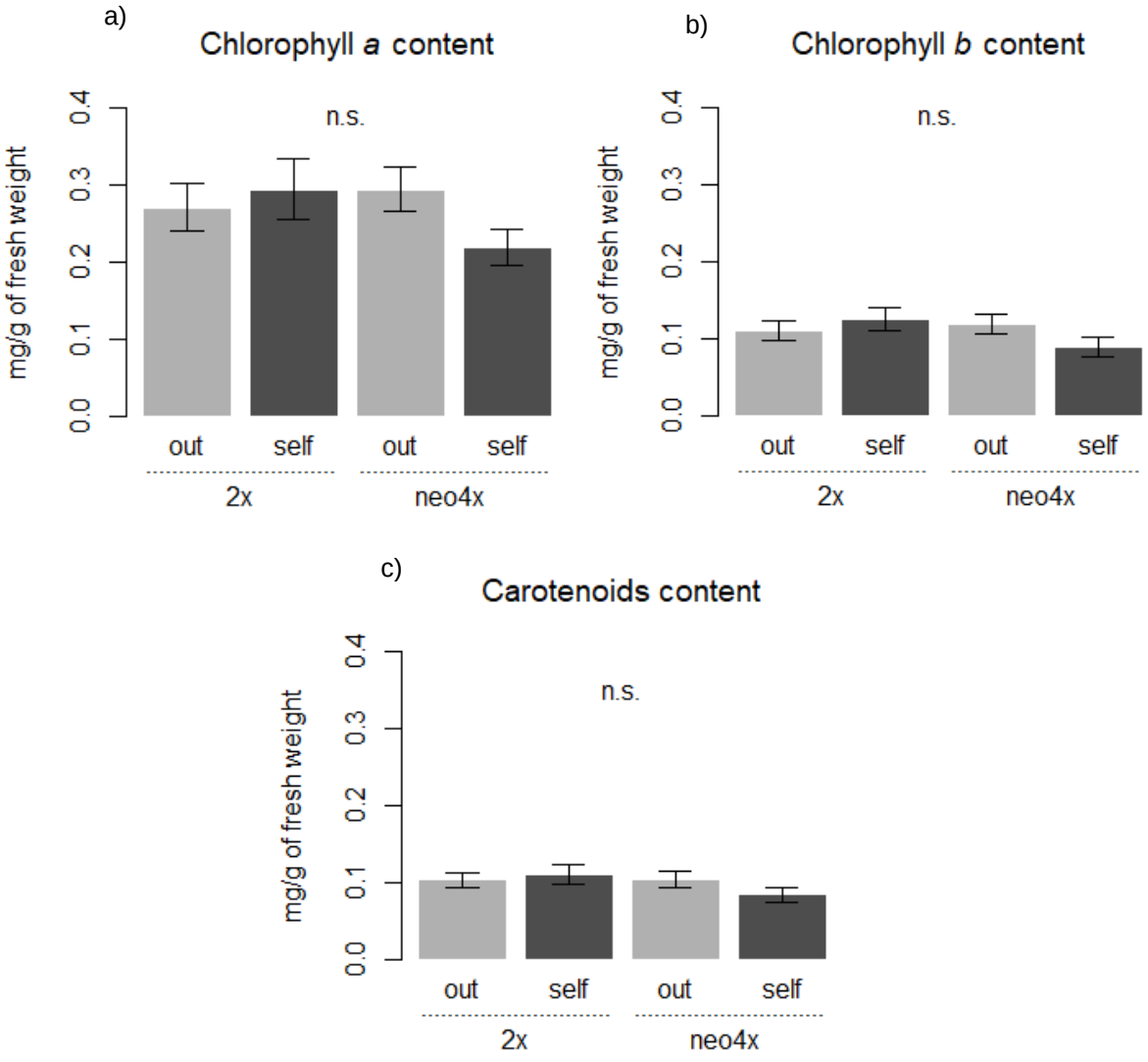

Figure 8: Plant performance of diploids (2*x*) and neotetraploids (neo4*x*) of *Jasione maritima*: (a) Chlorophyll *a* (mg / g fresh weight); (b) Chlorophyll *b* (mg/ g fresh weight) and (c) Carotenoids content (mg/ g fresh weight). Values are presented as mean and standard error of the mean. Different letters represent statistical differences at $P < 0.05$ for parameters without interaction between factors and according with the post-hoc analysis; n.s. denotes no statistical differences between levels at $P > 0.05$.

Total Soluble Sugars. Ploidy level affected TSS content, regardless of the crossing treatment. Outcrossing treatment within diploids presented significantly higher values than the selfing treatment (Table 4 and 5; Appendix 3.1; Figure 9a); however within neotetraploids, no differences were observed between crossing treatments (Table 4 and 5; Appendix 3.1; Figure 9a). Significant differences were observed between ploidies within outcrossing treatment, with neotetraploids presenting lower values; still, no significant differences were observed between ploidies within selfing treatment (Table 4 and 5; Appendix 3.1; Figure 9a)

Starch. Starch content was not influenced by ploidy level and crossing treatment (Table 4; Appendix 3.1), with no significant differences being observed in the comparisons made at all levels (Figure 9b).

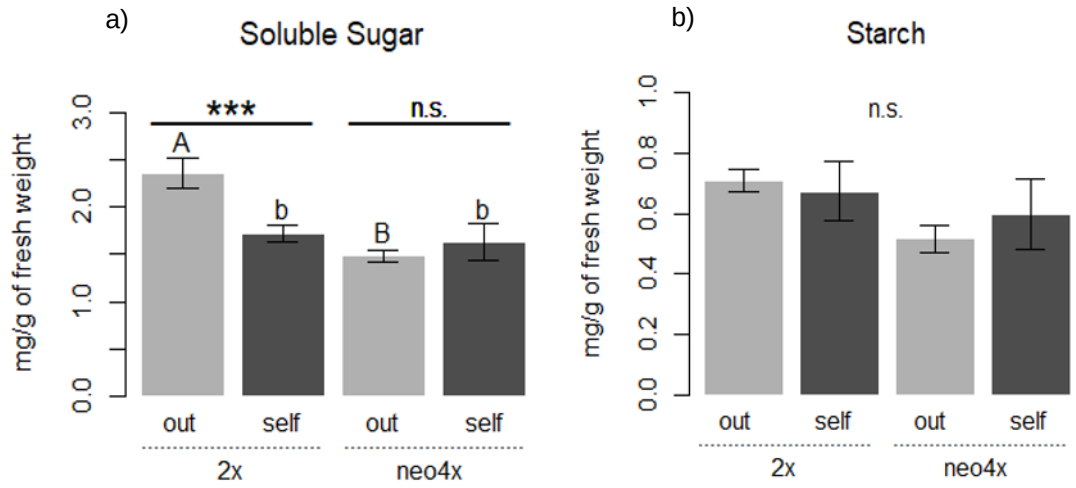

Figure 9: Plant performance of diploids (2*x*) and neotetraploids (neo4*x*) of *Jasione maritima:* (a) Total soluble sugars and (b) Starch. Values are presented as mean and standard error of the mean. Different letters represent statistical differences at $P < 0.05$ for parameters without interaction between factors and according with the post-hoc analysis; n.s. denotes no statistical differences between levels at $P > 0.05$. For total soluble sugars (with significant interaction), asterisk (*) indicates statistical differences between crosses within cytotypes; different uppercase letters represents differences between cytotypes on outcrossing treatments and different lowercase letters differences between cytotypes on selfing treatments. * $0.01 < P < 0.05$; ** $0.01 < P < 0.001$ and *** $P < 0.001$;

Relative Water Content (RWC). Ploidy level and crossing treatment did not affect RWC. This physiological trait was higher on neotetraploids than in the diploids (Table 4 and 5; Appendix 3.2; Figure 10a). No significant differences were observed between crosses on both diploids and neotetraploids (Figure 10a). No differences were observed between ploidies of the outcrossing treatment, contrarily to the selfing treatment, where

neotetraploids presented significantly higher RWC (Table 4 and 5; Appendix 3.2; Figure 10a).

Relative Electrolyte Leakage (REL). For REL, no differences were observed between ploidy levels and cross treatments (Table 4 and 5; Appendix 3.2; Figure 10b). No differences occurred between crosses within both cytotypes (Figure 7d). However, within outcrossing treatment, REL was significantly higher in the neotetraploids. No differences were observed between cytotypes within selfing treatment (Figure 10b).

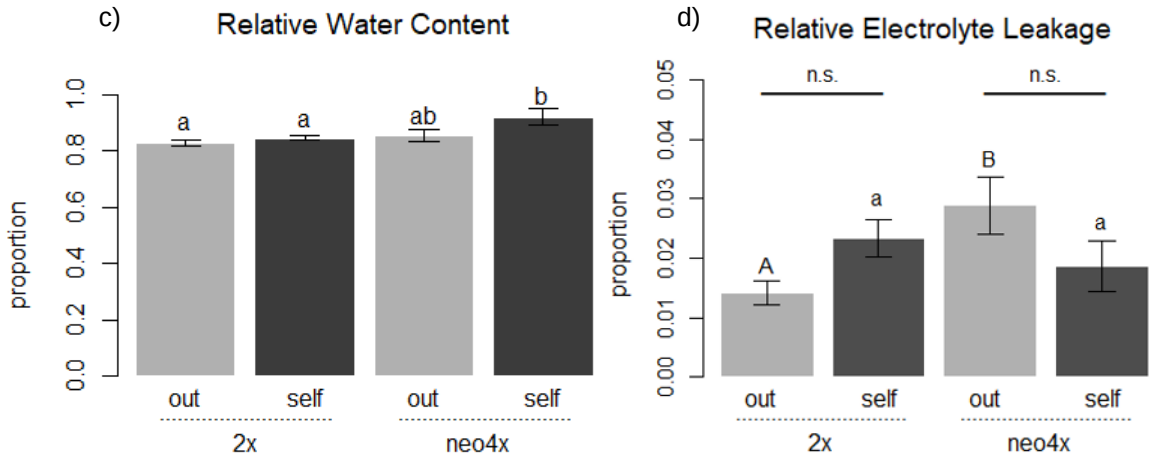

Figure 10: Plant performance of diploids (2*x*) and neotetraploids (neo4*x*) of *Jasione maritima:* (a) Relative water content (RWC) and (b) Relative electrolyte leakage (REL). Values are presented as means and standard error. Different letters represent statistical differences at $P < 0.05$ for parameters without interaction between factors and according with the post-hoc analysis; For REL (with significant interaction), different uppercase letters represents differences between cytotypes on outcrossing treatments and different lowercase letters differences between cytotypes on selfing treatments; n.s. denotes for no statistical differences between levels at $P > 0.05$.

Maximum quantum yield of photosystem II (F_v/F_m). F_v/F_m was significantly affected by ploidy level, with neotetraploids presenting higher RWC, but no differences were observed between crosses (Table 4; Appendix 3.2). Also, no differences were observed between the comparison of all the levels (Figure 11a).

Effective quantum yield of photosystem II (Φ_{PSII}). ΦPSII was significantly higher in the neotetraploids, but no differences occurred between crosses (Table 4; Appendix 3.2). No differences were observed between ploidy levels within both outcrossing and selfing treatment (Figure 11b).

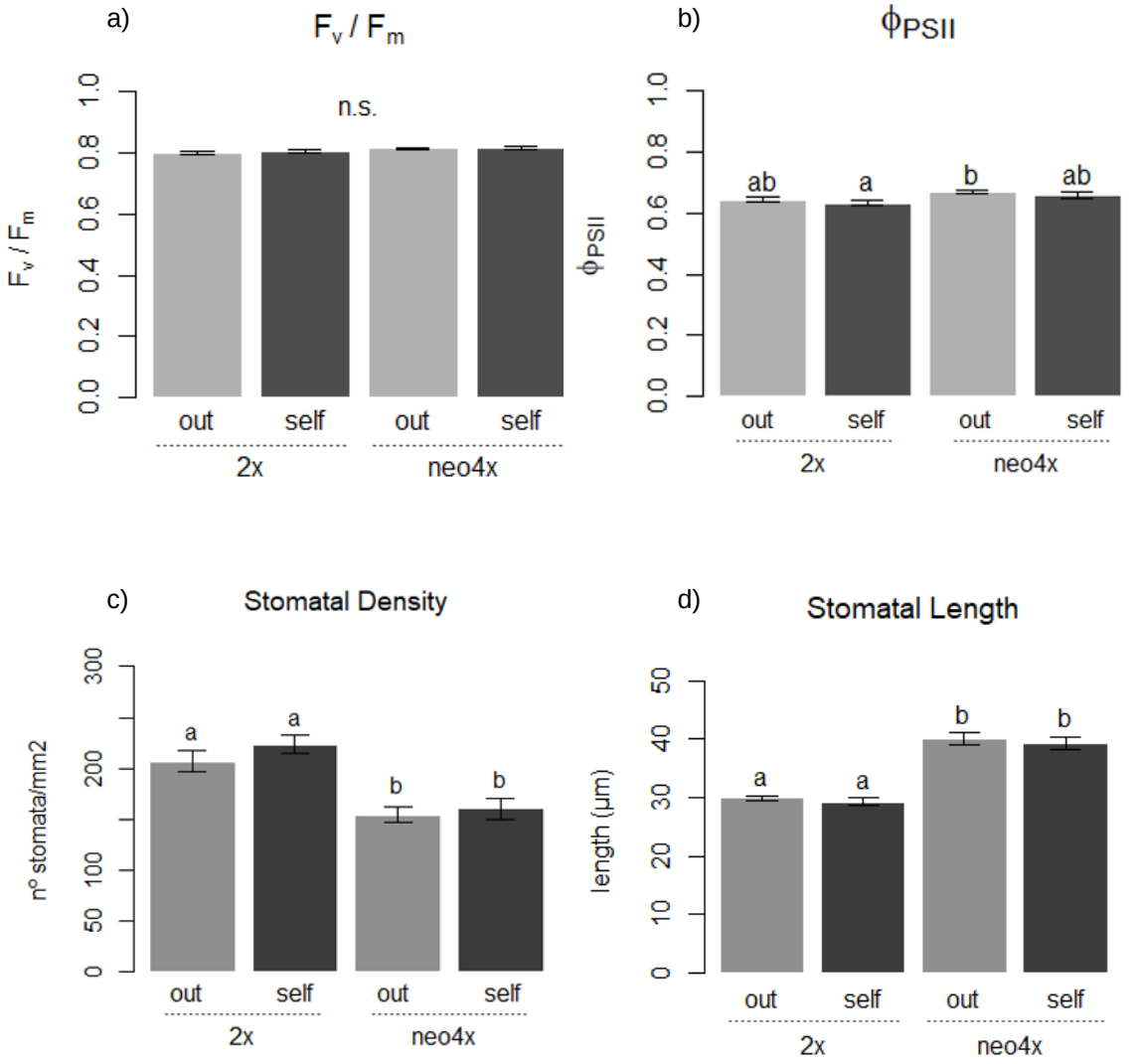

Figure 11: Plant performance of diploids (2*x*) and neotetraploids (neo4*x*) of *Jasione maritima*: (a) F_v/F_m, (b) ϕ_{PSII}; (c) Stomata density and (d) Stomata length. Values are presented as means and standard error. Different letters represent statistical differences at $P < 0.05$ for parameters without interaction between factors and according with the post-hoc analysis.

Stomata traits. Stomata density was significantly higher in the diploids in comparison with the neotetraploids (Table 4; Appendix 3.2; Figure 11c). By contrast, for stomata length, neotetraploids presented significantly higher values than diploids (Table 4; Appendix 3.2; Figure 11d). For both cases, these traits were not significantly affected by the crossing treatment (Table 4; Appendix 3.2; Figures 11c-d).

Table 4: Effect of ploidy level and crossing treatment on physiological plant performance (Chlorophyll *a*, Chlorophyll *b*, Carotenoids, Starch, Soluble Sugar, RWC, REL, F_v/F_m, ϕ_{PSII}, Stomata Density, Stomata Length) of diploids and neotetraploids of *Jasione maritima*. Obtained values of F-statistic and *P* value of the nested GLM's are showed. Significant *P* values are highlighted in bold.

Physiological traits	Ploidy level	Crossing	Crossing * Ploidy
Chlorophyll *a*	*F* = 0.691, *P* = 0.413	*F* = 0.675, *P* = 0.418	*F* = 2.520, *P* = 0.124
Chlorophyll *b*	*F* = 0.961, *P* = 0.335	*F* = 0.310, *P* = 0.582	*F* = 2.856, *P* = 0.102
Carotenoids	*F* = 1.504, *P* = 0.230	*F* = 0.339, *P* = 0.565	*F* = 1.643, *P* = 0.210
Starch	*F* = 2.694, *P* = 0.112	*F* = 0.075, *P* = 0.786	*F* = 0.489, *P* = 0.490
Total Soluble Sugars	*F* = 12.570, ***P* = 0.001**	*F* = 3.249, *P* = 0.082	*F* = 7.948, ***P* = 0.009**
RWC	*F* = 9.184, ***P* = 0.005**	*F* = 5.884, **P = 0.023**	*F* = 3.189, P = 0.086
REL	*F* = 1.741, P = 0.198	*F* = 0.023, P = 0.881	*F* = 6.697, **P = 0.015**
F_v/F_m	*F* = 7.224, ***P* = 0.008**	*F* = 0.260, *P* = 0.611	*F* = 0.337, *P* = 0.562
ϕ_{PSII}	*F* = 9.003, ***P* = 0.003**	*F* = 1.544, *P* = 0.216	*F* = 0.010, *P* = 0.920
Stomata Density	*F* = 37.754, ***P* <0.001**	*F* = 1.479, *P* = 0.228	*F* = 0.274, *P* = 0.602
Stomata Length	*F* = 172.769, ***P* <0.001**	*F* = 0.805, *P* = 0.372	*F* = 0.015, *P* = 0.904

Table 5: Effect of ploidy level and crossing treatment on physiological plant performance (Total Soluble Sugars, Relative Water Content, Relative Electrolyte Leakage) of diploids and neotetraploids of *Jasione maritima*. Obtained values of *F*-statistic and *P* value of the nested GLM's are showed for parameters with significant interaction between factors (see table 3). Nested design, with crossing nested in ploidy and ploidy nested in crossing are represented. Significant *P* values are highlighted in bold.

Parameters	Ploidy level	Crossing	Ploidy:Crossing	Crossing:Ploidy
Total Soluble Sugars	*F* = 12.570, ***P* = 0.001**	*F* = 3.249, *P* = 0.082	*F* = 5.599, ***P* = 0.009**	*F* = 10.259, ***P* <0.001**
Relative Water Content	*F* = 1.044, *P* = 0.316	*F* = 0.026, *P* = 0.872	*F* = 4.527, ***P* = 0.020**	*F* = 5.035, ***P* = 0.014**
Relative Electrolyte Leakage	*F* = 1.741, *P* = 0.198	*F* = 0.023, *P* = 0.881	*F* = 3.360, ***P* = 0.049**	*F* = 4.219, ***P* = 0.025**

CHAPTER 4 – Discussion

4. Discussion

The results obtained in this study provide new insights of the potential role of the selfing on the establishment of neotetraploids within the polyploid *Jasione maritima*. The experiment developed under controlled conditions enabled the comparison of plant development under the same environmental conditions, thus reducing the effects caused by other variables. The controlled pollinations and the greenhouse experiment had the following major results: (1) *J. maritima* is a self-incompatible plant regardless of the cytotype and, thus, the great majority of the offspring is produced after outcrossing; (2) Genome duplication does not seems to disrupt the self-incompatibility system of *J. maritima*, with neotetraploids presenting similar incompatibility indexes when compared with the diploids; (3) In general, neotetraploids presented lower plant performance than the diploids, probably as a result of the profound changes in the genome; (4) Within the neotetraploids, crossing treatment did not affect the performance of the offspring and thus, the offspring obtained after selfing developed similarly as the offspring obtained after outcrossing.

These results provide new insights on the necessary conditions for the establishment of the new polyploid entities and are discussed in detail below.

4.1 How genome duplication affected reproductive fitness of neotetraploids of *Jasione maritima*?

The results confirm that both diploid and neotetraploid individuals of *J. maritima* are self-incompatible. The reproductive success of outcross pollinations was always higher than the success after self-pollinations, regardless of the variable measured and the cytotype. Although both diploids and neotetraploids are able to produce fruits after selfing, the number of seeds per fruit is extremely low in comparison with outcrossing. The presence of an incompatibility system is in agreement with previous results obtained for the species (Castro, 2018) and are also similar to the closest species, *J. montana*, for which controlled pollinations revealed that only after outcrossing diploid plants are able to produce offspring (Parnell, 1982).

The disruption of the self-incompatibility system may allow the newly formed polyploid to self-reproduce at initial stages under limited compatible mate availability, and thus selfing ability has been pointed as an important factor enabling polyploid establishment (Husband and Schemske, 1997; Petit *et al.*, 1997; Rosquist, 2001; Galloway *et al.*, 2003; Husband and Sabara, 2003; Buggs and Pannell, 2006; Borges *et*

al., 2012) and has been included in the models that attempted to determine the necessary conditions for the success of polyploid lineages (Rodríguez, 1996; Rausch and Morgan, 2005). It has been widely accepted that polyploids self-fertilize more than their diploid relatives (Mable, 2004; Barringer, 2007) due to factors that may select the use of selfing on polyploids. Alongside with the breakdown of the self-incompatibility system, it has been hypothesized that polyploidization may also attenuate the levels of inbreeding depression (Husband and Schemske, 1997; Miller and Venable, 2000; Mable, 2004), favouring the use of selfing. However, the experimental evidence of the role of selfing on polyploids establishment is still scarce, with only a few examples being available in the literature (Husband and Schemske, 1997; Petit *et al.*, 1997; Rosquist, 2001; Galloway *et al.,* 2003; Husband and Sabara, 2003; Buggs and Pannell, 2006; Borges *et al.*, 2012). Surprisingly, regardless of the benefits that polyploidization could provide after selfing, no differences were observed in the incompatibility system between diploids and neotetraploids of *J. maritima* (namely, for the SCI calculated based on the fruit and seed set), suggesting that self-incompatibility is not disrupted by polyploidization on the neopolyploids of this species.

Even with a low production of viable seeds by the neotetraploids of *J. maritima*, the use of selfing might be advantageous, depending on the opportunities for reproduction of the neotetraploid. When reproduction by cross-pollination is not possible due to the absence of compatible mates (in this case, plants of the same ploidy level), the use of selfing becomes of major importance for the neotetraploids (Lloyd, 1992) even with the loss of reproductive fitness. For example, in *Kosteletzkya virginica*, the use of selfing was usually associated with strong inbreeding depression. However, the use of this mating system was still advantageous once it allowed reproductive assurance (Ruan *et al.*, 2011). Thus, in the initial stages after the emergence of *J. maritima* neotetraploid, the production of a few seeds after selfing could have enabled the neotetraploid to increase its number within the parental population.

Interestingly, the occurrence of selfing on *J. maritima* necessarily requires pollinator's visitation. The individual flowers of *J. maritima* are dichogamous, separating male and female functions of a given flower in time (Lloyd, 1992), and pollinators are essential for the exchange of pollen between flowers within the inflorescence. Field observations showed that pollinators of *J. maritima* are generalist, including species from different order of insects (C. Siopa and S. Mendes, field observations). The floral visitors move along the inflorescence and mediate pollen movement between the flowers of the capituliform inflorescence (i.e. geitonogamy). Neotetraploids have also been described

to have bigger inflorescences (Castro, 2018). Bigger inflorescences are frequently more attractive for pollinators and may increase visitation rate. Therefore, at initial stages after neotetraploid emergence, increased pollen exchanges within the same plant or between the few neotetraploid individuals in the population may occur. However, geitonogamy causes pollen and seed discounting due to reduction of pollen exchanges with outcrossing partners (Lloyd, 1992). This reinforces the fact that selfing may act as a short-term solution for reproductive assurance on the first stages of neotetraploid emergence where mates are scarce.

Another clear result from this study is the overall lower reproductive fitness of neotetraploids in comparison with the diploid individuals (clear when comparing outcrossing results). To balance the increase of genome content after polyploidization, a reorganization of the entire genome and changes on its functions at both genetic and epigenetic levels occurs (Comai, 2005). The inherent genetic instability after polyploidization may lead to negative effects that reduces neopolyploid fitness in the first generations. Additionally, increased cell volume frequent after polyploidization (Melaragno, 1993), may lead to unbalances on bidimensional and tridimensional components of the cells, which may negatively affect important processes related with cell division. This leads to epigenetic instability that may negatively affect polyploids, due to chromosome remodelling and subsequent gene silencing (Wang, 2004; Adams and Wendel, 2005). Another disadvantage is the tendency for polyploids to produce aneuploids (i.e. cells with a number of chromosomes that is not an exact multiple of the basic chromosome number; Comai, 2005) during meiosis. This may happen, for example, by the formation of multivalent chromosomes on meiosis that may lead to abnormal segregation of chromosomes. A lower fitness for polyploids was observed in other studies. For example, on rye (*Secale cereale*), diploids presented a seed set of 76.5%, while tetraploids presented mean values of 59.5%, showing a lower fertility after polyploidization (Müntzing, 1951). Also, seed germination on *Spartina pectinata* was lower on hexaploids (30.8%) in comparison with the tetraploids (50.8%) (Kim *et al.*, 2012). Although neopolyploid emergence seems to be very common in nature, factors as those referred above together with the minority cytotype disadvantage can act as obstacles for polyploids establishment, probably causing high polyploid extinction rates (Soltis and Soltis, 2000).

In most of the polyploid complexes, unravelling the direct effects of polyploidy are only possible using synthetic neopolyploids because it is not possible in a contemporary timeframe to find them in natural populations. However, the use of synthetized neopolyploids (obtained for example by colchicine treatment) may also have limitations.

In particular, the treatment with c-mitotic agents might have direct consequences on plant development that are not directly attributed to polyploidization effects. In Münzbergová (2017), colchicine application positively affected plant performance of first and second generation neopolyploids, probably due to strong selection of the fittest plants. This was not observed in here, but, overall, it reinforces the need of include neopolyploids in order to understand not only the effects of c-mitotic agents on the neopolyploids fitness but also to develop methodologies that are able to control its effects.

4.2 How is offspring performance of *Jasione maritima* affected by different pollination treatments?

Interestingly, within *J. maritima* neotetraploids, the fitness of selfed individuals was comparable to that of outcrossing treatment. This suggests that the use of selfing does not seem to negatively affect neotetraploids offspring fitness in *J. maritima* for the traits and life-stages studied. Again, empirical studies regarding this are very scarce. Husband *et al.* (2008) compared the inbreeding depression of synthetic neotetraploids and diploids and observed that the cost of selfing were null on *Chamerion angustifolium* neotetraploids on the first stages (Husband *et al.*, 2008). Johnston and Schoen (1996) studied the correlation between the use of selfing and inbreeding depression on the genus *Amsinckia* and discovered that tetraploids presented higher values of inbreeding depression; however, the authors also concluded that the two processes are determined more by other factors than by each other, highlighting the importance of understanding the role of ecological and genetic factors on these features.

Additionally, neotetraploids of outcrossing and selfing treatment showed no advantage when compared with the outcrossed diploids for most of the parameters. No differences were observed on pigment content. Similar results were observed in in *Atriplex confertifolia* (Warner and Edwards 1989), with different ploidy levels having similar values of chlorophyll content. Contrarily, on Dong *et al.* (2017), tetraploids of *Chrysanthemum nankingense* presented higher values of chlorophyll *a/b* than diploids. In our study, ϕ_{PSII} and F_v/F_m had higher values on neotetraploids in relation to diploids, on both crosses, although only F_v/F_m presented significant differences with outcrossed diploids. Chlorophyll florescence parameters represent a non-intrusive way to assess the primary reaction of photosynthesis (light dependent reactions of photosynthesis), so it is possible to assume that neotetraploids had a tendency to a higher efficiency of the process on the first stages (Sayed, 2003). However, the increase of photosynthetic efficiency was not accompanied by a higher sugar content (soluble sugars and starch)

of the plants, as no differences were observed between neotetraploids and diploids. This can be probably explained by the second part of the photosynthetic process. The Calvin Cycle may be affected by genome duplication due to, for example, changes on RuBisCO enzyme expression and activity (Warner and Edwards, 1993; Wang *et al.*, 2013) or morpho-anatomical differences on stomata (e.g. more close stomata) that decrease stomata gas-exchange and consequently decrease CO_2 availability for photosynthesis (Del Pozo and Ramirez-Parra, 2014). If this is the case, the increase of photosystem II efficiency on neotetraploids was counteracted in the Calvin Cycle. Once no differences were found on carbohydrates content, as expected, no significant differences were observed on total dry weight. Plant height was also not affected. However, neotetraploids of both crosses presented significant lower number of leaves than outcrossed diploids. This may reflect a trade-off between size and density of leaves. As neotetraploids had a lower number of leaves in comparison with diploids, despite this parameter was not measure, bigger leaves are expected to counterbalance it, resulting in a similar biomass.

Finally, neotetraploids of both crosses presented lower density of stomata but bigger stomata than outcrossed diploids. The use of selfing did not affect these parameters. The results observed after polyploidization corroborates the pattern usually observed for polyploids (Hetherington and Woodward, 2003; Kim *et al.*, 2012; Segraves, 2017). Stomata parameters may affect RWC, once lower water loss is often observed with increased stomata size and decreased stomata density (Venora and Calcagno, 1991; Wang and Clarke, 1993). Del Pozo and Ramirez-Parra (2014) observed, along with bigger stomata and lower density, that tetraploids guard cells in *Arabidopsis* also presented a more pronounced closure of the stomata, with consequently lower water loss than diploids. In fact, in neotetraploids of *J. maritima* higher values of RWC in relation to the outcrossed diploids were observed, despite significant differences were only detected for selfed neotetraploids. Outcrossing treatment did not present significant differences with outcrossed diploids. This may be explained by the REL. Neotetraploids of outcrossing treatment presented the highest value for this physiological parameter. Water stress is usually related with higher values of cell membrane damage, detected by REL (Farooq and Azam, 2006). Once outcrossed neotetraploids presented significantly higher level of cell membrane damage it is expected a negative effect on other physiological features as RWC, which may explain the observed differences between outcrossed and selfed neotetraploids in this parameter.

For the establishment of neotetraploids of *J. maritima*, a set of characteristics that increase its fitness are fundamental. Polyploidization by itself does not seem to bring the fitness advantage that neotetraploids require to compete with diploids in the first stages,

although it also does not seem to constitute a disadvantage. The use of selfing as mating system enables the production of offspring even with no mate availability, however, with a trade-off, as reproductive fitness is negatively affected by the use of this reproductive strategy. Although reproductive success is negatively affected, the seeds produced by selfing are capable to reach the adult stage and have, in many traits, similar fitness than diploid offspring.

4.3 Could selfing enable the establishment of neotetraploids offspring?

Even with low seed production by the neotetraploids after selfing, the seeds that germinated were capable to reach the adult stage without a significant loss of fitness in comparison with the outcrossing treatment. Thus, the use of selfing may allow the neopolyploids to avoid fertilization with diploids and to form offspring. The reproductive assurance hypothesis, suggests that the need for offspring production makes selfing still advantageous even if inbreeding depression is strong (Rodríguez, 1996; Mable, 2004). Several studies with different plant species support this hypothesis (Eckert and Schaefer, 1998; Lloyd, 1992; Schoen and Brown, 1991). In polyploid complexes, for example, the conditions after genome duplication were favourable for the occurrence of selfing in *C. angustifolium*, as the neotetraploids presented lower values of inbreeding depression when compared with the diploids (Ozimec and Husband, 2011). These authors thus suggest that selfing strategy might have been one of the factors that was involved with the establishment of tetraploid in nature. Similar results were observed by Barringer and Geber (2008) in the polyploid *Clarkia*, where lower inbreeding depression was detected on selfing species in comparison with outcrossing species, as well as in polyploid species in comparison with diploids.

In *J. maritima*, the need for reproductive assurance on first stages of emergence of the neotetraploids may have suited self-reproduction as vital for neotetraploids establishment, even with (1) loss of reproductive fitness and (2) no gain of fitness for the offspring. However, our results also suggest that polyploidization and the use of selfing may not be sufficient to allow for neotetraploids establishment. Therefore, other mechanisms and ecological determinants were probably needed to overcome the minority cytotype exclusion. Several models tried to understand how the different determinants may be important for neotetraploids establishment (Levin, 1975; Felber, 1991; Burton and Husband, 2000; Husband, 2000; Fowler and Levin, 2008), including the use of selfing (Rodríguez, 1996; Rausch and Morgan, 2005; Ozimec and Husband, 2011). Rodríguez (1996) concluded that high rates of selfing makes the neopolyploids

establishment more probable. However, other processes as an increase in fecundity or niche separation also incremented the probability of establishment by reducing the disadvantage of the minority cytotype. Rausch and Morgan (2005) concluded that determinants as the production of 2*n* gametes, reduced inbreeding depression and small population size favour neopolyploids establishment. Finally, Husband (2000) assessed how the hypothesis of the minority cytotype may affect polyploid establishment and observed that the fitness of tetraploids of *C. angustifolium* was frequency dependent, as opposed to seed set that was independent of the frequency of tetraploids due to assortative mating mediated by bee visitation. Clearly, there seems to be a cumulative effect of several factors that contribute to the success of the neopolyploid in a given scenario, and most probably the factors with be dependent on the plant species, historical processes and context at the timeframe of neopolyploid emergence.

CHAPTER 5 – Conclusions

5. Conclusions

5.1 General conclusions

With this thesis we were able to understand how selfing acts as reproductive assurance and how this process affects the offspring of neotetraploids of *J. maritima*. Despite the negative effect of selfing on reproductive fitness, especially on seed set, the reproductive assurance given by this reproductive strategy might be essential on the first stages after neotetraploid emergence due to the lack of compatible mates. The SCI values obtained also corroborate that this plant, for both diploids and neotetraploids, is a self-incompatible species.

Additionally, in general, polyploidization and the use of selfing did not bring fitness advantage for the neotetraploids offspring. Neotetraploids showed, overall, lower plant performance in relation to the diploids. However, within neotetraploids, no differences on plant performance were observed between crosses. This was not expected, once it is largely accepted that the use of selfing brings negative costs to the offspring produced due to inbreeding depression. Therefore, our results show that the offspring obtained by selfing can reach adult stage without a clear fitness disadvantage against the diploids. In first stages, selfing could be one the factors involved with neotetraploids establishment, although other traits need also to be involved in the success of the new cytotype, including perenniality, high 2*n* gametes production, high dispersal capacity or barriers that favour assortative mating in mixed-ploidy populations.

The use of synthetized neopolyploids contributes with important information about the effect of polyploidization *per se*, not only on the process of selfing but also on the effects over several morphological, fitness and physiological traits measured along this study.

5.2 Future directions

In future studies it would be interesting to include established tetraploids, as these entities would enable to predict how the different reproductive, morphological and physiological characteristics may had developed through the maintenance of the new cytotype. Additionally, because the use of selfing by itself might not be sufficient to allow for neotetraploids establishment, it would be interesting to understand the role of other processes/features beyond selfing such as life cycle duration, dispersal capacity and/or

the acquisition of breeding barriers that promote assortative mating of first stages. These studies would allow to understand in more detail the dynamics of the neotetraploids establishment in *J. maritima*. Additionally, further knowledge on the effects of polyploidization on genetic and genomic attributes of polyploids may also be important to better understand the mechanisms behind the success of polyploid lineages. In fact, polyploidization effects include a wide range of areas, from genetics and genomics towards ecology, being important to have a concerted knowledge at all these biological levels.

CHAPTER 6 – References

Adams, K. and Wendel, J. (2005) 'Novel patterns of gene expression in polyploid plants', *Trends in Genetics*, 21(10), pp. 536–539. doi: 10.1016/j.tig.2005.08.002.

Baack, E. (2005) 'To succeed globally, disperse locally: Effects of local pollen and seed dispersal on tetraploid establishment', *Heredity*, 94(5), pp. 538–546. doi: 10.1038/sj.hdy.6800656.

Barker, M., Arrigo, N., Baniaga, A., Li, Z. and Levin, D. (2016) 'On the relative abundance of autopolyploids and allopolyploids', *New Phytologist*, 210(2), pp. 391–398. doi: 10.1111/nph.13698.

Barringer, B. (2007) 'Polyploidy and self-fertilization in flowering plants', *American Journal of Botany*, 94(9), pp. 1527–1533. doi: 10.3732/ajb.94.9.1527.

Barringer, B. and Geber, M. (2008) 'Mating system and ploidy influence levels of inbreeding depression in *Clarkia* (Onagraceae)', *Evolution*, 62(5), pp. 1040–1051. doi: 10.1111/j.1558-5646.2008.00361.x.

Blanc, G. and Wolfe, K. (2004) 'Widespread Paleopolyploidy in Model Plant Species Inferred from Age Distributions of Duplicate Genes', *the Plant Cell Online*, 16(7), pp. 1667–1678. doi: 10.1105/tpc.021345.

Borges, L., Souza, L., Guerra, M., Machado, I., Lewis, G. and Lopes, A. (2012) 'Reproductive isolation between diploid and tetraploid cytotypes of Libidibia ferrea (= *Caesalpinia ferrea*) (Leguminosae): Ecological and taxonomic implications', *Plant Systematics and Evolution*, 298(7), pp. 1371–1381. doi: 10.1007/s00606-012-0643-3.

Bretagnolle, F. and Lumaret, R. (1995) 'Bilateral polyploidization in *Dactylis glomerata* L. subsp. lusitanica: occurrence, morphological and genetic characteristics of first polyploids', *Euphytica*, 84(3), pp. 197–207. doi: 10.1007/BF01681812.

Bretagnolle, F. and Thompson, J. (1995) 'Gametes with the Stomatic Chromosome Number: Mechanisms of Their Formation and Role in the Evolution of Autopolypoid Plants', *New Phytologist*, 129(1), pp. 1–22. doi: 10.1111/j.1469-8137.1995.tb03005.x.

Bretagnolle, F. and Thompson, J. (1996) 'An experimental study of ecological differences in winter growth between sympatric diploid and autotetraploid *Dactylis glomerata*', *Journal of Ecology*, 84(3), pp. 343–351. doi: 10.2307/2261197.

Bretagnolle, F., Thompson, J. and Lumaret, R. (1995) 'The influence of seed size variation on seed germination and seedling vigour in diploid and tetraploid *Dactylis glomerata* L.', *Annals of Botany*, pp. 607–615. doi: 10.1006/anbo.1995.1138.

Brochmann, C., Brysting, A., Alsos, I., Borgen, L., Grundt, H., Scheen, A.-C. and Elven,

R. (2004) ‘Biological relevance of polyploidy : ecology to genomics Polyploidy in arctic plants’, *Biological Journal of the Linnean Society*, 82(4), pp. 521–536. doi: 10.1111/j.1095-8312.2004.00332.x.

Buggs, R. and Pannell, J. (2006) ‘Rapid Displacement of a Monoecious Plant Lineage Is Due to Pollen Swamping by a Dioecious Relative’, *Current Biology*, 16(10), pp. 996–1000. doi: 10.1016/j.cub.2006.03.093.

Buggs, R. and Pannell, J. (2007) ‘Ecological differentiation and diploid superiority across a moving ploidy contact zone’, *Evolution*, 61(1), pp. 125–140. doi: 10.1111/j.1558-5646.2007.00010.x.

Burton, T. and Husband, B. (2000) ‘Fitness Differences Among Diploids, Tetraploids, and Their Triploid Progeny in *Chamerion Angustifolium*: Mechanisms of Inviability and Implications for Polyploid Evolution’, *Evolution*, 54(4), pp. 1182–1191. doi: 10.1554/0014-3820(2000)054[1182:FDADTA]2.0.CO;2.

Castro, M. (2018) *Evolutionary ecology of polyploids: understanding species coexistence at the contact zones*.

Castro, S., Münzbergová, Z., Raabová, J. and Loureiro, J. (2011) ‘Breeding barriers at a diploid-hexaploid contact zone in *Aster amellus*’, *Evolutionary Ecology*, 25(4), pp. 795–814. doi: 10.1007/s10682-010-9439-5.

Castro, S. and Loureiro, J. (2014) ‘El papel de la reproducción en el origen y la evolución de las plantas poliploides’, *Ecosistemas*, 23(3), pp. 67–77. doi: 10.7818/ECOS.2014.23-3.09.

Comai, L. (2005) ‘The advantages and disadvantages of being polyploid’, *Nature*, 6, pp. 836–846. doi: 10.1038/nrg1711.

Dhawan, O. and Lavania, U. (1996) ‘Enhancing the productivity of secondary metabolites via induced polyploidy: a review’, *Euphytica*, 87(2), pp. 81–89. doi: 10.1007/BF00021879.

Dias, M., Oliveira, H., Costa, A. and Santos, C. (2014) ‘Improving elms performance under drought stress: The pretreatment with abscisic acid’, *Environmental and Experimental Botany*, 100, pp. 64–73. doi: 10.1016/j.envexpbot.2013.12.013.

Dolezel, J., Greihuber, J. and Suda, J. (2007) *Flow Cytometry with Plant Cells: Analysis of Genes, Chromosomes and Genomes*. John Wiley & Sons.

Doležel, J., Sgorbati, S. and Lucretti, S. (1992) ‘Comparison of three DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants’, *Physiologia Plantarum*,

85(4), pp. 625–631. doi: 10.1111/j.1399-3054.1992.tb04764.x.

Dong, B., Wang, H., Liu, T., Cheng, P., Chen, Y., Chen, S., Guan, Z., Fang, W., Jiang, J. and Chen, F. (2017) 'Whole genome duplication enhances the photosynthetic capacity of *Chrysanthemum nankingense*', *Molecular Genetics and Genomics*. Springer Berlin Heidelberg, 292(6), pp. 1247–1256. doi: 10.1007/s00438-017-1344-y.

Eckert, C. and Schaefer, A. (1998) 'Does self-pollination provide reproductive assurance in *Aquilegia canadensis* (Ranunculaceae)?', *American Journal of Botany*, 85(7), pp. 919–924. doi: 10.2307/2446357.

Eigsti, O. (1938) 'A cytological study of colchicine effects in the induction of polyploidy in plants', *Proceedings of the National Academy of Sciences*, 24(2), pp. 56–63.

Farooq, S. and Azam, F. (2006) 'The use of cell membrane stability (CMS) technique to screen for salt tolerant wheat varieties', *Journal of Plant Physiology*, 163(6), pp. 629–637. doi: 10.1016/j.jplph.2005.06.006.

Felber, F. (1991) 'Establishment of a tetraploid cytotype in a diploid population: Effect of relative fitness of the cytotypes', *Journal of Evolutionary Biology*, 4(2), pp. 195–207. doi: 10.1046/j.1420-9101.1991.4020195.x.

Fowler, N. and Levin, D. (2008) 'Ecological Constraints on the Establishment of a Novel Polyploid in Competition with Its Diploid Progenitor', *The American Naturalist*, 124(5), pp. 703–711.

Galloway, L., Etterson, J. and Hamrick, J. (2003) 'Outcrossing rate and inbreeding depression in the herbaceous autotetraploid, *Campanula americana*', *Heredity*, 90(4), pp. 308–315. doi: 10.1038/sj.hdy.6800242.

Grant, V. (1956) 'The Influence of Breeding Habit on the Outcome of Natural Hybridization in Plants', *The American Naturalist*, 90(854), p. 319. doi: 10.1086/281939.

Grant, V. (1971) *Plant Speciation*. Columbia University Press.

Gray, A., Marshall, D. and Raybould, A. (1991) 'A Century of Evolution in *Spartina anglica*', *Advances in Ecological Research*, 21(C), pp. 1–62. doi: 10.1016/S0065-2504(08)60096-3.

De Haan, A., Maceira, N.O., Lumaret, R. and Delay, J. (1992) 'Production of In Gametes in Diploid Subspecies of *Dactylis glomerata* L. 2. Occurrence and Frequency of In Eggs', *Annals of Botany*, 69, pp. 345–350.

Hao, G., Lucero, M., Sanderson, S., Zacharias, E. and Holbrook, N. (2013) 'Polyploidy enhances the occupation of heterogeneous environments through hydraulic related

trade-offs in *Atriplex canescens* (Chenopodiaceae)', *New Phytologist*, 197(3), pp. 970–978. doi: 10.1111/nph.12051.

Harlan, J. and DeWet, J. (1975) 'On Ö. Winge and a prayer: the origins of polyploidy', *The Botanical Review*, 41(4), pp. 361–390.

Hegarty, M., Abbott, R. and Hiscock, S. (2012) 'Allopolyploid Speciation in Action: The Origins and Evolution of *Senecio cambrensis*', in *Polyploidy and genome evolution*. Springer, Berlin, Heidelberg, pp. 245–270. doi: 10.1007/978-3-642-31442-1.

Hetherington, A. and Woodward, F. (2003) 'The role of stomata in sensing and driving environmental change', *Nature*, 424(6951), pp. 901–908.

Husband, B. (2000) 'Constraints on polyploid evolution: A test of the minority cytotype exclusion principle', *Proceedings of the Royal Society B: Biological Sciences*, 267(1440), pp. 217–223. doi: 10.1098/rspb.2000.0990.

Husband, B., Ozimec, B., Martin, S. and Pollock, L. (2008) 'Mating Consequences of Polyploid Evolution in Flowering Plants: Current Trends and Insights from Synthetic Polyploids', *International Journal of Plant Sciences*, 169(1), pp. 195–206. doi: 10.1086/523367.

Husband, B., Baldwin, S. and Suda, J. (2013) 'Plant Genome Diversity Volume 2 Plant Genome Diversity Volume 2', in *Plant Genome Diversity*, pp. 255–276. doi: 10.1007/978-3-7091-1160-4.

Husband, B. and Sabara, H. (2003) 'Reproductive isolation between autotetraploids and their diploid progenitors in fireweed, *Chamerion angustifolium* (Onagraceae)', *New Phytologist*, 161(3), pp. 703–713. doi: 10.1046/j.1469-8137.2003.00998.x.

Husband, B. and Schemske, D. (1995) 'Magnitude and timing of inbreeding depression in a diploid population of *Epilobium angustifolium* (Onagraceae)', *Heredity*, 75(2), pp. 206–215. doi: 10.1038/hdy.1995.125.

Husband, B. and Schemske, D. (1997) 'The effect of inbreeding in diploid and tetraploid populations of *Epilobium angustifolium* (Onagraceae): Implications for the genetic basis of inbreeding depression', *Evolution*, 51(3), pp. 737–746. doi: 10.2307/2411150.

Husband, B. and Schemske, D. (2000) 'Ecological mechanisms of reproductive isolation between diploid and tetraploid *Chamerion angustifolium*', *Journal of Ecology*, 88(4), pp. 689–701. doi: 10.1046/j.1365-2745.2000.00481.x.

Irigoyen, J., Einerich, D. and Sánchez-Díaz, M. (1992) 'Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*)

plants', *Physiologia Plantarum*, 84(1), pp. 55–60. doi: 10.1111/j.1399-3054.1992.tb08764.x.

Johnston, M. and Schoen, D. J. (1996) 'Correlated evolution of self-fertilization and inbreeding depression: an experimental study of nine populations of *Amsinckia* (Boragainaceae)', *Evolution*, 50(4), pp. 1478–1491.

Kihara, H. and Ono, T. (1926) 'Chromosomenzahlen und systematische gruppierung der Rumex-arten', *Zeitschrift für Zellforschung und mikroskopische Anatomie*, 4(3), pp. 475–481.

Kim, S., Rayburn, A., Boe, A. and Lee, D. (2012) 'Neopolyploidy in *Spartina pectinata* Link: 1. Morphological analysis of tetraploid and hexaploid plants in a mixed natural population', *Plant Systematics and Evolution*, 298(6), pp. 1073–1083. doi: 10.1007/s00606-012-0617-5.

Levin, D. (1975) 'Minority Cytotype Exclusion in Local Plant Populations', *Taxon*, 24(1), pp. 35–43.

Levin, D. (2002) The role of chromosomal change in plant evolution, Oxford Series in *Ecology abd Evolution*. doi: 10.1017/CBO9781107415324.004.

Lim, K., Matyasek, R., Kovarik, A. and Leitch, A. (2007) 'Parental origin and genome evolution in the allopolyploid *Iris versicolor*', *Annals of Botany*, 100(2), pp. 219–224. doi: 10.1093/aob/mcm116.

Lim, K., Kovarik, A., Matyasek, R. and Leitch, A. (2007) 'Sequence of events leading to near-complete genome turnover in allopolyploid *Nicotiana* within five million years', *New Phytologist*, 175(4), pp. 756–763. doi: 10.1111/j.1469-8137.2007.02121.x.

Liu, G., Li, Z. and Bao, M. (2007) 'Colchicine-induced chromosome doubling in *Platanus acerifolia* and its effect on plant morphology', *Euphytica*, 157(1–2), pp. 145–154. doi: 10.1007/s10681-007-9406-6.

Lloyd, D. (1992) 'Self- and Cross-fertilization in Plants. II. The selection of self-fertilization', *International Journal of Plant Sciences*, 153(3), pp. 370–380.

Loureiro, J., Rodríguez, E., Doležel, J. and Santos, C. (2007) 'Two new nuclear isolation buffers for plant DNA flow cytometry: a test with 37 species', *Annals of Botany*, 100, pp. 875–888.

Mable, B. (2004) 'Polyploidy and self-compatibility: Is there an association?', *New Phytologist*, 162(3), pp. 803–811. doi: 10.1111/j.1469-8137.2004.01055.x.

Maceira, N., De Haan, A., Lumaret, R., Billon, M. and Delay, J. (1992) 'Production of 2*n* Gametes in Diploid Subspecies of *Dactylis glomerata* L. 1. Occurrence and Frequency of 2*n* Pollen', *Annals of Botany*, 69(March), pp. 345–350.

Madlung, A. (2013) 'Polyploidy and its effect on evolutionary success: Old questions revisited with new tools', *Heredity*. Nature Publishing Group, 110(2), pp. 99–104. doi: 10.1038/hdy.2012.79.

Maherali, H., Walden, A. and Husband, B. (2009) 'Genome duplication and the evolution of physiological responses to water stress', *New Phytologist*, 184(3), pp. 721–731. doi: 10.1111/j.1469-8137.2009.02997.x.

Marques, I., Loureiro, J., Draper, D., Castro, M. and Castro, S. (2018) 'How much do we know about the frequency of hybridisation and polyploidy in the Mediterranean region?', *Plant Biology*, 20(October), pp. 21–37. doi: 10.1111/plb.12639.

Melaragno, J. (1993) 'Relationship between Endopolyploidy and Cell Size in Epidermal Tissue of *Arabidopsis*', *the Plant Cell Online*, 5(11), pp. 1661–1668. doi: 10.1105/tpc.5.11.1661.

Miller, J. and Venable, D. (2000) 'Polyploidy and the Evolution of Gender Dimorphism in Plants', *Science*, 289(5488), pp. 2335–2338.

Molin, W., Meyers, S., Baer, G. and Schrader, L. (1982) 'Ploidy Effects in Isogenic Populations of Alfalfa: II. Photosynthesis, Chloroplast Number, Ribulose-1, 5-Bisphosphate Carboxylase, Chlorophyll, and DNA in Protoplasts', *Plant Physiology*, 70(6), pp. 1710–1714.

Müntzing, A. (1951) 'Cyto-genetic properties and practical value of tetraploids rye', *Hereditas*, 37(1–2), pp. 17–84.

Münzbergová, Z. (2017) 'Colchicine application significantly affects plant performance in the second generation of synthetic polyploids and its effects vary between populations', *Annals of Botany*, 120(2), pp. 329–339. doi: 10.1093/aob/mcx070.

Osaki, M., Shinano, T. and Tadano, T. (1991) 'Redistribution of carbon and nitrogen compounds from the shoot to the harvesting organs during maturation in field crops', *Soil Science and Plant Nutrition*, 37(1), pp. 117–128. doi: 10.1080/00380768.1991.10415017.

Otto, S. and Whitton, J. (2000) 'Polyploid Incidence and Evolution', *Annual Review of Genetics*, 34(1), pp. 401–437.

Ozimec, B. and Husband, B. (2011) 'Effect of recurrent selfing on inbreeding depression

and mating system evolution in an autopolyploid plant', *Evolution*, 65(7), pp. 2038–2049. doi: 10.1111/j.1558-5646.2011.01259.x.

Parnell, J. (1982) 'Cytotaxonomy of *Jasione montana* L. in the British Isles', *Watsonia*, 151, pp. 147–151.

Petit, C., Lesbros, P., Ge, X. and Thompson, J. (1997) 'Variation in flowering phenology and selfing rate across a contact zone between diploid and tetraploid *Arrhenatherum elatius* (Poaceae)', *Heredity*, 79(1), pp. 31–40. doi: 10.1038/hdy.1997.120.

Del Pozo, J. and Ramirez-Parra, E. (2014) 'Deciphering the molecular bases for drought tolerance in *Arabidopsis* autotetraploids', *Plant, Cell and Environment*, 37(12), pp. 2722–2737. doi: 10.1111/pce.12344.

Ramsey, J. (2011) 'Polyploidy and ecological adaptation in wild yarrow', *Proceedings of the National Academy of Sciences*, 108(17), pp. 7096–7101. doi: 10.1073/pnas.1016631108.

Ramsey, J. and Schemske, D. (1998) 'Pathways, Mechanisms, and Rates of Polyploid Formation in Flowering Plants', *Annual Review of Ecology and Systematics*, 29(1), pp. 467–501. doi: 10.1146/annurev.ecolsys.29.1.467.

Ramsey, J. and Schemske, D. (2002) 'Neopolyploidy in Flowering Plants', *Annual Review of Ecology and Systematics*, 33(1), pp. 589–639. doi: 10.1146/annurev.ecolsys.33.010802.150437.

Rausch, J. and Morgan, M. (2005) 'The effect of self-fertilization, inbreeding depression, and population size on autotetraploid establishment', *Evolution*, 59(9), pp. 1867–1875. doi: 10.1554/05-095.1.

Redman, R., Haraldson, J. and Gusta, L. (1986) 'Leakage of UV-absorbing substances as a measure of salt injury in leaf tissue of woody species', *Physiologia Plantarum*, 67, pp. 87–91.

Rieseberg, L. and Willis, J. (2007) 'Plant speciation', *Science*, 317(5840), pp. 910–914. doi: 10.1126/science.1137729.

Rodríguez, D. (1996) 'A model for the establishment of polyploidy in plants', *The American Naturalist*, 147(1), pp. 33–46. doi: 10.2307/2463222.

Rosquist, G. (2001) 'Reproductive Biology in Diploid *Anthericum ramosum* and Tetraploid *A . liliago* (Anthericaceae)', *Oikos*, 92(1), pp. 143–152.

Ruan, C., Qin, P. and Teixeira da Silva, J. (2011) 'Relationship between reproductive

assurance and mixed mating in perennial *Kosteletzkya virginica*', *South African Journal of Botany*. SAAB, 77(2), pp. 280–291. doi: 10.1016/j.sajb.2010.08.012.

Sayed, O. (2003) 'Chlorophyll fluorescence as a tool in cereal crop research', *Photosynthetica*, 41(3), pp. 321–330. doi: 10.1023/B:PHOT.0000015454.36367.e2.

Schoen, D. and Brown, A. (1991) 'Whole-and part-flower self-pollination in *Glycine clandestina* and *G. argyrea* and the evolution of autogamy', *Evolution*, 45(7), pp. 1651–1664.

Segraves, K. (2017) 'The effects of genome duplications in a community context', *New Phytologist*, 215(1), pp. 57–69. doi: 10.1111/nph.14564.

Segraves, K. and Thompson, J. (1999) 'Plant Polyploidy and Pollination : Floral Traits and Insect Visits to Diploid and Tetraploid *Heuchera grossulariifolia*', *Society*, 53(4), pp. 1114–1127.

Sims, D. and Gamon, J. (2002) 'Relationship between leaf pigment con- tent and spectral reflectance across a wide range species, leaf structures and development stages', *Remote Sensing of Environment*, 81, pp. 337–354. doi: 10.1016/S0034-4257(02)00010-X.

Soltis, D., Buggs, R., Doyle, J. and Soltis, P. (2010) 'What we still don't know about polyploidy', *Taxon*, 59(5), pp. 1387–1403. doi: 10.2307/20774036.

Soltis, D., Visger, C., Marchant, D. and Soltis, P. (2016) 'Polyploidy: Pitfalls and paths to a paradigm', *American Journal of Botany*, 103(7), pp. 1146–1166. doi: 10.3732/ajb.1500501.

Soltis, P. and Soltis, D. (2000) 'The role of genetic and genomic attributes in the success of polyploids', *Proceedings of the National Academy of Sciences*, 97(13), pp. 7051–7057. doi: 10.1073/pnas.97.13.7051.

Soltis, P. and Soltis, D. (2009) 'The Role of Hybridization in Plant Speciation', *Annual Review of Plant Biology*, 60(1), pp. 561–588. doi: 10.1146/annurev.arplant.043008.092039.

Stebbins, G. (1971) 'Chromosome Evolution in Higher Plants', *Columbia University Press*.

De Storme, N., Copenhaver, G. and Geelen, D. (2012) 'Production of Diploid Male Gametes in *Arabidopsis* by Cold-Induced Destabilization of Postmeiotic Radial Microtubule Arrays', *Plant Physiology*, 160(4), pp. 1808–1826. doi: 10.1104/pp.112.208611.

Suda, J., Kron, P., Husband, B. and Trávníček, P. (2007) 'Flow cytometry and ploidy: applications in plant systematics, ecology and evolutionary biology.', *Willey*, pp. 103–130.

Thébault, A., Gillet, F., Müller-Schärer, H. and Buttler, A. (2011) 'Polyploidy and invasion success: Trait trade-offs in native and introduced cytotypes of two Asteraceae species', *Plant Ecology*, 212(2), pp. 315–325. doi: 10.1007/s11258-010-9824-8.

Trojak-Goluch, A. and Skomra, U. (2013) 'Artificially induced polyploidization in *Humulus lupulus* L. and its effect on morphological and chemical traits', *Breeding Science*, 63(4), pp. 393–399. doi: 10.1270/jsbbs.63.393.

Venora, G. and Calcagno, F. (1991) 'Study of stomatal parameters for selection of drought resistant varieties in *Triticum durum* Desf', *Euphytica*, 57(3), pp. 275–283. doi: 10.1007/BF00039674.

Wang, H. and Clarke, J. (1993) 'Genotypic, intraplant, and environmental variation in stomatal frequency and size in wheat', *Canadian Journal of Plant Science*, 73(3), pp. 671–678. doi: 10.4141/cjps93-088.

Wang, J. (2004) 'Stochastic and Epigenetic Changes of Gene Expression in *Arabidopsis* Polyploids', *Genetics*, 167(4), pp. 1961–1973. doi: 10.1534/genetics.104.027896.

Wang, Z., Wang, M., Liu, L. and Meng, F. (2013) 'Physiological and proteomic responses of diploid and tetraploid black locust (*Robinia pseudoacacia* L.) subjected to salt stress', *International Journal of Molecular Sciences*, 14(10), pp. 20299–20325. doi: 10.3390/ijms141020299.

Warner, D. and Edwards, G. (1989) 'Effects of Polyploidy on Photosynthetic Rates, Photosynthetic Enzymes, Contents of DNA, Chlorophyll, and Sizes and Numbers of Photosynthetic Cells in the C(4) Dicot *Atriplex confertifolia*.', *Plant physiology*, 91(3), pp. 1143–1151. doi: 10.1104/pp.91.3.1143.

Warner, D. and Edwards, G. (1993) 'Effects of polyploidy on photosynthesis', *Photosynthesis Research*, 35(2), pp. 135–147. doi: 10.1007/BF00014744.

Weyers, J. and Travis, A. (1981) 'Selection and preparation of leaf epidermis for experiments on stomatal physiology', *Journal of Experimental Botany*, 32(4), pp. 837–850. doi: 10.1093/jxb/32.4.837.

CHAPTER 7 – Appendices

Appendix 1: Descriptive statistics for fruit set, seed set, seed germination and their respective self-compatible indexes, with mean, standard error of the mean (SE), standard deviation of the mean (SD) and sample size (n) being given for each cytotype and treatment for fruit set, seed set and seed germination and for each cytotype for the SCI of the three parameters.

Reproductive traits	n	Ploidy level	Crossing treatment	mean	± SE	± SD
Fruit set (pi)	132	2*x*	Outcrossing	0.776	0.028	0.163
			Selfing	0.738	0.037	0.211
		Neo-4*x*	Outcrossing	0.520	0.046	0.274
			Selfing	0.574	0.051	0.282
Seed set (nº seeds / fruit)	132	2*x*	Outcrossing	9.454	1.295	7.440
			Selfing	0.689	0.309	1.777
		Neo-4*x*	Outcrossing	3.182	0.785	4.642
			Selfing	0.396	0.126	0.699
Seed germination (pi)	154	2*x*	Outcrossing	0.877	0.032	0.198
			Selfing	0.673	0.052	0.326
		Neo-4*x*	Outcrossing	0.669	0.047	0.305
			Selfing	0.331	0.054	0.318
SCI – Fruit set (f.s. self / f.s. out)	64	2*x*	--	0.950	0.047	0.272
		Neo-4*x*	--	1.102	0.097	0.541
SCI – Seed set (s.s. self / s.s. out)	64	2*x*	--	0.073	0.033	0.188
		Neo-4*x*	--	0.124	0.039	0.220
SCI – Seed germination (s.g. self / s.g. out)	74	2*x*	--	0.767	0.060	0.372
		Neo-4*x*	--	0.495	0.080	0.475

Appendix 2: Descriptive statistics for the developmental fitness parameters with mean, standard error of the mean (SE), standard deviation of the mean (SD) and sample size (n) being given for each cytotype and treatment.

Developmental and fitness traits	n	Ploidy level	Crossing treatment	mean	± SE	± SD
Number of leaves	173	2*x*	Outcrossing	231.477	20.114	133.695
			Selfing	152.619	14.044	91.015
		Neo-4*x*	Outcrossing	92.667	9.821	75.394
			Selfing	76.947	7.603	46.866
Height (cm)	173	2*x*	Outcrossing	5.947	0.849	5.632
			Selfing	3.936	0.199	1.293
		Neo-4*x*	Outcrossing	4.636	0.385	2.497
			Selfing	4.226	0.239	1.475
Belowground biomass (mg)	134	2*x*	Outcrossing	173.331	20.536	123.218
			Selfing	139.221	16.148	94.160
		Neo-4*x*	Outcrossing	131.409	16.077	93.744
			Selfing	92.460	9.871	54.065
Aboveground biomass (mg)	134	2*x*	Outcrossing	418.922	43.374	260.241
			Selfing	276.718	30.023	175.062
		Neo-4*x*	Outcrossing	288.353	31.889	185.946
			Selfing	223.493	24.031	131.622
Total biomass (mg)	134	2*x*	Outcrossing	583.217	59.066	354.394
			Selfing	415.938	44.560	259.828
		Neo-4*x*	Outcrossing	414.815	46.213	269.465
			Selfing	315.953	32.013	175.341

Appendix 3.1: Descriptive statistics for fruit set, seed set and seed germination, with mean, standard error of the mean (SE), standard deviation of the mean (SD) and sample size (n) being given for each cytotype and treatment.

Physiological traits	n	Ploidy level	Crossing treatment	mean	± SE	± SD
Chlorophyll *a* (mg / g fresh weight)	32	2*x*	Outcrossing	0.271	0.089	0.089
			Selfing	0.295	0.109	0.109
		Neo-4*x*	Outcrossing	0.295	0.080	0.080
			Selfing	0.220	0.067	0.067
Chlorophyll *b* (mg / g fresh weight)	32	2*x*	Outcrossing	0.110	0.012	0.035
			Selfing	0.125	0.015	0.044
		Neo-4*x*	Outcrossing	0.120	0.012	0.035
			Selfing	0.090	0.012	0.035
Carotenoids (mg / g fresh weight)	32	2*x*	Outcrossing	0.104	0.010	0.027
			Selfing	0.111	0.013	0.037
		Neo-4*x*	Outcrossing	0.104	0.010	0.028
			Selfing	0.085	0.009	0.026
Soluble sugar (mg / g fresh weight)	32	2*x*	Outcrossing	2.357	0.160	0.452
			Selfing	1.723	0.087	0.245
		Neo-4*x*	Outcrossing	1.484	0.061	0.172
			Selfing	1.624	0.196	0.554
Starch (mg / g fresh weight)	32	2*x*	Outcrossing	0.710	0.036	0.101
			Selfing	0.675	0.099	0.281
		Neo-4*x*	Outcrossing	0.518	0.046	0.129
			Selfing	0.599	0.119	0.337

Appendix 3.2: Descriptive statistics for fruit set, seed set and seed germination, with mean, standard error of the mean (SE), standard deviation of the mean (SD) and sample size (n) being given for each cytotype and treatment.

Physiological traits	n	Ploidy level	Crossing treatment	mean	± SE	± SD
Relative Electrolyte Leakage pi)	32	2*x*	Outcrossing	0.014	0.002	0.006
			Selfing	0.023	0.003	0.009
		Neo-4*x*	Outcrossing	0.029	0.005	0.014
			Selfing	0.019	0.004	0.012
Relative Water Content (pi)	30	2*x*	Outcrossing	0.828	0.011	0.031
			Selfing	0.845	0.009	0.027
		Neo-4*x*	Outcrossing	0.853	0.020	0.052
			Selfing	0.920	0.029	0.076
Maximum quantum yield of photosystem II (F_v/F_m)	171	2*x*	Outcrossing	0.800	0.029	0.029
			Selfing	0.805	0.035	0.035
		Neo-4*x*	Outcrossing	0.815	0.020	0.020
			Selfing	0.815	0.031	0.031
Effective quantum yield of photosystem II (Φ_{PSII})	171	2*x*	Outcrossing	0.644	0.029	0.056
			Selfing	0.633	0.035	0.059
		Neo-4*x*	Outcrossing	0.633	0.020	0.046
			Selfing	0.659	0.031	0.058
Stomatal density (nº of stomata / mm^2)	82	2*x*	Outcrossing	206.930	10.383	47.580
			Selfing	223.269	9.118	40.775
		Neo-4*x*	Outcrossing	153.893	7.738	34.607
			Selfing	160.357	10.113	45.227
Stomatal length (µm)	82	2*x*	Outcrossing	29.889	0.399	1.828
			Selfing	29.275	0.571	2.552
		Neo-4*x*	Outcrossing	39.999	0.993	4.443
			Selfing	39.379	1.053	4.826