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Cytotype Associations, Ecological Divergence and Genetic Variation in the Apomictic Complex *Paspalum intermedium* Munro Ex Morong (Poaceae)

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Paspalum intermedium
Photo by Diego Hojsgaard

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ABSTRACT

Polyploidization (whole genome duplication – WGD) is a recurrent process in plants and provides greater potential for diversification. Neopolyploids in natural populations should go under substantial structural changes in their genetics, reproductive mode (e.g. apomixis – asexual reproduction via seeds), and ecological preferences to ensure their successful establishment. Apomixis in plants provides reproductive assurance, and superior colonizing abilities respect to sexuals, but it also constrains genetic variation and clonal plants are expected to have restricted adaptive capabilities. These complex rearrangement processes and adaptations in polyploid complexes are well reflected by their genetic variation. However, there is a lack of non-model systems that exhibit successful changes with pronounced reflection for studies. *Paspalum intermedium* is a grass species with diverging genetic systems (diploidy vs. autopolyploidy, allogamy vs. autogamy and sexuality vs. apomixis) with substantial ecological differentiation between cytotypes occurring in allopatry, sympatry and parapatry, hence provides an ideal platform to study polyploidization, apomixis and their ecological and genetic importance in plant evolution.

Therefore, in this thesis, I used *P. intermedium* as a model system to recognize the causality of biogeographic patterns, adaptation and ecological flexibility of cytotypes, to study variations in the expression of sexuality and apomixis, to analyze developmental competition between reproductive modes, and their effects on reproductive fitness, and to study genetic variation and its significance in polyploid complexes. I used chromosome counts, flow cytometry, and embryological analyses to characterize within-species genetic systems diversity. Environmental niche modelling was performed to evaluate intraspecific ecological attributes and to assess correlations among ploidy, and ecological conditions ruling species' population dynamics, range expansion, adaptation and evolutionary history. Proportions of sexuality and apomixis *in situ* were analyzed against local climatic conditions to study the influence of environmental factors on reproductive modes. Total seed set and germinability analyses were used to estimate the reproductive fitness. Analysis of genetic markers AFLPs was used to assess the genetic variation between and within cytotypes and within and among populations. To get insights into the genetic structure variation depending on the reproductive mode and how it explains the niche variation between cytotypes, the results were compared with the geographical distribution patterns and different ecological preferences of the cytotypes. My results show that the two dominant cytotypes of *P. intermedium* are non-randomly distributed along local and regional geographical scales and displayed niche differentiation. Polyploidy and contrasting reproductive traits between cytotypes have promoted shifts in niche optima, and increased ecological tolerance and niche divergence. Ecologically specialized diploids maintain cytotype stability in core areas

by displacing tetraploids, while broader ecological preferences and a shift from sexuality to apomixis favored polyploid colonization in peripheral areas promoting range expansion. The expression of sex and apomixis in tetraploid populations shows high variation both within and among populations. Even though ovule and seed analyses show apomictic development has higher competitive ability, fitness of apomictic individuals is depleted compared to sexual individuals and populations, indicating asexuality suffering higher seed abortion. Environmental modulation of reproduction was evident at population level where sex increased with higher mean diurnal range (MDR) while apomixis decreased. Thus, a *Tug of War* situation was identified between factors intrinsic to apomixis and environmental stressors promoting sex, suggesting a crucial role of local ecological conditions in sexual expression and adaptation of apomictic populations. Population structure analyses show that apomictic autotetraploids are of multiple independent origin. Although diploids show higher genetic variation, within and among population genetic variation equally make up the observed variation in all cytotypes. All individuals fall into three genetic clusters with substantial genetic admixture, and geographical distribution of genetic variation is in accordance with niche differentiation. The contact zone of the two cytotypes is primary in origin where tetraploids may frequently occur in mix ploidy populations. Polyploidization in *P. interemedium* is a recurring phenomenon and the newly arisen polyploids successfully establish themselves by acquiring enough genetic variation that allows them to adapt to new environments. Genetic variation analysis points to a slight deviation from the known *General Purpose Ghenotype* and the *Frozen Niche Variation* concepts as there is neither a common genotype nor are the diploids occupying a part of diploid sexuals' niche.

CHAPTER 1

1. INTRODUCTION

1.1. Polyploidization and Plant Evolution

Whole genome duplication (WGD), commonly referred to as polyploidy has been recognized as a major driving force of plant evolution; A phenomenon which was previously considered as evolutionary noise, unimportant to the main evolutionary processes, an evolutionary dead end (e.g. Stebbins 1950, Wagner 1970), and "blind alleys" (Arrigo and Barker 2012) leading studies to nothing else but stalemate. However, with the courtesy of new genomic and computational tools, recent studies show that not only polyploidy is recurrent but also more frequent than expected in nature (Soltis and Soltis 1999, 2000). It has been estimated that the formation of polyploids is relatively higher than the genetic mutation rate (Ramsey and Schemske 1998). Moreover, a crucial step of polyploidization, unreduced gamete formation (see below), was found to be occurring at a high rate of approximately 0.5% per gamete (Ramsey and Schemske 1998, Wood et al. 2009). Studies show that approximately 15% of plant speciation events resulted from polyploidy (Wood et al. 2009) and that polyploidy is substantially associated to higher plant diversity (Symonds et al. 2010, Jiao et al. 2011). For instance, extensive analysis of the *Arabidopsis thaliana* genome indicates two WGDs events in Brassicaseae and one triplication event shared in all eudicots (Vision et al. 2000, Barker et al. 2008). Furthermore, genome doubling is present not only in plants but also in other eukaryotes including yeast (Kellis et al. 2004) and other vertebrate and invertebrate groups (reviewed in Levin 2002, Gregory and Mable 2005).

1.1.1. Types of polyploidy and mechanisms of their formation

Two major types of polyploids have been recognized depending on their origin, which are characterized by the segregation pattern of chromosomes during meiosis: Allopolyploids arise through outcrossing of two closely related species (i.e. hybridization) and followed by chromosome doubling, autopolyploid originate from within species parents (e.g. genome duplication). A third type called segmental allopolyploids ranging between the major tow originates from parents with partially non-homologous chromosome arrangements where some chromosome regions between parents are homologous and others are not (Soltis and Soltis 2000). Bivalent formation at meiosis is characteristic of allopolyploids due to fixed (i.e. non-segregating) heterozygosity resulting from divergent parental genomes, as a result disomic inheritance operate at each locus. The autopolyploids are characterized by multivalent formation at meiosis as a result of polysomic inheritance. Irregularities such as univalent, trivalent and other

multivalent during meiosis is characteristic of segmental allopolyploidy (Soltis and Soltis 2000, Boff and Schifino-Wittmann 2003, Wu et al. 2004, Xu et al. 2013). Nevertheless, mechanisms of formation and post-polyploidization changes such as chromosome rearrangements and reshuffling of homologs and homeologs are unclear in all recognized polyploids (see Soltis et al. 2010). Studies demonstrated that autotetraploids are much more common in nature than previously expected (Soltis and Soltis 2000).

There are two largely recognized mechanisms explaining the formation of polyploids in natural population: i) one-step process involving the fusion of an unreduced egg with an unreduced pollen, ii) two-step process via a triploid bridge (Husband 2004) involving the fusion of a normal haploid gamete (e.g. haploid egg) with an unreduced gamete (e.g. unreduced/diploid pollen) forming a triploid, followed by the fusion of a triploid gamete (e.g. typically an unreduced egg cell from the triploid mother) with a haploid gamete (e.g. haploid pollen) (reviewed in Soltis et al. 2010). Nevertheless, despite recent findings, the frequency, dominance, and the importance of these two processes is still to be fully understood (reviewed in Hojsgaard 2018). In *Arabidopsis thaliana*, studies have characterized the gene (AtPS1) implicated in the formation of unreduced diplogametes and mutants in this gene lead to the generation of F1 triploids (D'Erfurth et al. 2008). Even though it is evident that the mechanisms of unreduced gamete formation are of preeminent importance to discern the polyploid formation, we are only starting to untangle the complex processes involved.

1.1.2. Evolutionary importance of polyploidy

Polyploidization events in natural populations, on the one hand, can act as a mechanism for instantaneous sympatric speciation, due to barriers that prevent gene flow between the new polyploid and the progenitor species (Hendry 2009). On the other hand it can lead to isolated individuals in a population destined for extinction due to reproductive isolation (Minority cytotype exclusion, Levin 1975), hence, polyploidization is a double-edged sword. Although newly arisen polyploids are most likely to go extinct after the emergence in a population even before they are detected (Ramsey and Schemske 1998, Soltis et al. 2010), once they reproduce and become locally established, and survive while adapting to different environments, they can achieve long-term evolutionary success (see Soltis et al. 2015). Recent studies demonstrated numerous ancient polyploidy and that all extant angiosperms have gone through at least one round of polyploidy (e.g. Jiao et al. 2011) (Figure 1.1). Furthermore, apart from ancient polyploidy, there is abundant evidence that polyploidy has been a major contributor for diverisification of many plant taxa (e.g. *Ranunculus* – Paun et al. 2006, Hörandl 2008; *Nicotiana* – Leitch and Leitch 2008; *Suaveolentes* – Marks et al. 2011; *Opuntia* – Majure et al. 2012; *Triticum* – Bordbar et al. 2011; *Viola* – Marcussen et al. 2012; *Salix* – Serapiglia et al. 2015; *Paspalum* - Quarin 1992).

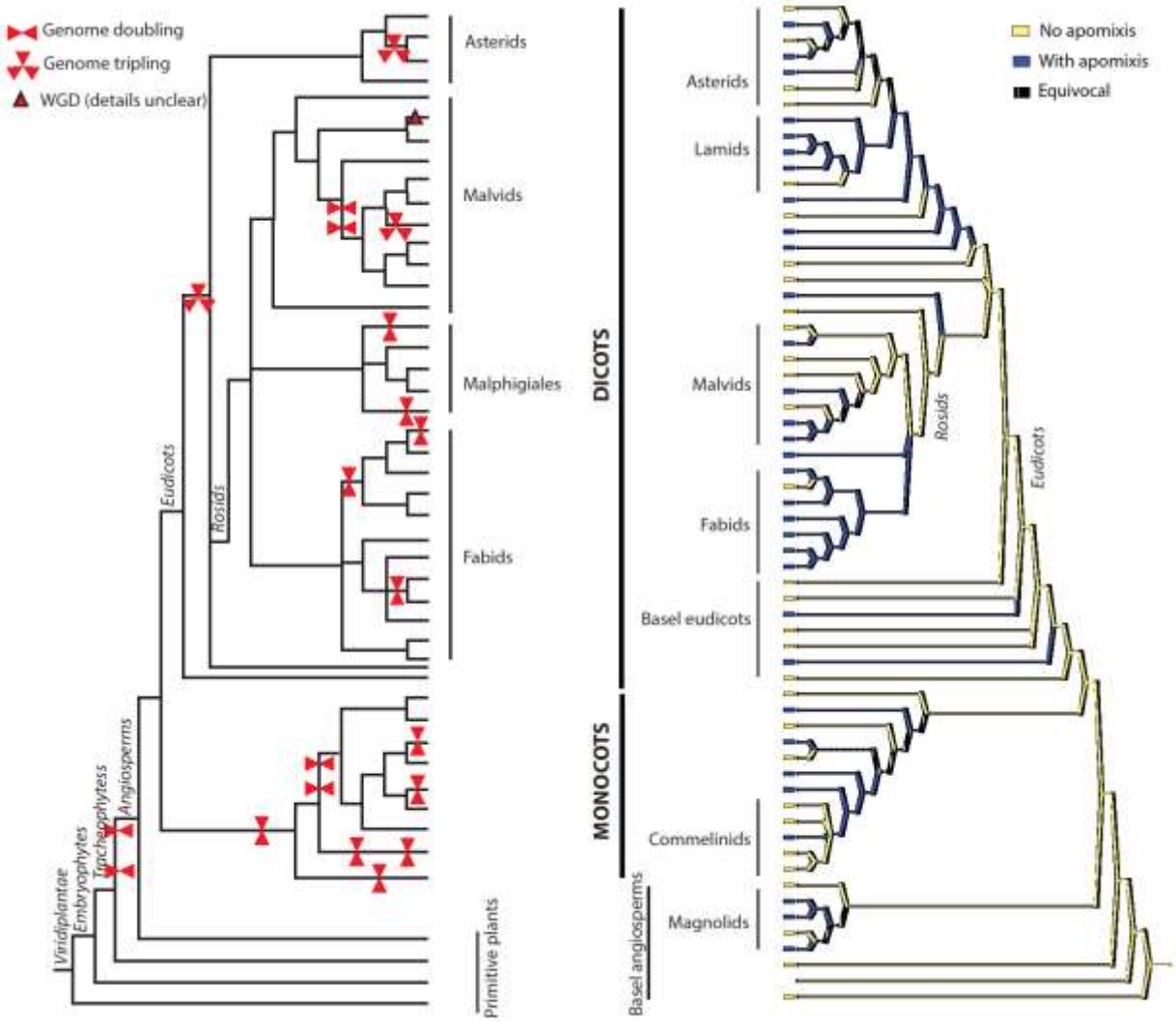

Figure 1.1. Phylogenetic trees showing (a) polyploidization events in plants, adopted from Campbell et al. (2016); (b) incidence of apomixis in angiosperms, adopted from Hörandl and Hojsgaard (2012). Branch lengths are not to scale.

Over the past few decades, it was evident from studies that polyploidy is highly dynamic and a recurrent process, even within the same population. Multiple origin of polyploids can maintain high genetic and genotypic variation, arising from different individuals of the diploid progenitor populations (e.g. Werth et al. 1985). However, the extent to which the variation is contained depends on several factors such as how much genetic variation of diploid progenitors passed on to the poplyploids and the eventual gene flow among different entities (e.g diploids to polyploids and among polyploids) (Soltis et al. 2010). Furthermore, there is compelling evidence that polyploid genomes are highly dynamic in its tendency for variation and genomic novelty (reviewed in Soltis et al. 2009). Polyploidization alters gene dosage and gene expression (Hegarty

and Hiscock 2005) which can lead to phenotypic changes such as self-compatibility thereby acquiring new features, for example the ability to colonize new marginal habitats (Stebbins 1950, Vogel et al. 1999, Pannell et al. 2004). Polyploid genomes can act as a "genomic playground" allowing new genomic and structural changes (e.g. mutations) and eventually fix them leading to trait innovations (see Madlung 2013, Soltis and Soltis 2016).

Polyploids can be predisposed to survival in new environments where they are preadapted to new habitats. For example, traits such as large cells and organs and slower rates of cell division in polyploids can affect polyploid populations' ecophysiology (Mcarthur and Sanderson 1999, Ramsey and Schemske 2002) resulting in superior adaptability to certain environmental conditions and adapting to new and harsh habitats and ecological differentiation (Baack 2005, Te Beest et al. 2012). Rigorous study of ancient polyploid genomes in model plants (e.g. *Arabidopsis thaliana, Brassica,* and Wheat) revealed that primary polyploids go through series of genomic modifications resulting in post-polyploid diploidization. These ploidy changes with chromosome rearrangements give rise to genomes that function like diploids' (reviewed in Mandáková and Lysak 2018). Post-polyploid diploidization is accompanied by a variety of processes (e.g. genome downsizing, loss/gain new gene functions, activation of transposable elements and epigenetic reprograming) for a successful diversification (e.g. Freeling 2009, Conant et al. 2014). Mandáková et al. (2010) and Mandáková and Lysak (2018) categorized polyploids into three groups, depending on the age of WGD and the diplodization rate as an attempt to study the evolutionary significance of different polyploidization events: paleopolyploids, mesopolyploids, and neopolyploids. The authors further stated that "Dysploidies (ploidy change) may lead to reproductive isolation among post-polyploid offspring and significantly contribute to speciation and cladogenetic events" (Mandáková et al. 2010, Mandáková and Lysak 2018), and they concluded demonstrating the importance of genetic and genomic studies on paleo-, meso-, and neo-polyploids to understand the role of dysploid changes preceded by polyploidization in genome evolution and speciation.

Intraspecific trait variation is known to affect the structure of the community, ecological opportunities and adaptive eco-evolutionary dynamics of the species (Bolnick et al. 2011, Wellborn and Langerhans 2015). In the case of trait variation associated with polyploidy, especially reproductive modes affects the plant physiology, ecological preferences and dispersal abilities and as a result altering the population density, species' niche preferences and the ecology of the plant community (Bolnick et al. 2011, Araújo et al. 2013). Therefore, it is essential to study such traits, especially in polyploid species, in oder to understand the local and regional population dynamics (Castro et al. 2012, Sonnleitner et al. 2016, Visger et al. 2016), to recognize the underlying mechanisms of species coexistance and evolutionary pathways, and to get insights into

ecological opportunity and adaptive diversification (Arrigo and Barker 2012, Wellborn and Langerhans 2015). During the past few years, there has been a rise of interest in the topic aided by new ecological modeling techniques studying the intraspecific diversity, especially in diploid-polyploid species (e.g. Raabová et al. 2008, Kirchheimer et al. 2016, 2018, Sonnleitner et al. 2016, Visger et al. 2016, Chumová et al. 2017, Paule et al. 2017). Nevertheless, our understanding of interploidy relationships, especially in terms of ecological divergence and opportunity is far from complete. Therefore, more comprehensive analyses focusing on intraspecific trait variations in relation to bioclimatic conditions are needed and essential to better understand the natural forces underlying plant adaptation and distribution in different regions of the world. Furthermore, the influence of the environmental factors (e.g. climate, stress) on one of the essential factors of polyploidization, the formation of unreduced gametes is yet to be fully understood (Ramsey and Schemske 1998). Therefore, it is unequivocally important to study the variation of geographical distribution to understand the the environmental impact on the formation of unreduced gametes thereby polyploids.

1.1.3. Cytotype contact zones and Mixed-ploidy populations

Geographic regions where polyploid hybrids and their diploid progenitors overlap, often referred to as Contact Zones, provide ideal platforms to study characteristics of the early stages of poplyploid establishment and to test hypotheses concerning dynamics and evolution of polyploid complexes (reviewed in Petit et al. 1999, Soltis et al. 2016). Moreover, contact zones are significantly important for testing biologically relevant questions regarding, for example the nature of interactions between cytotypes (e.g. competition), fine scale genetic variation, or the emergence of reproductive isolation and reinforcement mechanisms (Cosendai et al. 2013, Hopkins 2013, Sabara et al. 2013, Zozomová-Lihová et al. 2015) . In general, three important processes that takes place in cytotype contact zones have been discussed: a) reproductive restrains between cytotypes by sterile intermediate cytotypes (e.g. triploids), b) produce conditions for the establishment of new polyploids, and c) enhance the dynamics and further evolution of polyploid complexes (see Petit et al. 1999). Depending on the origin of the contact zone, they are categorized into two: i) primary – zones where the emergence of neopolyploids is within a diploid population (e.g. Castro et al. 2012), ii) secondary – zones where formerly allopatric diploids and polyploids come into contact. While primary zones are composed of genetically related individuals, secondary contact zones are mostly composed of individuals combining genetically distinct parental gene pools (e.g. Hardy et al. 2000, Weiss et al. 2002, Stuessy et al. 2004, Kolár et al. 2009). Even though the two processes seem exclusive from each other, there reports of both primary and secondary contacts occurring in the same zone (e.g. *Aster amellus* – Castro et al. 2012, *Knautia arvensis* – Kolár et al. 2009).

Distribution of cytotypes within contact zones are particularly relevant to understand the underlying evolutionary processes. Burton and Husband (1999) reported that the distribution of cytotypes in contact zones of *Galax urceolata* consisting mixed-ploidy populations is governed by a combination of genetic and ecological variables. Sympatric distribution of polyploids in mixed-ploidy populations in contact zones is regulated by ecological sorting in a heterogeneous physical environment (Husband and Schemske 1998). Fine scale shift of niche optima at contact zones and local adaptation to different ecological conditions propell the establishment of polyploid cytotypes in newly available habitats (e.g. Zozomová-Lihová et al. 2015, Kirchheimer et al. 2018), especially in the areas that were glaciated during the last Pleistocene (Bierzychudek 1987). Furthermore, fine scale-niche differentiation, phenological shifts and increased selfing are observed in zones of cytotype coexistence in sympatry as a result of coping coexistence (e.g. Felber-Girard et al. 1996, Petit et al. 1999, Soltis et al. 2016).

Despite overstated reproductive isolation of polyploidy from diploids, Stebbins (1971) pointed that gene flow can occur in two pathways: i) via sporadic hybrids forming triploids (also triploid bridge, Levin 2002) and eventually allowing gene flow from diploid progenitors to the polyploids, ii) via unreduced gametes (2n = 2x) formed in diploid progenitor populations followed by the crossed with reduced gametes (1n = 2x) of tetraploids. Both these pathways are unidirectional, allowing gene flow from diploids to higher ploidy although gene flow may occur in both directions (e.g. diploid *Betula nana* and tetraploid *B. pubescence* – Thórsson et al. 2001). Henry et al. (2005) stated that triploids of *Arabidopsis thaliana* can function as bridges between euploid types, hence mediate genetic link between diploids and tetraploids. Schinkel et al. (2017) using flow cytometry seed analysis showed that female triploid bridge via unreduced egg cell is a major pathway for polyploidization in *Runnunculus kuepferi,* allowing gene flow to polyploids. The observation of high percentage of mixed-ploidy populations in contact zones reaffirms these mechanisms of gene flow are relevant for plant evolution (e.g. Husband and Schemske 1998, Husband and Sabara 2003, Husband 2004, Cosendai et al. 2013). Therefore, detailed examination of dynamics of gene flow, genetic variation and mechanisms of polyploid generation in mixed-ploidy populations is unequivocally important to understand the early stages of polyploid establishment in nature.

Fine-scale analysis of patterns of genetic variability and gene flow are crucial to understand how independent formations of polyploid cytotypes shape the genetic structure and adaptation of plant populations. Such kind of fine-scale analysis require the study of both newly formed and recently established polyploid taxa in order to get a glimps on different times along the phases of polyploid evolution. Nevertheless, there is only a handful of known polyploid species formed recently (<500 years): *Spartina anglica* (Ainouche et al. 2004), *Tragopogon mirus* and

T.micellus (Ownbey 1950, Soltis et al. 2004), *Cardamine schulzii* (Urbanska et al. 1997), *Senecio cambrensis* and *S.eboracensis* (Abbott and Lowe 2004) that may bear genetic clues to formation of polyploids. Contact zones are a potential source of new polyploid formation and establishment at different stages. Therefore, it is indisputably important to study contact zones of different origin, ages, and with different underlying mechanisms of segregation.

1.2. Apomixis

Apomixis is a widely used term for asexual reproduction via seeds in flowering plants (Nogler 1984, Asker and Jerling 1992). After the initial discovery of apomixis in a higher plants (i.e. *Alchornea ilicifolia* – Smith, 1841), the term was vaguely used for all forms of asexual reproduction found in different plant groups. The current usage of the term is synonymous with "agamospermy," the formation of asexual seeds by a mechanism that avoids meiosis (apomeiosis) and fertilization of the egg cell (parthenogenesis), leading to asexual embryo development (Richards 1997). Apomixis is an effective form of asexual reproduction exploiting the benefits of seed dispersal (Mogie 1992). Studies on apomixis over the past decades revealed that this natural process plays a central role in plant evolution and diversification within apomictic systems (see Bicknell and Koltunow, 2004; Hojsgaard et al. 2014), apart from its potential utility in agricultural crop development (see Koltunow et al. 2013).

1.2.1. Mechanisms of apomixis

During sexual reproduction in angiosperms, a sequence of events must take place for viable seed production. i) Megaspore mother cell (MMC) differentiation followed by production of (three-) four megaspores (1n) via meiosis (megasporogenesis), ii) selection of one megaspore i.e. the subsequent programmed cell death of all but one megaspore, iii) The selected megaspore (1n) undergoes three mitotic divisions (megagametogenesis program) resulting in the development of an 8-nucleate embryo sac (ES) (one egg cell, two synergids at the micropylar end, two polar bodies that fuse to form a 2n central cell, and three antipodals at the chalazal end). Later when the flower opens and pollination occurs, iv) double fertilization (1. fusion of egg cell with one sperm, 2. fusion of central cell with the other sperm) takes place followed by embryo and endosperm development. Completion of all these processes is crucial in sexual seed production. This is the most common form of sexual ES formation observed in angiosperms, often known as the Polygonum type ES (Figure 1.2) (Willemse and van Went 1984); Other types bear varying number of reduced nuclei (e.g. less than four or ranging from 16-32) (Carman 1997). During apomixis, however, some of these processes are skipped or modified (Nogler 1984, Asker and Jerling 1992). Therefore, depending on the differential development, two major types of apomixis have been identified (Figure 1.2): Gametophytic – the seed embryo develops from an unreduced egg cell

without fertilization, and Sporophytic – the development of the embryo from a somatic cell (capable of embryogenesis without fertilization) of different tissues inside the ovule (e.g. the nucellus, integuments).

The sporophytic type of apomixis is also known as adventitious or nucellar embryony and they develop alongside sexual embryos. As in the sexual embryo development, adventitious embryony requires the formation of the endosperm. Therefore, the adventitious embryony utilizes the endosperm from sexual embryo sacs. As a result, adventitious embryony produces multiple embryos in a single seed, thus known as polyembryony. This type of apomixis is commonly seen in tropical trees and orchids (Naumova 1992).

Gametophytic apomixis is divided into two major developmental pathways, based on the cell type that gives rise to the ES: i) Apospory – the unreduced ES develops from a somatic cell in the nucellus of the ovule called aposporous initial cell (AI). The differentiation of AIs can occur at various times of ovule development. As a result, one meiotically produced ES and one or more aposporous ESs can coexist in the same ovule. Apospory can be further divided into two types: a) the Hieracium type – produces an unreduced eight nucleate ES cytologically similar to sexual Polygonum type; b) Panicum type – produces an unreduced four nucleate ES commonly without antipodals. ii) Diplospory – MMC gives rise to the unreduced ES where the MMC undergos restitutional meiosis or mitotic-like division. Diplospory is also further divided into two: a) meiotic diplospory – the MMC begins meiosis but does not complete, instead a restitution meiosis takes place followed by mitosis (e.g. *Erigeron annuus*); b) mitotic diplospory – the MMC undergoes direct mitosis without entering meiosis (e.g. *Tripsacum dactyloides*). Mitotic diplospory is the most common type of diplospory observed in plants (Nogler 1984, Crane 2001).

The endosperm formation is essential for embryo development in higher plants, except for a few plant groups that seek other forms for acquiring nutrition (e.g. Orchids). This is achieved either by fertilization of the polar nuclei in the central cell by a sperm nucleus of the pollen grain (pseudogamy), or it can develop independently (autonomous development). In sexual ovules, the two central cells in the ESs are fertilized by a reduced pollen nucleus (sperm), maintaining a maternal to paternal genome ratio of 2:1. Interestingly, while deviations of any kind in the paternal contributions to the formation of the endosperm in Polygonum type ESs will drastically alter the development of the endosperm and a viable seed, in apomictic plants, ESs are cytologically and anatomically different and hence male and female contributions are asymmetric but deviations to the 2:1 paternal contribution to the endosperm are tolerated (Talent 2009). In Panicum type apomixis, the central cell is formed by only one unreduced maternal nucleus (2n=4x or higher), which is often fertilized by a reduced (n) sperm allowing the maternal and paternal

ratios of 2:1. However, in most apomicts, the central cell is formed by two unreduced maternal nuclei which might fuse before fertilization by a reduced pollen nuclei resulting in an endosperm nucleus with a 4:1 maternal to paternal genome ratio.

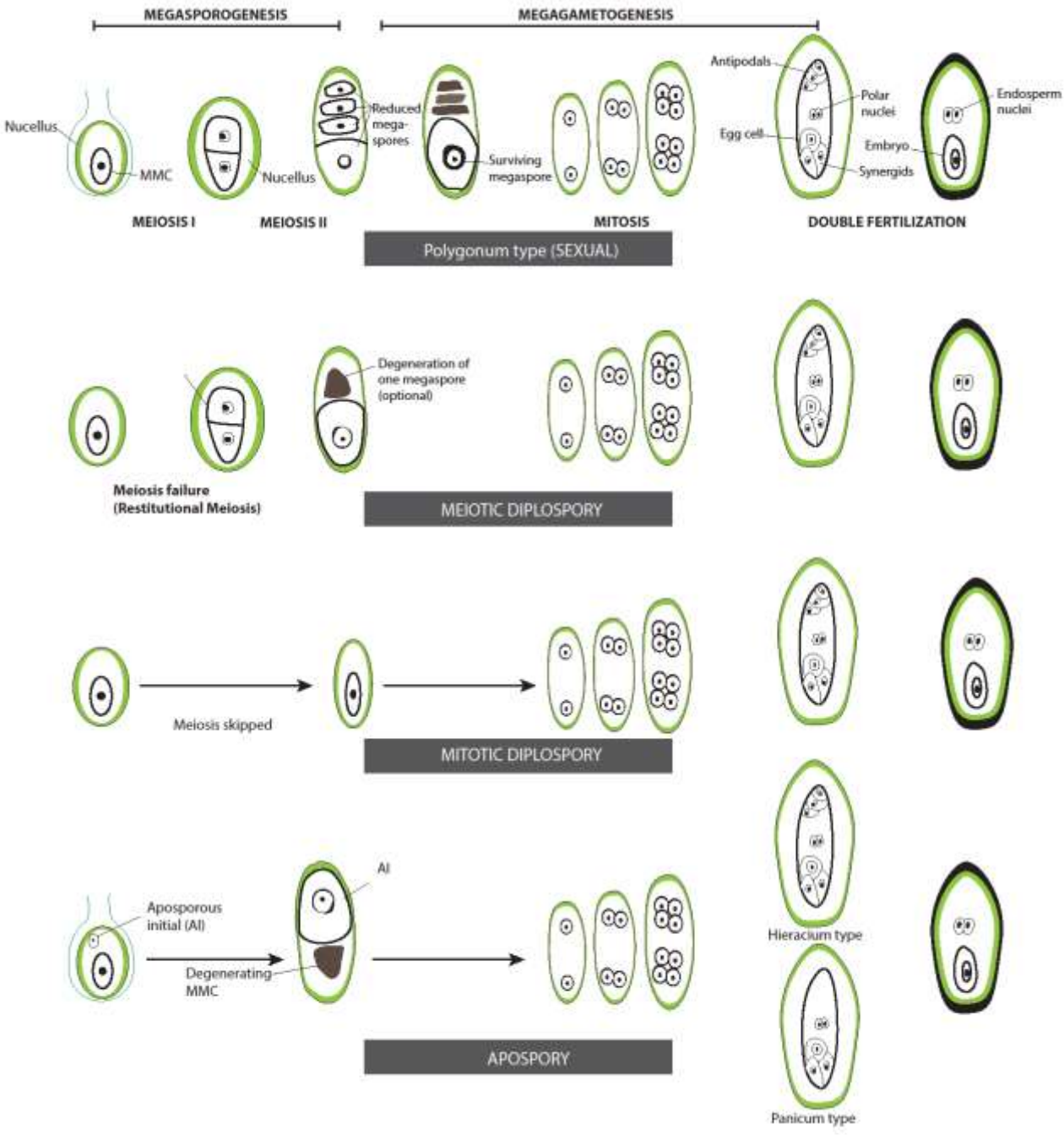

Figure 1.2. The mechanisms of apomixis, mitotic/meiotid diplospory and apospory compared with Polygonum type sexual embryo sac development. The illustration is primarily based on Nogler (1984). The common pathways are aligned in the same verticle line. Sporophytic apomixis (adventitious embryony) is not shown.

1.2.2. Genetic control of apomixis

Apomixis is a heritable trait and can be expressed facultatively with sex (Ozias-Akins and van Dijk 2007). However, the genetic control of apomixis is still poorly known despite the increased interest. Considering different apomictic mechanisms and the occurrence of apomixis in angiosperm families (discussed below), it is evident that apomixis exhibits multiple independent origin (Carman 1997, van Dijk and Vijverberg 2005).

Apomixis was previously thought of a consequence of polyploidy. This however does not explain the existence of non-apomictic polyploids (Carman 1997) although studies have pointed the strong connection of polyploidy and hybridization to activate a switch from sex to apomixis in plants (Hörandl and Hojsgaard 2012, Lovell et al. 2013). Nevertheless, studies have attempted to decipher complex nature ofthe genetic control of apomixis. Apomixis in flowering plants has been shown to be inherited as a dominant trait (reviewed in Hand and Koltunow, 2014). Although earlier studies suggested that apomixis is controlled by a single dominant locus, later, it was found that several developmental components of apomixis in some taxa are controlled by independent loci (e.g. *Taraxacum* – van Dijk et al. 1999, *Poa* – Albertini et al. 2001, *Hieracium* – Catanach et al. 2006, *Hypericum* – Schallau et al. 2010, *Cenchrus* – Conner et al. 2013). In *Hieracium praealtum,* the deletion of *LOA* (*LOSS OF APOMEIOSIS*) or *LOP* (*LOSS OF PARTHENOGENEIS*) loci reactivated the sexual pathway (Catanach et al. 2006), implying that apomixis in *Hieracium* is superimposed on the sexual pathway. Therefore, Koltunow et al. 2013 stated that apomixis is not completely independent of the genetic control of the sexual pathway. This is supported by the observation that apomixis and sexuality are not exclusive and they coexists. This rather seems different in the case of diplospory because sexual pathway is altered in a way that the meiosis is not completed (Rodrigues et al. 2010), rather than having a completely independent control of apomixis.

The suppression of recombination, frequently found around the apomixis loci, poses a challenge to identify apomictic loci. This recombination frequency distortion in many apomictic species, points to the assumption of increased divergence of alleles involved in apomixis. The hemizygosity of the apomixis associated loci has been found to be causing such divergence in some extreme cases e.g. *Hieracium* – (Okada et al. 2011) and *Pennisetum* – (Akiyama et al. 2004). In other instances, apomixis loci have been associated to heterochromatin e.g. *Pennisetum squamulatum* (Akiyama et al. 2004) and/or with increased repetitive or transposon-rich genome regions e.g. apospory-specific genome region (ASGR) in *Pennisetum, LOA* in *Hieracium*, and apomictic controlling locus (*ACL*) in *Paspalum*) (Calderini et al. 2006). As a result, much attention has been given to these repetitive sequences as they may explain the deviation of sexual pathway by rearrangement of the repetitive regions enabling apomixis

(Koltunow and Grossniklaus 2003). However, this was not evident in progeny tests of crosses between *Hieracium pilosella* and *H.praealtum* when tested with *LOA*-linked markers as they lacked the repetitive structure (Kotani et al. 2014). This has led to the hypothesis that chromosomal restructuring and recombination degree presumably are an indication of the age of apomixis in a species (reviewed in Hand et al. 2014). Nevertheless, while most of the studies concluded without much luck with identifying apomictic genes, a few led to the identification of several candidate genes with a potential role in the induction and maintenance of apomixis (see Koltunow et al. 2013, Hand and Koltunow 2014).

Rapid development of transcriptomic analysis has also allowed numerous studies to assess gene expression in apomixis. Comparative analysis of gene expression has revealed massive differential expression of genes, including genes putatively responsible for apomictic and sexual pathways, (Albertini et al. 2005, Polegri et al. 2010, Sharbel et al. 2010, Okada et al. 2013, Ortiz et al. 2017). However, aforementioned studies have not been yet able to identify a master candidate gene for apomixis. Further, studies have identified apomeiosis-like phenotypes in mutants that replace meiosis with a mitosis and thus, they mimick apomeiosis (e.g. *MiMe – Mitosis instead of Meiosis* in *Arabidopsis thaliana* – D'Erfurth et al. 2008). In a different study, inactivation of DNA methyltransferase in maize produced diploid gametes and multiple ES (Garcia-Aguilar et al. 2010), suggesting epigenetic influence of the regulation of sexual and apomictic pathways (see Kumar 2017).

Recent studies have identified environmental conditions to be an important factor that may directly influence the reproductive pathway (e.g. Knight et al. 2006, Liu et al. 2011). In this regard, environmental stress (e.g. temperature fluctuation, drought, etc.) has been pointed out as a key environmental factor that affects both sexual and apomictic pathways (Gounaris et al. 1991, Rodrigo et al. 2017, Klatt et al. 2018). There are several reports of increase in sexuality in facultative apomictic plants under stress conditions (Carman et al. 2011, Mateo De Arias 2015) as well as vice versa; For instance, drought conditions increased the production of sexual ES in *Boechera* (Mateo De Arias 2015) while cold treatments increased the apomictic seed formation in *Ranunculus kuepferi* (Klatt et al. 2018). Nevertheless, apart from a handful of studies, our understanding of the environmental influence on the modulation of reproductive pathways within apomictic complexes is far from being satisfactory.

1.2.3. Importance of apomixis

Apomixis in flowering plants is tightly linked to polyploidy even though the mechanisms from which they arise are not necessarily similar. Most interestingly, sexual counterparts of the same or closely related taxa are usually diploids (Asker and Jerling 1992, Koltunow 1993). Apomixis coupled with polyploidy not only provides reproductive assurance to

polyploids by aiding them to overcome minority cytotype disadvantages (Levin 1975), but also enhances dispersal colonizing new habitats, and reinforce founder events (Baker 1955). Simulation of reproductive mode in apmictic complexes showed that a switch to apomixis overemphazises the superiority of polyploids in their colonizing abilities (e.g. Kirchheimer et al. 2018). Due to the avoidance of meiosis, apomixis counteracts genetic drift and maintain higher heterozygosity (Paun et al. 2006, Cosendai et al. 2013). Furthermore, processes such as, spontaneous mutation, genetic restructuring, and residual sexuality introduces additional genetic variation to apomictic populations (Hörandl and Paun 2007, Hojsgaard and Hörandl 2015), further diversifying the apomictic taxa. It has been also suggested that apomicts can reverse to obligate sexuals (Carman 1997, Hörandl and Hojsgaard 2012, Hojsgaard and Hörandl 2015) and they may diversify more rapidly than their sexual diploid progenitors as they accumulate new traits with genomic rearrangements along the way and expanded distribution allowed by superior colonizing abilities of pro-apomictis (Soltis et al. 2016, Mandáková and Lysak 2018).

Many studies on apomixis have and are being focused on its potential utility in crop development as it is, not only a convenient mechanism of clonal propagation via seeds but also it has shown to maintain hybrid vigor in progenies over generations (reviewed in Koltunow 1993, Bicknell and Koltunow 2004, Ortiz et al. 2013, Kumar 2017). Nevertheless, the focus of my project is to assess the evolutionary consequences and importance of apomixis.

Over the years since the first description of apomixis, studies continuously found apomixis in different plant taxa increasing the number of species, genera and families containing apomixis. Stebbins (1941) – 23 families, 44 genera; Asker and Jerling (1992) – 108 genera, Naumova (1992) – 116 genera and Carman (1997) – 222 genera are the major revisions along the history. Previously it was hypothesized that a predisposition of apomixis occurring in three large families: Asteraceae, Poaceae, and Rosaceae (Carman 1997, Richards 1997, Ozias-Akins and van Dijk 2007). However, in the last comprehensive study on the occurrence and distribution of apomixis in angiosperms, Hojsgaard et al. (2014) reported the presence of apomixis in 73 families (19% of all described plant families) and 293 genera (ca. 2.2% of all plant genera), and showed that apomixis is scattered among all angiosperms thus founding is no support for a "predisposition hypothesis". The most common type of apomixis was adventitious embryony, found in 148 genera, followed by apospory (110 genera) and diplospory (68 genera). Interestingly, combinations of all three types of apomixis occur in several genera (ca.17 genera). Furthermore, their study also showed that total numbers of genera in families were highly correlated to the frequency of apomictic-containing genera, suggesting that apomixis is associated to biodiversity (see Hojsgaard et al. 2014). A comparison of the distribution of polyploidy and apomixis among major plant groups is illustrated in the Figure 1.1. Despite the considerable lack of studies on

apomixis in plants covering all regions and climatic zones, apomixis appear to occur in all climatic zones of the earth, including Arctic (except in Antarctic) and provides a clear advantage for exploiting new habitats, environments and niches (Carman 1997, Whitton et al. 2008, Tucker and Koltunow 2009, Hojsgaard et al. 2014b, Firetti 2018).

Another phenomenon associated with apomixis is *geographical parthenogenesis*, where asexuals exhibit a wider distribution than their sexual progenitors (Hörandl 2006, 2008). This is commonly seen along latitudinal gradients and previously glaciated areas (Kearney 2005, Hörandl 2008). In this regard, it is argued that the ability of asexual plants to found a new population (uniparental reproduction) is a major advantage (Mogie et al. 2007, Hörandl 2009). Superior colonizing abilities of apomictic taxa combined with polyploidy have allowed them to spread into new habitats and occupy novel niches, acquiring broader distributions and species expansion (Chapman et al. 2003, Suda et al. 2004, Brochmann et al. 2004, Hörandl 2006, 2008, Soltis et al. 2010). Three of many hypotheses on geographical parthenogenesis have been often tested: i) General Purpose Genotype model – this model assumes that a highly flexible genotype emerges as a result of heterogeneous environmental conditions (Lynch 1984), ii) The Frozen Niche Variation model suggests that different apomictic descendants produced by sexual hybridization freezes a part of the genetic, genotypic and the niche variation of the parents (Vrijenhoek 1994), iii) The Baker's Law is based on the assumption that plant characteristics such as selfing and apomixs, that enhance uniparental reproduction and founder events will maintain superior colonizing abilities and range expansion (Baker 1955, 1967). These three major concepts have received both positive and negative support from studies. I also test these hypotheses and draw inferences on the geographical parthenogenesis observed in my model system.

Although the evolutionary significance of geographical parthenogenesis is not well understood yet, it is often seen as a consequence of the short term success provided by asexuality (e.g. Van Dijk, 2003). It has been also suggested that parthenogenesis in several cases may have more of a secondary role stabilizing strongly selected hybrid genotypes; hence parthenogenesis rather conveys the role of hybridization than sex *per se* (Kearney 2005). In contrast, formation of autopolyploids accompanied by apmixis have helped the range expantion and niche divergence allowing the species to occupy new habitats which otherwise would have been unavailable (e.g. Cosendai et al. 2013, Kirchheimer et al. 2018). Despite the increased interest and enormous efforts by researchers to understand all possible causes and consequences of apomixis, our understanding of the complex dynamics of apomixis and its advantages in plant diversification both in short term and the long run remain unclear.

1.3. *Paspalum intermedium* Munro ex Morong

The species of my model system in this project is *Paspalum intermedium*, a perennial Panicoid grass of the genus *Paspalum* L., one of the ten largest genera within Poaceae, with a centre of origin in tropical South America (Zuloaga and Morrone 2005). The genus is a well-known model system for biosystematics and reproductive biology studies (e.g. Quarin 1992, Giussani et al. 2009, Rua et al. 2010, Ortiz et al. 2013). Cytogenetic evaluation of different accessions shows that ploidy levels in *Paspalum* species range from diploid to hexadecaploid (2x - 16x) (e.g. Honfi et al. 1990, Pagliarini et al. 2001, Hojsgaard et al. 2009). In *P. intermedium* we found two cytotypes with contrasting reproductive modes occurring in nature, sexual self-sterile diploids (2n = 2x = 20) and apomictic self-fertile auto-tetraploids(2n = 4x = 40) (diploids and), intermingled in sympatry, parapatry or allopatry (Burson and Bennett 1970, Norrmann et al. 1989).

The centre of diversification of *P. intermedium* is considered to be the neo-subtropics, where they inhabit marshy grasslands in diverse phytogeographic formations along ecological gradients in Argentina, Paraguay, Bolivia and Brazil (Zuloaga et al. 2012). Overall, the species occupy a wide range of ecological and climatic gradients (e.g. latitudinal gradient) in Sub-tropical and temperate regions of Argentina (Zuloaga et al. 2012, Karunarathne et al. 2018).

Therefore, *P. intermedium* not only provides a unique venue for testing various hypotheis on polyploidization, its consequences and geographical parthenogenesis but also serves as an ideal model system for studying cytotype coexistence, ecological and biological factors governing intraspecific trait variation along climatic, geographic and ecological gradients, population dynamics and adaptation at local and regional geographic scales. Previous studies have demonstrated the utility of the species as a convenient non-model plant for such studies; For instance,

i. Meiotic and apomeiotic processes had been well-characterize by embryology, cytogenetic and molecular studies. Most studies shows apospory as main type of functional apomixis found in tetraploids (e.g. Martínez and Quarín 1999, Martínez et al. 2001, Hojsgaard et al. 2008). On the other hand, anatomical features of meiotic and apomictic embryo sacs are different, which makes it easy to identify and calculate relative proportions of functional reproductive pathways (e.g. Hojsgaard et al. 2013). Furthermore, specific structure of embryo sacs observed in the species allow for discrimination of reproductive origin of seeds and functional reproductive pathways by Flow Cytometry (e.g. Hojsgaard et al. 2013), hence, *P. intermedium* is a convenient model species to study not only the reproductive biology of polyploid complexes but also the competition and the environmental influence on the reproductive success of different cytotypes with varying reproductive pathways.

ii. The species exhibit divergent reproductive systems and cytotypes (i.e. allogamy vs. autogamy, sexuality vs. apomixis and diploidy vs. polyploidy) which has been reported as substantial contributors for the genetic diversity of plants that facilitate ecological diversification and evolutionary potential of species and plant communities (Tilman and Lehman 2001, Pauls et al. 2013, Allan et al. 2015); more on ecological and niche divergence in *P.intermedium* is published under this project (Karunarathne et al. 2018 – presented in Chapter 2).

iii. The tetraploids are autopolyploid (Norrmann et al. 1989), thus avoid potential suppression of alleles due to genomic asymmetry after hybridization (e.g. Feldman et al. 2012). Further, the polyploid complex is also relatively new in terms of evolutionary time compared to other systems (Hojsgaard et al. 2009, Karunarathne et al. 2018), which represents an ideal opportunity to examine ecological and evolutionary mechanisms acting upon natural populations, like ecological niche divergence and sources of genetic variation, cytotype coexistence and recurrent polyploidy.

1.4. Aims of the Project

In this thesis project, I conducted a comprehensive and multidisciplinary analysis of intraspecific cytotype associations, ecological and niche divergence, reproductive pathway variation and genetic diversity among different genetic systems and cytotypic associations using *Paspalum intermedium*.

The first part of the study focused on deciphering natural factors and stressors governing intraspecific trait diversity, cytotype coexistence and their dynamics within and among populations. Therefore, in Chapter 2, I focus on the analysis of Niche divergence, changes in phenology and reproductive strategies between cytotypes to discern ecological consequences of polyploidy (Chapter 2). In chapter 3, I present a thorough population level analysis focusing on the influence of environmental factors (e.g. bioclimatic variables) on the expression of apomixis and meiosis. (Chapter 3).

In chapter 4, I present a study of population structure of polyploids in comparison to their diploid progenitors to assess the genetic variation within and among populations and between cytotypes (Chapter 4) since such studies in various systems (e.g. old and new polyploid complexes) have shown significant importance in terms of understanding the evolutionary history where it can generate a snapshot of the ancestor diploids and provide a fine scale resolution of the origin of different ploidy levels, as well as providing clues on the fate of the neopolyploids. Furthermore, it can also provide information on various factors that shape the distribution patterns observed in plants; for example, the influence of environmental factors and spatial separation on the coexistence and establishment of the polyploid complexes. In this regard, studies on newly

established polyploids or in the process of establishment such as *P. intermedium* are of increased importance as they can provide crucial insights into the genetic processes that take place soon after and/or during the process of establishment of polyploidization. Findings were also useful to recognize patterns of polyploid formation and dispersal and to evaluate the proposed hypotheses explaining the mechanism to geographical parthenogenesis.

Furthermore, following specific objectives are addressed in different chapters.

Chapter 2 – In this chapter, I (i) evaluate the natural prevalence of *P. intermedium* cytotypes at various spatial scales; (ii) evaluate reproductive and phenological shifts; (iii) assess climatic and ecological preferences between cytotypes of *P. intermedium*; (iv) determine the presence of singular ecological and biological signals driving cytotype distribution and dominance; and (v) provide evidence of niche differentiation between cytotypes and further insights into natural stressors governing the dynamic of cytotype associations, geographic displacement and range expansions that contribute to local adaptation and ecological opportunity.

Chapter 3 – The main objectives of the chapter 3 was to (i) assess the varied expression of apomixis in population level, (ii) get insights into the competition between meiotic and apomictic pathways in facultative apomictic complexes, (iii) evaluate the fitness levels of each reproductive mode in terms of fertility, (iv) examine the spatial and temporal variation of apomixis, and (v) determine the ecological and environmental influence on the expression of apomixis.

Chapter 4 – In the chapter 4, I present the findings obtained using *P. intermedium* with flow cytometry, genetic marker AFLPs, and ecological and geographical data, (i) to assess the genetic structure of populations of two cytotypes showing niche divergence, (ii) to determine the origin and genetic variability of within and among autotetraploid populations, (iii) to examine the genetic composition of mix ploidy and contact zone populations, and finally (iv) to draw inferences on the distribution patterns and ecological amplitude of the two cytotypes based on the genetic variability.

2. DISTRIBUTION PATTERNS AND ECOLOGICAL DIFFERENTIATION IN DIFFERENT CYTOTYPES OF *PASPALUM INTERMEDIUM*

This chapter presents the results of cytotype determination, regional and local assemblage patterns and niche differentiation of the polyploid complex *P. intermedium*. The findings are published in the research article ***Karunarathne et al., 2018. Intraspecific ecological niche divergence and reproductive shifts foster cytotype displacement and provide ecological opportunity to polyploids. Annals of Botany 121: 1183–1196***.

2.1. ABSTRACT

Niche divergence between polyploids and their lower ploidy progenitors is one of the primary mechanisms fostering polyploid establishment and adaptive divergence. However, within-species chromosomal and reproductive variability have usually been neglected in community ecology and biodiversity analyses even though they have been recognized to play a role in the adaptive diversification of lineages. I used *Paspalum intermedium*, a grass species with diverging genetic systems (diploidy vs. autopolyploidy, allogamy vs. autogamy and sexuality vs. apomixis), to recognize the causality of biogeographic patterns, adaptation and ecological flexibility of cytotypes. Chromosome counts and flow cytometry were used to characterize within-species genetic systems diversity. Environmental niche modelling was used to evaluate intraspecific ecological attributes associated with environmental and climatic factors and to assess correlations among ploidy, reproductive modes and ecological conditions ruling species' population dynamics, range expansion, adaptation and evolutionary history. Two dominant cytotypes non-randomly distributed along local and regional geographical scales displayed niche differentiation, a directional shift in niche optima and signs of disruptive selection on ploidy related ecological aptitudes for the exploitation of environmental resources. Ecologically specialized allogamous sexual diploids were found in northern areas associated with higher temperature, humidity and productivity, while generalist autogamous apomictic tetraploids occurred in southern areas, occupying colder and less productive environments. Four localities with a documented shift in ploidy and four mixed populations in a zone of ecological transition revealed an uneven replacement between cytotypes. Polyploidy and contrasting reproductive traits between cytotypes have promoted shifts in niche optima, and increased ecological tolerance and niche divergence. Ecologically specialized diploids maintain cytotype stability in core areas by displacing tetraploids, while broader ecological preferences and a shift from sexuality to apomixis favoured polyploid colonization in peripheral areas where diploids are displaced, and fostered the ecological opportunity for autotetraploids supporting range expansion to open southern habitats.

2.2. INTRODUCTION

Polyploidization events in plants have been recurrently associated with niche divergence and ecological differentiation of cytotypes as an important mechanism for the establishment of new polyploids in nature. This facilitates both the maintenance of intraspecific cytotype diversity as well as sympatric speciation events among closely related taxa (Soltis et al. 2004, Schluter 2009, Givnish 2010, Glennon et al. 2014, Anacker and Strauss 2014, Visger et al. 2016). Thus, polyploidy alters the ecological niche of a species by broadening environmental tolerance and providing ecological and evolutionary flexibility (e.g. Dubcovsky and Dvorak 2007, Fawcett et al. 2009). Although there are many concepts of 'niche' (Soberón and Nakamura, 2009), the Grinnellian niche, defined by the sub-set of scenopoetic (non-interactive) environmental conditions under which populations of a species have positive growth rates (Grinnell 1917, Soberón 2007), is the one extensively used in recent years. This concept has become popular also because data for niche-defining variables (e.g. topography, average temperature, solar radiation, precipitation, etc.) are progressively becoming available for the entire planet (e.g. Turner et al., 2003). At present, increasing availability of public databases [e.g. the Global Biodiversity Information Facility (GBIF); WorldClim] and information gateways [e.g. Geographic Information Systems (GIS)], and a renewed interest in plant polyploidy allow modern biogeography to use mathematical models [species distribution modelling (SDM)/ ecological niche modelling (ENM)] (e.g. Elith and Leathwick 2009, Soberón 2010) to better understand how polyploidy and associated features influence niche evolution, habitat suitability and organism distributions.

Natural intraspecific trait variation associated with polyploidy, in particular reproductive modes, is widely known to affect plant physiology, ecology and dispersal abilities. Experimental studies indicate that intraspecific trait variation can have a significant effect on community ecology (Bolnick et al. 2011, Araújo et al. 2013). Intraspecific trait variation is expected to alter population density, niche breadth and the strength of the interaction among phenotypes, affecting the structure of the community, ecological opportunities and adaptive eco-evolutionary dynamics (Bolnick et al. 2011, Wellborn and Langerhans 2015). The study of traits such as cytotype diversity, dispersal ability, phenology, different reproductive modes and associated environmental signals is essential for understanding local and regional population dynamics (e.g. Castro et al. 2012, Sonnleitner et al. 2016, Visger et al. 2016), provides insights into evolutionary pathways and forces driving species coexistence, ecological opportunity and adaptive diversification (Arrigo and Barker 2012, Wellborn and Langerhans 2015). For example, reproductive shifts toward self-fertility and apomixis (asexual reproduction via seeds) are frequently linked to polyploid cytotypes (Asker and Jerling 1992, Robertson et al. 2010) and drastically affect species' dispersal abilities and distribution patterns. Such features provide

reproductive assurance to polyploids by enabling them to overcome density-dependent reproductive limitations (e.g. minority cytotype disadvantage; Levin 1975) and gamete incompatibility (Asker and Jerling 1992, Hojsgaard et al. 2014b), and facilitate 'founder events' (e.g. Baker's Law; Baker 1955). Consequently, polyploids with better colonizing abilities may capitalize on ecological opportunities, achieve wider distributions leading to phenomena such as geographical parthenogenesis (e.g. Hörandl 2006, Vrijenhoek and Parker-Jr. 2009) and enhance diversification abilities via reversals to sex (Hojsgaard et al. 2014a, Hojsgaard and Hörandl 2015).

Despite the increased interest in the topic, studies examining intraspecific diversity and modelling ecological divergence in diploid–autotetraploid species (e.g. Visger et al., 2016), diploid–allopolyploid species (e.g. Sonnleitner et al. 2016) or other diploid–polyploid associations (e.g. Raabová et al. 2008, Chumová et al. 2017, Paule et al. 2017) are just starting to be feasible as high-resolution climatic data sets are becoming available. More studies carrying comprehensive analyses of intraspecific traits and bioclimatic conditions are needed and essential to better understand the natural forces underlying plant adaptation and distribution in different regions of the world. Here, we utilize the grass species *Paspalum intermedium* Munro ex Morong to decipher natural factors and stressors governing intraspecific trait diversity, cytotype coexistence and their dynamics within and among populations. Niche divergence and changes in phenology and reproductive strategies that may provide a platform for ecological opportunity are also studied to discern ecological consequences of polyploidy.

In this part of the study, I (1) evaluate the natural prevalence of *P. intermedium* cytotypes at various spatial scales; (2) evaluate reproductive and phenological shifts; (3) assess climatic and ecological preferences between cytotypes of *P. intermedium*; (4) determine the presence of singular ecological and biological signals driving cytotype distribution and dominance; and (5) provide evidence of niche differentiation between cytotypes and further insights into natural stressors governing the dynamic of cytotype associations, geographic

2.3. MATERIALS AND METHODS

2.3.1. Sampling sites and collection of plant materials

Plant materials were collected from Eastern Gran Chaco, Central and Northern Mesopotamia (core distribution areas of the species), and Northern Pampas and Western Gran Chaco (peripheral distribution of the species) in Argentina (Table 2.1; Figure 2.1; Supplementary Data Table S2.1) (see Zuloaga et al. 2012). Additional information on cytotype occurrences was gathered from the literature and from material examined at different herbaria (MNES, CTES, BAA, SI, B, GOET, HUH and PE) (acronyms follow Thiers 2017) (see Supplementary Data Table S2.2).

Sampling was done during two different time periods (November/December and February/ March) to avoid seasonal bias on cytotype frequencies and evaluate phenological differentiation between cytotypes. Changes in phenology were evaluated by grouping observations into early (October–December) and late (January–March) flowering followed by testing for independence. Collection sites were categorized into (1) sites for ploidy determination only (up to three individuals were collected) and (2) sites to evaluate cytotype diversity and dynamics at the population level (on average 30 individuals per population were collected) (Supplementary Data Table 1). For the latter, sampling sites were selected to attain a maximum representation of the distribution range of the species (i.e. North–South and East–West), and include both macro-scale (among populations) and micro-scale (within populations) trends. Overall, samples were collected from 75 localities, out of which 35 were selected for population evaluations (Table 2.1; Supplementary Data Table S2.1). A transect spanning the longest length available across the population was followed to obtain information on local dispersal of cytotypes. An even representation of individuals within the population was attained by uniform sampling (i.e. the distance between two consecutive individuals was maintained the same, and varied between 4 and 12 m depending on the spatial dimensions of each population). Young (i.e. smaller bushes with a diameter <40 cm with no or a few flowering stems) and mature individuals (i.e taller bushes with a diameter >60 cm with many flowering stems) were distinguished and collected to account for individual turnover and overlapping generations. Several vouchers from all locations were prepared and deposited at different herbaria (CTES, MNES, BAA and SI).

Table 2.1. Summary of ecoregions, collection sites, number of individuals and ploidy levels of the *P. intermedium* plants analysed.

Geographic region	Number of collection sites for		Total n° of individuals	Ploidy (*x*=10)
Mesopotamia	populations	5	119	2*x*
		17	460	4*x*
	solitary individuals	4	5	2*x*
		6	10	4*x*
Gran Chaco	populations	10	301	2*x*
		10	286	4*x*
	solitary individuals	6	6	2*x*
		6	6	4*x*
Pampas	populations	-	-	2*x*
		1	30	4*x*
	solitary individuals	-	-	2*x*
		1	1	4*x*
Total		68	1224	

2.3.2. Assessment of ploidy and reproductive trait variation

The ploidy level of each sample was determined by flow cytometry (FC) estimations of relative nuclear DNA contents in comparison with a P. intermedium plant with known ploidy (2x = 2n = 20). An AT-specific DNA fluorochrome, DAPI (4',6-diamidino-2-phenylindole) was used for FC with a CyFlow® Ploidy Analyser (Sysmex Partec GmbH, Görlitz, Germany). The protocol described by Suda and Trávníček (2006) for dried leaf materials was followed, with modifications (detailed in Supplementary Data Method S1). Histograms with a relative fluorescence intensity of around 5000 nuclei were analysed with CyView™ v. 1.5 data acquisition and data analysis software (Sysmex Partec GmbH, Münster, Germany). A maximum coefficient of variation (CV) value of 5 % was accepted for each sample peak (G0/G1 peak). FC ploidy determinations were cross-checked with (1) repetitions of FC measurements in selected samples and (2) chromosome counts in cells at the mitotic division of 17 samples recognized as diploids or tetraploids through FC analyses (following Hojsgaard et al. 2009). Mitotic metaphase cells were observed under a Leica DM5500B microscope (Leica Microsystems GmbH, Wetzlar, Germany) for chromosome counts.

Analysis of reproductive modes was conducted using FC. Open pollinated seeds from three randomly selected individuals per population were collected from a total of 20 P. intermedium populations with unknown ploidy (other populations did not bear mature seeds during the fieldwork). After ploidy determination, only three out of 20 were determined as diploid populations. A total of 500 seeds belonging to 15 populations (three diploids and 12 tetraploids) were used to assess variations in reproductive modes at geographic and/or cytotype levels following the methodology of Hojsgaard et al. (2014a) with a few modifications (details in Supplementary Data Method S2.2). The relative fluorescence intensity of around 3000 nuclei was analysed with CyView™, and discrete peaks were assigned to embryo and endosperm seed tissues. A maximum CV value of 5 % was accepted for each peak. Reproductive pathways were determined according to the rationale by Matzk et al. (2000) and following considerations for *Paspalum* spp. as in Hojsgaard et al. 2013). Sexually derived seeds have a diploid embryo (2n; 2C-value) and a triploid endosperm (3n; 3C-value), whereas seeds derived from apomixis carry a diploid embryo and a pentaploid endosperm (5n; 5C-value) (for details, see Hojsgaard et al. 2013).

2.3.3. Cytotype localities, environmental and climatic data

Since scenopoetic variables (abiotic variables that do not interact with each other) are regarded as being associated with heritable components of the physiology of species (Kearney and Porter 2009), it was assumed that (1) geographic distribution reflects adaptation and underlying ecological tolerance and (2) the occurrence data assembled here are a non-biased representation of intraspecific diversity and variability. Thus, the absence of any cytotype in a

geographic area was considered to be a result of natural processes underlying ecological signals, adaptation and evolutionary mechanisms acting within the species.

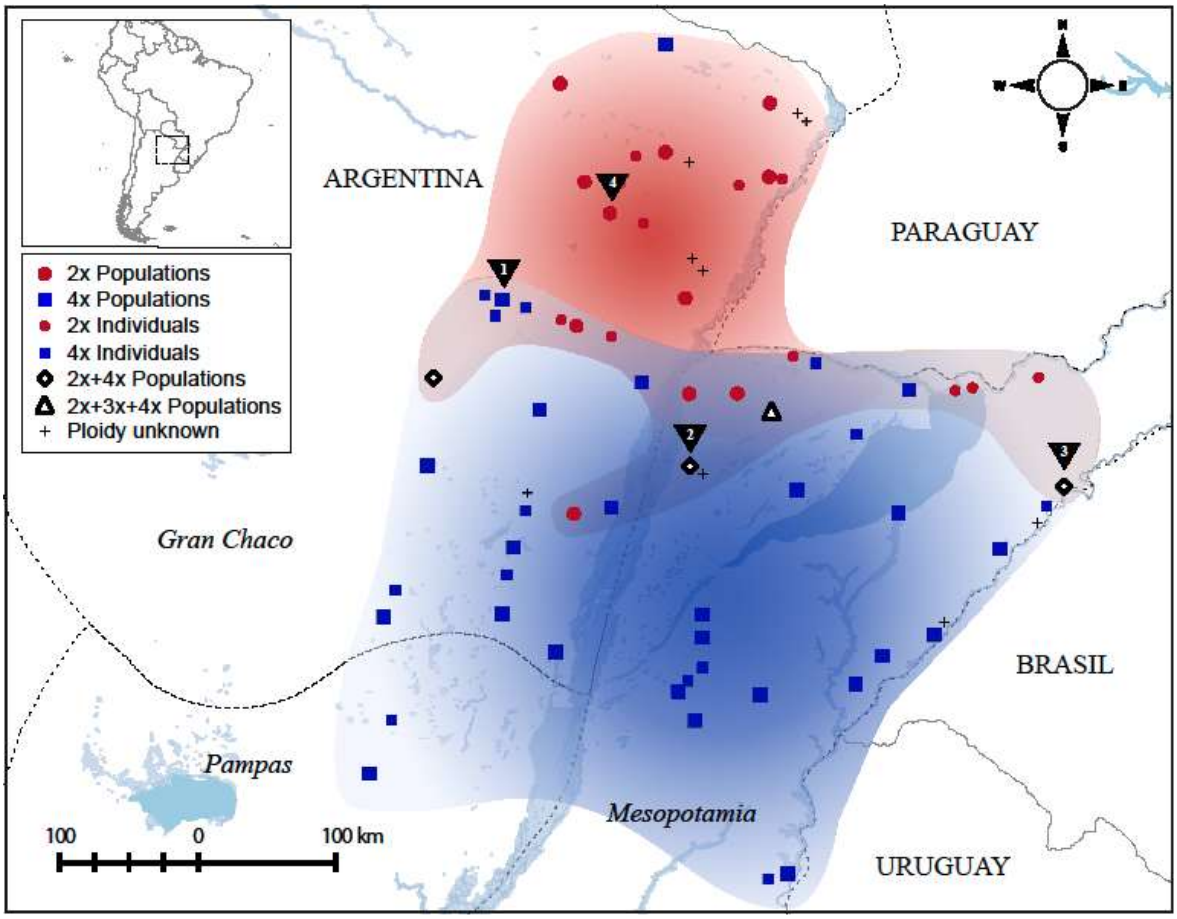

Figure 2.1. Map displaying all collection localities of *P. intermedium* and ploidy levels determined in the present study. The North–South cytotype cline is apparent, together with an East–West transition zone where cytotypes occur intermingled in pure and mixed populations. Ploidies at sites of populations are represented by data from at least 30 individual plants. Ploidies at sites of individuals are represented by data from 1–3 individuals. Triangles 1, 2, 3 and 4 indicate populations (geographical sites) representing *vis-à-vis* ploidy shifts between previous and present records (see the Discussion). Grey lines demarcate country boundaries (block letters) and dotted lines separate ecoregions (*italic letters*)

Ecological data of 26 bioclimatic variables were downloaded from various open-source databases. Nineteen commonly used bioclimatic variables were retrieved from the WorldClim data set (1950–2000; version 1.4) at 2.5 arc-min resolution (approx. 5 km2) (Hijmans et al. 2005; http://www.worldclim.org). The elevation data were downloaded from the Shuttle Radar Topography Mission (SRTM; http://srtm.csi.cgiar.org/) elevation data set at 30 arc-s (approx. 1 km2) resolution. Photosynthetically available radiation (PAR) data were downloaded from the

Moderate Resolution Imaging Spectroradiometer (MODIS) database (Myneni et al. 2015; https://lpdaac.usgs.gov). The annual mean UV-B radiation data set was downloaded from glUV (a global UV-B radiation data set for macro ecological studies) at 15 arc-min resolution (Beckmann et al. 2014; www.ufz.de/gluv). Cloud cover percentage, vapour pressure and frost day frequency data were downloaded from CGIAR CSI (www.cgiar-csi.org) at 30 arc-s resolution. Finally, soil type data (soil taxonomy) were downloaded from the SoilGrids database (ISRIC, 2015; www.soilgrids.org) in 30 arc-s resolution. Data sets with different resolutions were either aggregated or disaggregated to 2.5 arc-min accordingly using the bilinear method (Hijmans and Van Etten 2015) to match WorldClim data. R packages 'sp' (Bivand et al. 2013) 'maptools' (Bivand and Lewin-Koh 2013) and 'raster' (Hijmans and Van Etten 2015) were used in these steps.

For the analysis of past ecological niches of cytotypes, bioclimatic variables for past climatic conditions [Last Glacial Maximum (LGM) – approx. 21 000 years before present (ybp) and Mid-Holocene (MH) – approx. 6000 ybp] were retrieved from WorldClim for two different scenarios (BCC-CSM1-1 and CCSM4; see www.worldclim.org) at 2.5 arc-min resolution.

2.3.4. Environmental niche modeling

Species and cytotype distribution models were constructed using MaxEnt v. 3.3.3k (Phillips et al. 2006). A raster grid stack of all 26 bioclimatic variables for the entire South American continent was generated and the relevant data at each collection point for cytotype distribution analysis were extracted using the R package 'dismo' (Hijmans et al. 2016). A multiple logistic regression was performed to test ecological preferences and associations between ploidy and environmental variables. The R-package 'nnet' (Venables and Ripley 2002) was used for the analysis. Principal component analysis (PCA) was performed for the 26 variables using the R-package 'vegan' (Oksanen et al. 2016) to determine the main drivers of the niche space and cytotype differentiation. To avoid overfitting the data and minimize niche aggregation of cytotypes, we removed predictor variables exhibiting high pair-wise correlation values (Fisher weighted mean r values >0.85) and high collinearity on multiple logistic regression and PCA ordination output. Based on these criteria, 15 bioclimatic and environmental variables were retained and used as predictors to calibrate distribution models in MaxEnt (see Table 2.2).

For reconstructing past niches, data on PAR, UV-B, cloud cover percentage, vapour pressure, frost day frequency and soil type were not available. Therefore, only eight out of 15 selected bioclimatic predictors together with elevation data (see Table 2.2) were used for model calibration. Distribution of both cytotypes based on their realized Grinnellian niches was modelled with the present data and simulated into two past climatic periods, the MH and the LGM. The accuracy of past predictions was assessed against predictions for the present data using

the area under the model's receiver operator characteristic (ROC) curve (AUC values), a threshold-independent ROC analysis that measures the performance of models (Hanley and McNeil 1982).

Table 2.2. Bioclimatic and environmental variables retained (after multivariate analysis and binomial logistic regression) for cytotype distribution and niche analysis in *P. intermedium*, its significance values, and PCA contributions.

Environmental Variable	Code	*p*-value	PC1	Co^2	PC2	Co^2	AC
Annual Mean Temperature (°C)	BIO1	0.004	-0.9313	0.867404	0.31867	0.101556	8.796792
Isothermality (BIO2/BIO7) (*100)	BIO3	0.001	-0.6329	0.400577	0.53660	0.28795	6.974405
Temperature Seasonality (SD *100)	BIO4	<0.001	0.9615	0.924507	-0.0252	0.000638	8.07602
Min Temperature of Coldest Month (°C)	BIO6	0.007	-0.9018	0.813358	-0.18677	0.034883	7.5197
Temperature annual range (°C)	BIO7	0.005	0.6018	0.362187	0.56517	0.31942	7.01954
Mean Temperature of Wettest Quarter (°C)	BIO8	0.013	-0.2564	0.065749	0.8668	0.751362	9.65046
Mean Temperature of Driest Quarter (°C)	BIO9	<0.001	0.9687	0.938464	0.21164	0.044792	8.73122
Mean Temperature of Warmest Quarter(°C)	BIO10	<0.001	-0.8491	0.721005	0.41351	0.170999	8.35803
Mean Temperature of Coldest Quarter (°C)	BIO11	0.001	-0.9621	0.925642	0.22777	0.05188	8.70494
Precipitation seasonality (CV)	BIO15	0.008	0.2617	0.068509	0.91909	0.84474	10.80257
UV-B radiation (J/m^2/day)	U	0.001	-0.7011	0.806261	0.67793	0.133702	9.84241
Photosynthetically active radiation (PAR)	P	0.002	-0.8979	0.49162	0.36565	0.45959	8.65153
Frost day frequency (days per year)	F	0.017	0.6853	0.874856	0.07113	0.060288	4.16024
Surface vapor pressure (hPa)	S	0.002	-0.9353	0.469697	0.24553	0.00506	8.36329
Elevation	E	0.023	-0.5258	0.27651	0.22309	0.049773	3.01441

p-value: significance values ($\alpha = 0.05$) of the binomial multiple logistic regression analysis on climatic and ecological preferences between diploids and tetraploids; AC: Sum of absolute contributions of variables to principal components one and two; PC1 and PC2: eigenvalues of first two axes of the PCA for the ordination of variables demarcating the niche space between the two cytotypes. Co2: squared coordinates of variables (higher values indicate better representation of variables in the principal components)

2.3.5. Niche breadth and overlap

Niche characteristics were extracted using parametric generalized models. Coarse spatial resolution of ecological and geographic properties of the species was used to define Grinnellian niches of cytotypes. Niche breadth and niche shifts were computed as 1.5 s.d. of the Euclidian distance from the centroid of an individual's cloud for each cytotype and weighted by the Eigenvalues of PCA ordination axes, respectively. The amplitude of cytotype-specific habitat distribution and ecological requirements was considered as a measure of Grinnellian realized specialization (Devictor et al. 2010). Schoener's D index was used to assess the overall overlap of the environmental niche space between cytotypes. Therein, the similarity of the niches was summarized from 0 (no similarity) to 1 (complete similarity). The obtained niche overlap was plotted against a randomly simulated niche overlap generated with the assumptions of both niche equivalency and similarity (as described in Broennimann et al. 2012). In order to avoid uninformative data extraction for background environment, environmental data were extracted from random points (500 for diploids and 800 for tetraploids; the number of points was empirically chosen based on the highest AUC values of the SDM) drawn from a circular area around the

observed data points. Simulations for niche similarity and equivalency were performed in 1000 replicates each, using the R package 'ecospat' (Di Cola et al. 2017). A new approach was used to visualize density distributions of each cytotype in a collective environmental gradient (CEG). The CEG was computed utilizing all the selected environmental variables (predictors). The data set was transferred into a table with predictors in columns and geographic points in rows. A z-transformation was applied to all the predictors (columns) to create a CEG for each cytotype where all variables are collapsed into one single gradient. The transformed values for each locality and cytotype were summed up and used to obtain a 'collective' value representing the overall ecological setting for that particular geographical point assuming that all predictors contribute to the occurrence of the cytotype at a given location.

Collective values were then mapped into the CEG to obtain a kernel density estimation (KDE) (probability density) and to visualize any trend on main ecological preferences relative to each cytotype. For the KDE, a bandwidth of 0.5 *s.d.* was applied to achieve a moderate smoothing of the resulting density curves (Figure 2.2). The CEG was structured into quartile and interquartile points to assess the significance of the data and to better visualize the relative ecological differentiation between cytotypes along the collective environmental gradient (Figure 2.2).

2.3.6. Statistical analyses

Complete spatial randomness (CSR) of all the occurrences was tested with K-function (also Ripley's K-function) in the R package 'spatstat' (Baddeley and Turner 2005), prior to all the statistical analyses and modelling. Furthermore, a χ^2 dispersion test for spatial point patterns based on quadrat counts (quadrattest; Baddeley and Turner 2005) was used to test spatial separation of cytotypes in the sampling area (further details are given in Supplementary Data Fig. S5). All the statistical analyses and mapping were performed in R version 3.3.2 (R Core Team 2016) unless mentioned otherwise. QGIS [QGIS Development Team. QGIS Development Team. Open Source Geospatial Foundation (2016)] was used for visualization and creating maps.

2.4. RESULTS

2.4.1. Ploidy level variation, local and regional spatial separation

The ploidy evaluation of a total of 1224 individuals revealed two major cytotypes: diploids (2n = 2x = 20; N = 431; 35.2 %) and tetraploids (2n = 4x = 40; N = 793; 64.8 %) (Supplementary Data Fig. S2.1a, b; Method S2.1). In addition, one triploid individual (2n = 3x = 30) was also recorded (Supplementary Data Table S2.1). Thirty-one out of the 35 (88.6 %) populations were uniform, consisting of pure diploid or pure tetraploid plants; the remaining four (11.4 %) were mixed-ploidy populations (Hojs456, Hojs470, Hojs481 and Hojs487). Populations

consisting of multiple cytotypes are rare in *Paspalum*, and this is the first record for *P. intermedium*. The tetraploid was the most common cytotype, present in 28 (24 pure tetraploid) populations, while the diploid cytotype was found in 15 (11 pure diploid) populations (Figure 2.1; Table 2.1). The rare triploid cytotype was found in a mixed-ploidy (2x–4x) population (Figure 2.1). On comparison with previous records by various authors (Supplementary Data Table S2.2), we observed a shift of ploidy in four localities: three along a contact zone between cytotypes and one in the core distribution area of diploids (details in Figures 2.1 and 2.2; Supplementary Data Tables S2.1 and S2.2). The within-population sampling strategy unveiled localscale distribution patterns of cytotypes in mixed populations (Supplementary Data Figure S2.2). While one population (Hojs456) had only five diploid individuals restricted to one end of the population, the rest had various numbers of diploid and tetraploid cytotypes mixed in different patterns along the sampling line (Supplementary Data Figure S2.2). A Mann–Kendall rank test for randomness (in the R-package 'randtests'; Caeiro and Mateus, 2014) indicated a non-random distribution in the occurrence of cytotypes along the sampling transects (*p-value* ≤ 0.01 in all cases), suggesting that the local-scale distribution patterns and turnover followed a certain clustering order. In addition, in population Hojs470, we collected eight young ndividuals (seven tetraploids and one diploid) widespread among mature individuals (Supplementary Data Figure S2.2).

The spatial randomness test for the recorded occurrences of the two cytotypes with Ripley's K-function showed deviations of $\lambda K(r)$ (the expected vs. observed number of points per unit area) from the Poisson (theoretical) distribution (paired-end *t-test p-value* < 0.001) (Supplementary Data Figure S2.3a). This indicates a non-random distribution of ploidies among all geographically dispersed data points. In addition, a Pearson χ^2 goodness-of-fit test using quadrat counts showed a deviation (χ^2 = 161.14, *p-value* = 0 .0001; Supplementary Data Figure S2.3b) in the observed distribution compared with the null distribution, thus confirming a clustering of cytotypes along the observed North–South spatial separation and East–West contact zone of sympatric and parapatric occurrences (mixed-ploidy populations were considered both diploid and tetraploid in the analysis; triploids were not considered).

2.4.2. Cytotype reproduction modes and ploidy as a proxy for reproductive biology

A total of 500 seeds originating from 45 individuals (100 seeds from nine diploids and 400 seeds from 36 tetraploids) were analysed to assess reproductive modes of *P. intermedium* (Table 2.3). Single-seed histograms produced two types of peak configurations, corresponding to different embryo to endosperm DNA content ratios: peak configurations 2C:3C correspond to sexual seeds, carrying a diploid embryo and a triploid endosperm; peak configurations 2C:5C correspond to clonal seeds, carrying a parthenogenetic diploid embryo and a pentaploid endosperm (see details in Supplementary Data Method S2.2; Figure S2.1c). In diploid plants, only

seeds with a 2C:3C peak configuration were observed, and therefore diploids are considered as obligate sexuals (all seeds were produced after syngamy of meiotic gametes). Tetraploid plants presented a moderately low proportion of sexual seeds (<30 %; Table 2.3) and a larger amount of clonal seeds (>70 %; Table 2.3). Hence, tetraploids are considered as facultative apomicts.

Since ploidy levels in *P. intermedium* (Norrmann et al. 1989; this study) as well as in other *Paspalum* spp. and grasses (e.g. Galdeano et al. 2016) are tightly connected to divergent reproductive syndromes, our reproductive screenings validate the use of ploidy as a priori information and a proxy for reproductive biology in *P. intermedium*.

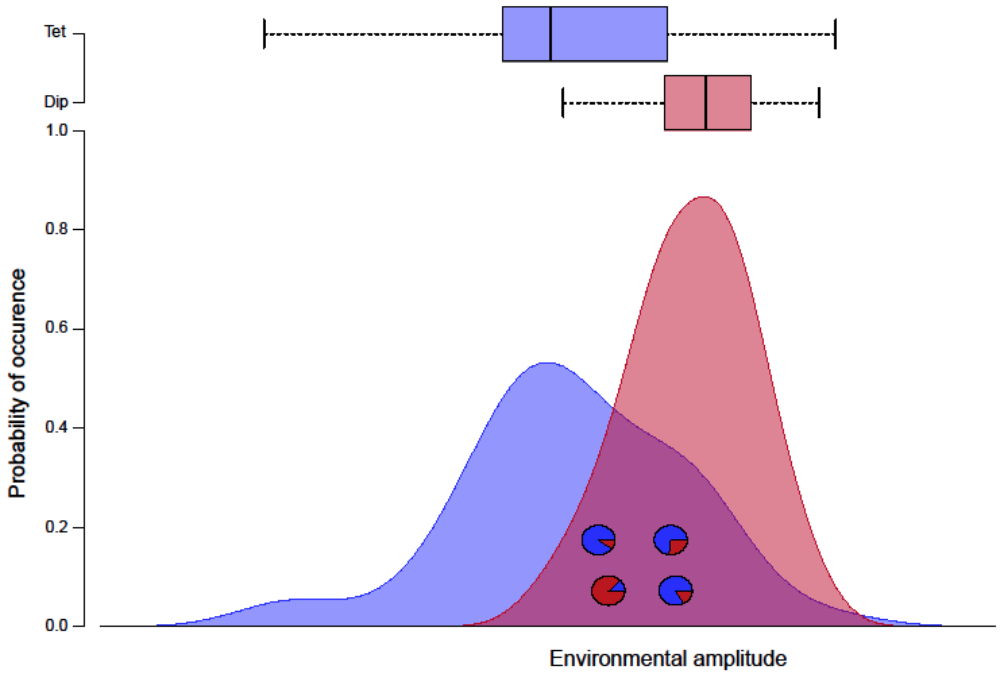

Figure 2.2. Niche breadth of diploid (red) and tetraploid (blue) *P. intermedium* cytotypes depicted as probability density function for occurrences along the collective environmental gradient of the ecological requirements of species. Boxplot-ranked sets of data (quartiles and interquartile range) further illustrate ecological differentiation between cytotypes. The ecological gradient of species coalesce the observed environmental heterogeneity after pooling (z-transformed) data of environmental variables showing significant differences ($P < 0.05$). Pie charts indicate the relative position of mixed-ploidy populations within the environmental gradient (x-axis) (upper left, Hojs487; lower left, Hojs481; upper right, Hojs470; lower right, Hojs456), and slices represent percentages of each cytotype (red = diploids, blue = tetraploids).

2.4.3. Phenological shift and intraspecific ecological differentiation of cytotypes

In the present analysis of phenology, I recorded a total of 38 sites in flowering during the early and late season trips and added 33 records of materials with known ploidy from herbaria. Three out of four mixed-ploidy populations were flowering; however, ploidy levels of individuals were unknown during collection. The probability of blooming incidence per ploidy indicates that diploids tend to flower early in the season (n = 24; *p-value* = 0.0074; df = 1) while the probability of finding tetraploids with flowering stems was the same for both the early and the late summer periods (n = 47; *p-value* = 0.2108; df = 1).

Generalized linear models with multiple error distribution (logistic regression) detected 15 environmental variables having significant differences between cytotypes (*p-value* ≤ 0.0147 in all cases; Table 2.2), indicating unique ecological and climatic preferences. Thus, most extreme cytotype differences were found for Bio1, annual mean temperature (*p-value* = 0.00019); Bio6, minimum temperature in the coldest month (*p-value* = 0.00037); and Bio15, precipitation seasonality (*p-value* = 0.0114) (Supplementary Data Figures S2.4 and S2.5). PAR and UV-B radiation (mean value for diploids, PAR = 0.532 ± 0.009 Jm^{-2}, UVB = 415 ± 79 Jm^{-2}; tetraploids, PAR = 0.511 ± 0.01 Jm^{-2}, UVB = 402 ± 92 Jm^{-2}; Supplementary Data Figure S2.4) also showed strong association with differences in climatic preferences between cytotypes (*p-value* = 0.000138 and 0.00026, respectively). The elevation, however, did not show a strong correlation as the species distribution range is restricted to a topographically flat area (58–156 ± 18.2 m for 2x; 35–93 ± 12.9 m for 4x; Table 2.2; Supplementary Data Figures S2.4 and S2.5).

Scenopoetic variables gathered from all localities defined the realized niche of species and displayed divergent differences between cytotypes (Supplementary Data Figure S2.4), with tetraploids occupying broader environmental ranges than diploids. The diploid range was fully enclosed within the tetraploid range in eight out of 13 variables, and tetraploids included the core of the ecological preferences of diploids (i.e. interquartile ranges) in 12 out of 13 variables (Figures 2.2 and 2.3). In the PCA, two principal components explained the majority of environmental variation observed for the *P. intermedium* data set (see Supplementary Data Figure S2.5). PC1 (represented 44.1 % of the variation) was explained by temperature-related variables, vapour pressure, PAR, UV-B radiation, frost day frequency and soil type, and defined the Euclidean space of diploids. PC2 (represented 31.8 % of the variation) was best explained by precipitation-related variables (Table 2.2; Supplementary Data Figure S2.5). The PCA revealed a shift in the Euclidean space between cytotypes along the PC1 axis and a large overlap along the PC2 axis, with tetraploids having a greater niche breadth (Figures 2.2 and 2.3). The differentiation of niche optima in Fig. 3 is defined as the Euclidean distance between centroids of ellipses weighted by the inertia of the first two axes after decomposition of inertia (six axes). The observed niche overlap of

diploids and tetraploids (Schoener's D = 0.25) is significantly lower (*p-value* = 0.0099) than the simulated overlap (mean = 0.65) under niche equivalency (Supplementary Data Figure S2.6a). On the other hand, the niche similarity test indicated that the observed environmental space similarity is higher than expected on a random basis (*p-value* = 0.297) (Supplementary Data Figure S2.6b). These reject the null hypothesis that diploids and tetraploids occupy equal climatic niche spaces, and recognize that both cytotypes occupy habitats with slightly dissimilar climatic regimes and environmental resources.

Table 2.3. Reproductive mode variation between cytotypes of *P. intermedium* in the study area

Ploidy	*n* (pop.)	*n* (ind.)	Seeds	P.i.±SD	Rep. path	Proportion (%) ±SD
Diploid	3	9	100	1.45 ±0.039	Sex.	100% ±0.0
Tetraploid	12	36	400	1.49 ±0.089	Sex.	27.9% ±7.0
				2.40 ±0.071	Apo.	72.1% ±9.7

n (pop): number of populations; *n* (ind.): number of individuals; Sex.: sexuality (cross- and self-fertility); Apo.: pseudogamous apomixis; P.i.: Peak index or endosperm: embryo peak ratio in flow cytometry analyses; Rep. path: reproductive pathway; SD: Standard deviation

The collective environmental gradient further shows this tendency by visualizing the probability density of cytotypes along a continuous gradient representing all ecological settings of the species. Diploids are symmetrically centred and display inferior ecological amplitude compared with tetraploids (Figure 2.2). In contrast, tetraploids can grow in a wider range of environmental conditions, exhibiting a broader yet lower probability density along the gradient with a median value and main ecological preferences shifted away from those of diploids (Figure 2.2). Overall, tetraploids display wider ecological amplitude and are 'generalists', while diploids are 'specialists'. Environmental niche differentiation of *P. intermedium* cytotypes followed a latitudinal gradient where tetraploids grow under more extreme environmental conditions (e.g. lower temperatures and light radiation) and therefore can cope well with seasonal changes in southern areas.

2.4.4. Ecological niche modelling and past distribution

Model simulations produced high AUC scores (0.83 and 0.81 for diploids and tetraploids, respectively) and thereby highly accurate predictions of climatic niche spaces which reflect the realized range of distribution of each cytotype. Prediction scores >0.65 were considered strong signals for habitat suitability of the cytotypes (see Fig. 4). MaxEnt predictions show that the environmental niche spaces of both cytotypes nearly reflect their realized niches in the sampling area, with a few exceptions. For example, the climatic niche of diploids is marginally expanded toward the North-east of their realized distribution (Figure 2.4; Supplementary Data Table S2.2), and the climatic niche of tetraploids is expanded toward the North along a stretch of the Paraguay River, reaching Bolivia and Brazil (Figure 2.4). Likewise, a surprisingly large area predicting a

niche overlap around the core distribution of diploids is not realized for the distribution range of tetraploids. The complete absence of tetraploids in this region suggests a zone of cytotype exclusion (Figure 2.1 and 2.4).

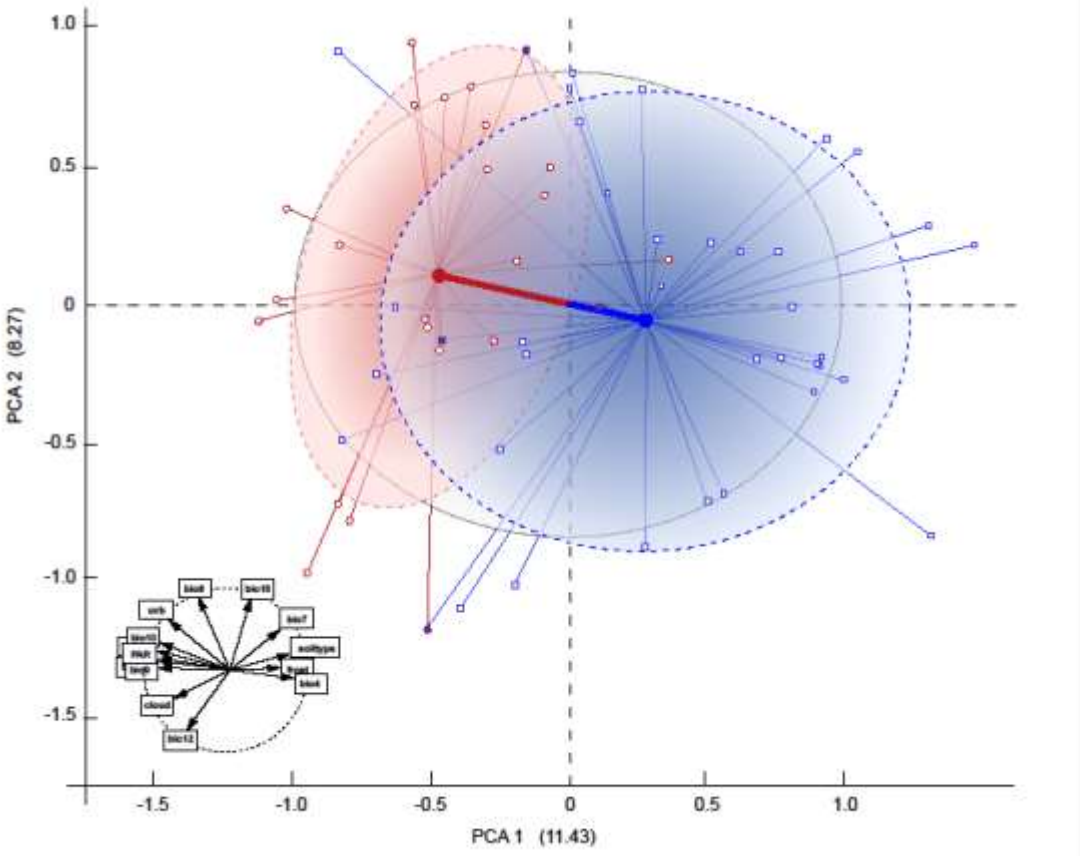

Figure 2.3. Multidimensional analysis of the ecological niche of P. intermedium and the shift in niche optima between cytotypes. The specialization of cytotypes and differentiation of niche optima is reflected by the multidimensional volume (here represented in a 2D space) represented by the spatial distribution of points (collection sites, each indicating a particular environmental setup), and the distance between centroids of ellipses. Main environmental variables used in the ordination are shown in the correlation circle (r=1; codes follow Table 2.2). Red circles symbolize diploids, blue squares tetraploids and red-filled blue squares heteroploid sites. Eigenvalues for first two axis inertia are given in parenthesis. Red and blue arrows indicate the direction of the shift in niche optima for diploid and tetraploid cytotypes. The grey-shaded ellipse represents the niche space of the species. Dotted ellipses indicate diploid (red) and tetraploid (blue) niches.

Projections of past environmental niche space of *P. intermedium* for both CCSM4 and BCC-CSM1-1 past climatic scenarios showed a temporo-spatial range shift in estimated spatial distributions of cytotypes. Both ecological models indicated the absence of suitable habitats for either cytotype in our current sampling area during the LGM (25 000 ybp), and the presence of suitable climatic conditions for diploids in northern Bolivia and central and southern parts of Brazil (Supplementary Data Figure S2.7). Towards the MH (approx. 6000 ybp), changes in environmental

conditions in the South American continent moved habitat suitability of diploids towards southern parts of Brazil, Paraguay and northern Argentina, thus expanding its distribution area, and priming the conditions for a successful establishment of tetraploids as projected for northern Argentina and southern Paraguay (Supplementary Data Figure S2.7).

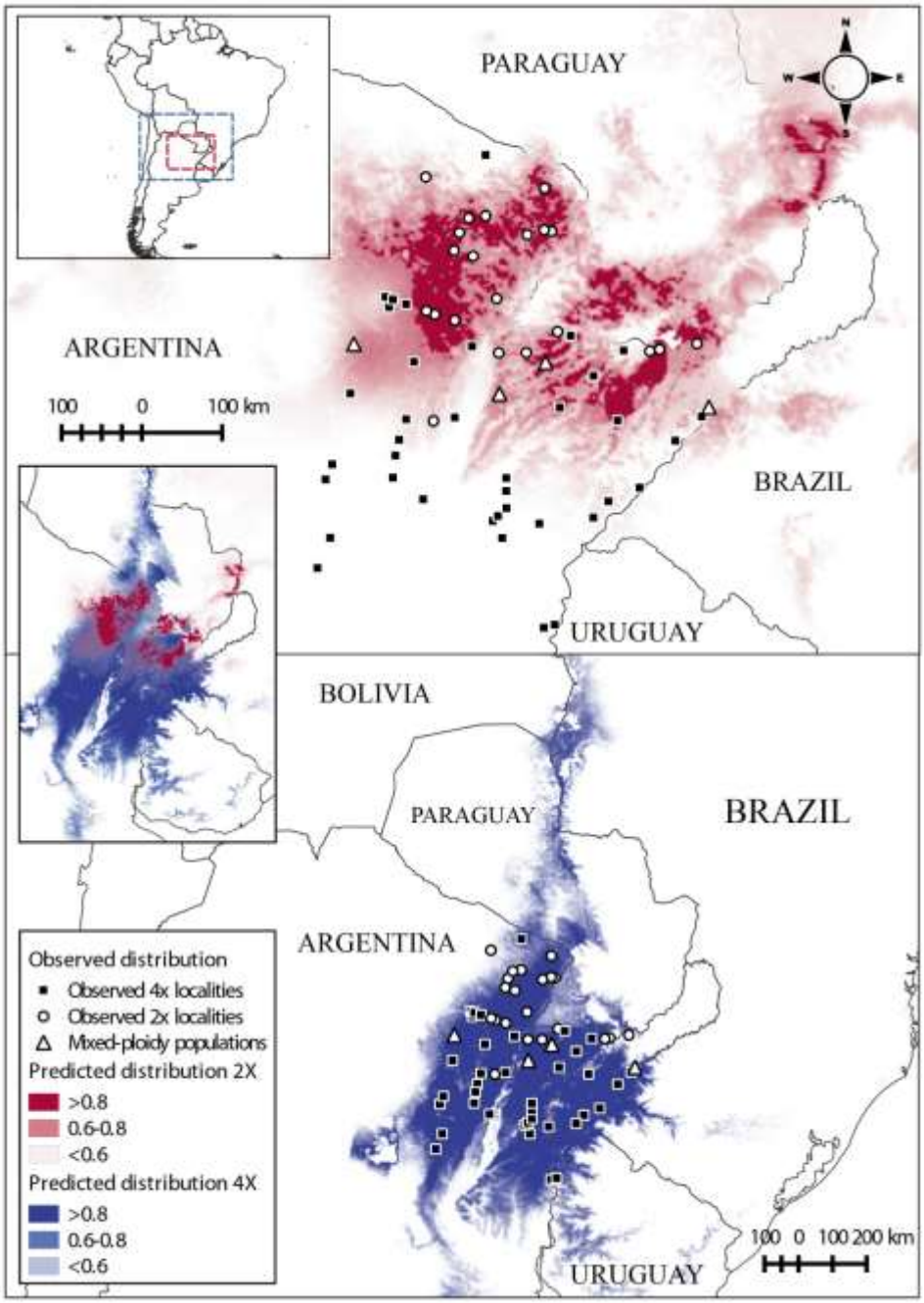

Figure 2.4. The output of species distribution modeling using MaxEnt. The map shows the realized and the potential distribution ranges of diploid (red) and tetraploid (blue) cytotypes of *P. intermedium* in the study area. The realized and the potential habitat suitability (the probability of occurrence inferred from the model output values of AUC) shows similar predicted distributions for diploids, but for tetraploids. The area inside the dotted line represents a zone where ecological conditions meet the requirements for the coexistence of diploids and tetraploids, yet it is only occupied by diploids

2.5. DISCUSSION

Knowledge on plant distributions and intraspecific trait variability is central to underpin ecological and physical factors affecting evolutionary dynamics and history of species. Studies on cytotype distributions and associated environmental and reproductive traits provide valuable insights into diploid–polyploid dynamics and factors responsible for contraction–expansion cycles (e.g. Cosendai and Hörandl 2010, Caperta et al. 2016, Sonnleitner et al. 2016). The present macro- and micro-scale study on *P. intermedium* is an attempt to recognize environmental factors and biological traits affecting cytotype coexistence, population dynamics and ecological adaptation outlining early events endowing polyploidization and speciation in plants.

2.5.1. Cytotype composition and distribution patterns

The distribution range of *P. intermedium* is centred in north and eastern Argentina with records spanning to Southern Brazil, eastern Paraguay and northern Uruguay (Zuloaga et al. 2012). The study revealed a North–South spatial segregation of the two cytotypes with a narrow East–West overlapping zone in the centre. Despite the fact that tetraploids are evolutionarily younger than diploids (see below), it has become the most common cytotype, occupying two-thirds of the species' geographic range.

Odd polyploids are known to be infrequent in nature; however, they provide crucial information on fundamental mechanisms of polyploid formation and establishment (e.g. Ramsey and Schemske 1998, Husband 2004). Previous and present findings of rare triploid cytotypes of *P. intermedium* do not provide much information, but rather an opportunity to study the role of triploids in polyploid formation and population dynamics that need to be addressed in a set of new experiments. The presence of one tetraploid population in the far North-west of the species' distribution may represent a case of polyploidization in the northern periphery of diploids, a glimpse of the progression of past cytotype displacement (further details below), a consequence of anthropogenic activities (extensive human intervention in the area is evident through agricultural activities) or a combination of such factors.

2.5.2. Ecological specialization and niche differentiation between cytotypes

In *P. intermedium*, the realized niche specialization based on environmental parameters and background similarity tests, niche breadth and cytotype densities showed significant differentiation on ecological requirements between cytotypes, indicating that diploids are adapted to a narrow range of ecological settings compared with tetraploids. By having a broader and transgressive niche breadth, tetraploids enclose the whole range of climatic preferences of diploids; a situation observed in other polyploid complexes as well (e.g. *Claytonia*

perfoliata complex, McIntyre 2012; *Tolmeia*, Visger et al. 2016), and expected in autopolyploids such as *P. intermedium*, recurrently originated from a diploid's gene pool. However, unlike in the *C. perfoliata* complex (McIntyre 2012) and other autopolyploid systems such as *Allium oleraceum* (Duchoslav et al. 2016), in *P. intermedium* we observe a significant separation of ecological optima between cytotypes, suggesting a segregation of ploidy-related ecophysiological aptitudes for the exploitation of environmental resources after polyploidization. Recently established polyploids usually display intermediate ecological preferences compared with diploids and established polyploids (e.g. Maherali et al. 2009, Levin 2011). Similarly, tetraploids from mixed populations in *P. intermedium* (presumably the product of recent polyploidization events and therefore younger than those occurring in southern areas) show ecological preferences intermediate to those observed in diploids and general tetraploids, which further reinforces our observations of cytotype dynamics. Different studies of niche shifts in autopolyploids support the common hypothesis that polyploids evolve to occupy wider or more extreme ranges than their progenitors (reviewed in Spoelhof et al. 2017). Accordingly, the directional and opposed shift in niche optima observed between *P. intermedium* cytotypes, the bimodal distribution of cytotypes and skewness observed for tetraploids along the collective environmental gradient suggest the action of past disruptive selection on established tetraploids favouring a divergent departure between the ecological preferences of cytotypes. Niche differentiation is a primary mechanism to avoid competitive exclusion by diploid progenitors and foster polyploid establishment (e.g. Levin 2003, Ramsey 2011). Thus, establishment and persistence of *P. intermedium* polyploids apparently took place at the expense of becoming (sub-) adapted to a broader range of ecological conditions and less competitive in areas where multidimensional space meets the niche optimum of diploids. The question remaining is whether the observed broader ecological tolerance is due to an effect of polyploidy (e.g. Kearney 2005) or caused by the fact that asexuality is probably freezing a range of genotypes among autopolyploid clones (carrying a sub-set of genes from the diploid ancestors) adapted to local narrow niches (Vrijenhoek 1979). While a shift from sexuality to apomixis can partition the use of resources in polyploids, it may not necessarily affect the niche dynamics (Dellinger et al. 2016). Nevertheless, achieving reproductive assurance through asexuality certainly shields polyploids from environmental stress (Freeling 2017; see next section) and confer higher colonizing ability to tetraploids (Hörandl 2006, Hojsgaard and Hörandl 2015). Molecular genetic analyses will benefit us to better understand the origin of tetraploids, the competitive dynamics between cytotypes within mixed populations, and the effect of asexuality and environmental stressors on partitioning genetic diversity and resource use.

2.5.3. Population dynamics, ploidy shifts and ecological displacement between cytotypes

The North–South distribution pattern of *P. intermedium* cytotypes is defined by a divergence in cytotype-specific ecological preferences. In core distribution areas, seasonal environmental variables foster cytotype stability. Dispersion of cytotypes following a seasonal–latitudinal gradient as observed in P. intermedium is not rare in nature (Španiel et al. 2008, Trávnícek et al. 2011, Zozomová-Lihová et al. 2015). Diploid–tetraploid coexistence is possible by different pre- and post-zygotic isolation barriers (Husband and Sabara 2003) or by character displacement and ecological differentiation (Beans 2014). Despite being rarely found in nature, niche displacement (i.e. when the niche of a cytotype is affected by the presence of another cytotype) through a shift in niche optima or breadth plays an important role in enabling closely related species to coexist. For example, Sonnleitner et al. (2016) found that contact zones in *Senecio carniolicus* were stabilized and reinforced by ecological differentiation of cytotypes as a result of habitat displacement. Unlike in other species, in *P. intermedium* the uneven replacement and local and regional spatial separation of cytotypes, the biased recruitment of young polyploid individuals in heteroploid populations and the discrepancy between predicted and observed distributions in tetraploids suggest a pattern of unstable temporal coexistence and directional turnover during which one cytotype is locally displaced reliant on ecological specialization and local environmental conditions.

The model prediction for the distribution of diploids is not significantly different from the observed distribution (Figure 2.4). However, the prediction for tetraploids indicates that polyploids should coexist along with diploids in its main distribution zone; a situation that has not been realized according to our field observations. This suggests the presence of a wide area of tetraploid exclusion. In addition, the current observation that only a pure diploid population is found in an area in the core zone of diploids where one tetraploid was collected 30 years ago (Norrmann et al. 1989), and that diploids are being replaced by tetraploids in peripheral areas of its distribution suggests that tetraploids may only overcome ecological competition in the marginal zones of diploids with greater environmental heterogeneity. Thus, tetraploids may fail to become locally established in areas where optimal niche requirements of diploids are successfully met. Reciprocal transplantation experiments would certainly provide more accurate conclusions on this observation.

By definition, boundaries of distribution in plants represent zones of ecological transition, i.e. areas where environmental conditions do not satisfy the main ecological preferences of a particular species or group (Grant 1981). Even when plants exhibit plasticity to environmental conditions, their performance at niche edges may decline due to the effects of biotic and abiotic factors on their reproductive success (Vergeer and Kunin 2013). Cytotypes

occupying habitats in areas of ecological transition, irrespective of the mode of coexistence (i.e. sympatry or parapatry), are prone to ecophysiological sub-adaptation. Changes in reproductive strategies (e.g. allogamy– autogamy, sexuality–apomixis) and ploidy levels are known to improve local and regional performance (Hörandl 2006). Mixed populations of *P. intermedium* appear in a region of spatial niche overlap between cytotypes, a zone of ecological transition between diploid–tetraploid niche optima (Figure 2.3) where competition is expected to be stronger and driven not only by ecological differences (as the ecological requirements of neither cytotype were fully met) and spatial segregation of cytotypes but also by reproductive changes. In fact, two out of four mixed populations (Hojs456 and Hojs470) found in the transition area where ecological conditions resembled more those of the niche optima of diploids (interquartile range; Figure 2.2) harbored a significantly higher number of tetraploids. Similarly, one of the other two populations (Hojs481) located in a transition area where conditions resembled more those of the niche optima of tetraploids was dominated by diploids, indicating that the reproductive mode might have an effect, even if temporary, on local cytotype success. Apomixis is known to shelter the polyploid from introgressive hybridizations, particularly heteroploid hybridizations (Hörandl and Temsch 2009). The observed incongruity between ecological conditions, niche preferences and population composition mentioned above, together with the documented ploidy shifts, suggests the existence of a temporal succession of polyploid establishment–diploid displacement events. In this case, whenever a tetraploid is successfully established in the peripheral areas of diploids, the new heteroploid population will eventually reach a situation of asymmetric turnover between cytotypes which will most probably drive diploids to a local extinction.

In *P. intermedium*, a shift to apomixis not only shelters the polyploid from introgression of diploids (only one triploid among 122 individuals in mixed populations), thus reducing fitness loss by infertile hybrids and avoiding minority cytotype disadvantages (Levin 1975), but also facilitates the multiplication of superior genotypes better adapted to local environmental conditions. In marginal areas where both cytotypes co-occur in sympatry, a generalist strategy with broader ecological tolerance and a capacity clonally to propagate rare and highly adapted genotypes may enhance the relative fitness of polyploids and their chances to displace diploids locally. Our observation of seven out of eight young *P. intermedium* plants sampled in a population with mixed ploidy being tetraploids and the non-random distribution of cytotypes within mixed populations supports the interpretation of non-random turnover and local displacement between cytotypes.

2.5.4. Reconstruction of past migrations, and evolutionary history of polyploid cytotypes

Reconstruction of past climatic niches indicated that only diploids of *P. intermedium* may have existed in northern Bolivia, and central and south Brazil during the LGM

around 21 000 ybp, in an area in southern Amazonia that was colder and drier than now, occupied by grasslands and savanna (e.g. Behling 2002). During late Quaternary, neither tropical climates nor vegetation were stable and, as climate started to warm up, deglaciation (14000 to 8000 ybp) transformed global vegetation distributions, even in tropical zones (Comes and Kadereit 1998, Williams 2009). Toward the MH (6000 ybp), the forest cover expanded and thermophilous taxa moved to higher altitudes and latitudes, reshuffling distributions of species with dramatic changes in some cases. For example, *Picea* suffered a biogeographic shift of around 2000 km northward from the central eastern USA (Williams 2009). Similar regional to continental shifts in distributions took place in different species in South America (e.g. *Araucaria* forest; Behling 2002), which may have affected the distribution of diploid *P. intermedium*. The present climatic niche modelling showed a shift of diploid occurrence during the Holocene, from central-east Brazil to southern areas in northern Argentina and Paraguay, perhaps a consequence of species' migration to track adaptive peaks as the fitness landscape changed (Supplementary Data Figure S2.7). The presence of fossil impressions of *P. intermedium* spikelets found in Gran Chaco region (northern Argentina) suggests that the species lived in the area around MH (Contreras et al. 2015), which agrees with model reconstructions of past vegetation types and distribution in South America (Cerling et al. 1997, Piovano et al. 2009).

Plant taxa primarily respond to climate variations via local changes in abundance and, consequently, climate change shapes vegetation dynamics in the long run (Williams 2009). In *P. intermedium*, climatic and fitness landscape changes seem to have prompted geographic shifts to new environments. The question remains of whether diploids and tetraploids coexisted in those areas adapted to similar climatic niches and diverged later, or whether tetraploids directly occupied vacant niches unfavorable for diploids while moving south. In either case, niche divergence facilitated the spatial segregation and establishment of both cytotypes. Since apomixis and selfing are known to provide superior colonization abilities in peripheral areas via uniparental reproduction (Baker's Law; Baker 1955), niche availability and segregating ecophysiological and reproductive traits may have delivered the appropriate background for polyploids to expand into southern habitats that are inaccessible to diploids where primary production and productivity measures are lower (Alcaraz-Segura et al. 2013). Broader ecophysiological tolerance of tetraploids and their habitat-associated characteristics featured by the Mesopotamian water system that drains toward Parana delta to the Atlantic Ocean certainly favored tetraploid dispersal, which eventually shaped the currently observed North–South distribution pattern.

3. Evolutionary implications of a TUG OF WAR between sexual and apomictic reproductive modes in *Paspalum intermedium* (Poaceae) leading to fitness variation in the polyploid complex

This chapter focuses on reproductive mode assessment, environmental influence on reproductive pathways and fitness assessment of diploids and tetraploids of *P. intermedium*. The findings have been compiled in a manuscript and submitted to *New Phytologist*, which at the time of writing this thesis is under review: **Piyal Karunarathne, Verena Reutemann, Mara Schedler, Adriana Glücksberg, Eric J Martínez, Ana I Honfi, Diego Hojsgaard. Sexual modulation and the evolutionary implications of a TUG OF WAR between sexual-apomictic reproductive modes in a polyploid grass species.**

3.1. ABSTRACT

In systems alternating between sexual and asexual reproduction, sex increases under unfavorable environmental conditions. In plants, capable of producing asexual (apomictic) seeds, the influence of the environment on sex is equivocal under experimental conditions and has not been studied in natural populations. Apomixis provides reproductive assurance, and superior colonizing abilities compared to sexuals, but it also constrains genetic variation and clonal plants are expected to have restricted adaptive capabilities. Thus, any influence that the surrounding conditions can have on the expression of sex in apomictic plants can play a major role in facilitating range expansion and local adaptation by introducing genetic variation. So far, studies evaluating the influence of bioclimatic variables into proportions of sex and reproductive fitness of natural apomictic populations are scarce. I used *Paspalum intermedium*, a species having two ploidy levels with contrasting reproductive modes and ecological differentiation, to study variation in the expression of sexuality and apomixis due to environmental influence, to analyze developmental competition between both reproductive modes, and their effects on reproductive fitness between cytotypes. Flow Cytometry and embryological analyses were used for ploidy and reproductive modes analyses. Proportions of sexuality and apomixis in situ were analyzed against local climatic conditions. Total seed set and germinability analyses were used to estimate the reproductive fitness of different cytotypes. The expression of sex and apomixis in tetraploid populations shows high variation both within and among populations. This variation is correlated to the number of ovules with both meiotic and apomictic embryo sacs existing in the same ovule. Even though ovule and seed analyses show that apomictic development has higher competitive ability, fitness of apomictic individuals is depleted compared to sexual individuals and populations, indicating asexuality results in higher seed abortion. Evidence was found for environmental modulation of embryo sac formation at population level by lower temperatures and mean diurnal range (MDR) whereby sexual ES formation increased with higher MDR while apomixis decreases. Thus, I identified a *Tug of War* situation between factors intrinsic to apomixis and environmental stressors promoting sex which influence the expression and distribution of sex in apomictic populations and suggest a crucial role of local ecological conditions in sexual expression and adaptation of apomictic populations.

3.2. INTRODUCTION

Sexual reproduction is inherent to all eukaryotes. Sexuality promotes the creation of genetically variable and physiologically flexible organisms capable of coping with spatial and temporal environmental heterogeneity. In different phylogenetic groups, changing environmental conditions induce stress often associated to a temporal suppression of sexuality and a shift to asexual reproduction (Neiman et al. 2014). This change of reproductive strategy, often referred to as facultative sexuality, produces both sexual and asexual seeds in the same generation in plants. Further, patterns of facultative and/or cyclical asexuality has been reported in different animals and plants capable of switching off sex, where individuals can produce offspring either sexually or asexually in the same or different generations. In higher plants the formation of asexual offspring (seeds) is done via a process called apomixis, and involves a complex developmental setup in which meiosis is converted into an apomeiosis and form unreduced female gametophytes, the egg cell develops by parthenogenesis into a clonal embryo, and the development of the endosperm may or may not require fertilization (Asker and Jerling 1992). Such developmental changes are associated to certain genetic and epigenetic backgrounds that fix apomixis transgenerationally (e.g. Grimanelli 2012, Rodriguez-Leal and Vielle-Calzada 2012, Verhoeven et al. 2018), except perhaps for the very early stages of the evolution in a new lineage (Hojsgaard 2018). Thus, in plants apomixis is not cyclical, but see possible cases of reversals from apomixis to sexuality (details in Hojsgaard et al. 2014a), though it is continuously expressed at different levels by facultative asexual individuals. Variable rates of sex (mostly low) had been found in different apomictic plant species (e.g. Espinoza et al. 2002, Bicknell and Koltunow 2004, Hojsgaard et al. 2013, Krahulcová et al. 2014) and are likely to have a genetic basis and be environmentally modulated. However, studies on natural populations are missing and experimental analyses of the influence of environmental factors on the incidence of apomixis and sexuality in individual plants are equivocal.

While most studies demonstrate a clear influence of different stressors on observed proportions of sexual and apomictic ovules, they have not analyzed or have failed to find any influence at seed and progeny levels. For example, Knox (1967) studying plants of *Dichanthium aristatum* artificially grown in a range of climatic conditions throughout 27 degrees of latitude revealed an association between photoperiods prevailing during development of the inflorescences and the proportion of apomixis. In a common garden experiment, Quarin (1986) found a similar quantitative response between the expression of apomixis and seasonal changes in length of day in *Paspalum cromyorrhizon* plants. Accordingly, experimental setups exposing facultative apomictic plants to diverse environmental stressors show an increase in the frequency of sexual gametophyte formation. For example, Gounaris et al. (1991) exposed daily apomictic

plants of *Cenchrus ciliaris* to a series of inorganic salts to observe abnormal features in pistils of salt-treated plants, including an increase in the number of sexual embryo sacs. Mateo De Arias (2015) found that some of the five apomictic *Boechera* species (and one sexual) subjected to drought stress and drought plus heat stress increased the frequency of sexual ovules significantly compared to those without stress, but did not find changes in the frequencies of sexual and apomictic seeds. Similarly, Klatt et al. (2016) grew different clones of the apomict *Ranunculus carpaticola* x *cassubicifolius* under a prolonged photoperiod and observed a significant increase in the frequency of ovules with functional meiotic megaspores yet without a significant increase in sexual seeds. Rodrigo et al. (2017) exposed apomict plants of *Eragrostis curvula* to drought stress conditions and showed that under water depravation, facultative apomictic plants increase the formation of sexual embryo sacs though without any influence on number of sexual offspring. Thus, even when varied environmental stressors including heat, drought, light and nutrients availability induce higher expression of sex during ovule development, their effects on increasing sexual offspring formation are still unclear.

In single ovules of many apomicts, both meiotic and apomictic pathways can run in parallel but they differ in spatiotemporal timing of developmental steps (e.g. Leblanc et al. 1995, Grimanelli et al. 2003). Flowers of apomictic plants exhibit highly asynchronous development and massive changes in gene expression patterns compared to sexual flowers (e.g. Sharbel et al. 2010, Pellino et al. 2013). Hence, modulation of sex during the development of the flowers in facultative apomictic plants is likely highly sensitive to environmental signals. Competition between meiotic and apomictic pathways within the ovule will affect the reproductive output and fitness of the plant. Studying different apomictic *Paspalum malacophylum* genotypes under homogeneous experimental conditions, Hojsgaard et al. (2013) showed gametophytic competition in ovules varies substantially among individuals, and observed a significant increase in efficiency of the apomictic pathway toward the formation of seeds and offspring. Yet, how environmental conditions affect the proportion of residual sexuality in natural populations of apomictic plants and its local and regional impact on plant fitness has not been analyzed.

In natural conditions, the existence of ecological modulation of sexual reproduction in otherwise clonal plants will provide a much-needed perspective on the question whether environmental variation facilitates the creation of genetic variability, local adaptation and survival of lineages traditionally condemned to extinction. In spite apomixis provides a colonizing advantage via uniparental reproduction (Baker's Law - Baker 1955) and clonality, without sex, apomictic plants will struggle to create the genotype diversity necessary for better use resources (Frozen Niche Variation Model - Vrijenhoek and Parker-Jr. 2009) and for local adaptation. Selection of genotypes best fitted to new conditions can promote niche shifts and departures from

areas of ecological competition to sexual counterparts (Karunarathne et al. 2018) endorsing range expansions (e.g. geographical parthenogenesis; Bierzychudek 1985, Hörandl 2006). In addition, sex is needed to purge clonal organisms from deleterious mutations (Muller´s ratchet; Muller 1964). Therefore, understanding whether rates of functional sex are environmentally modulated, its distribution at local and regional scales, and how functional reproductive pathways affect relative fitness in facultative apomictic plants will shed light on mechanistic causes determining the success of sexuality vs. asexuality in natural populations.

Here, I analyze levels of functional sexuality in geographically widespread populations of a facultative apomict under a variety of ecological conditions, and their relative contribution to plant fitness. I aim at 1) assessing the expression of sexuality in facultative apomictic populations, 2) evaluating the efficiency of both meiotic and apomictic pathways in the formation of seed offspring, 3) examining ecological and environmental factors possibly influencing the expression of sexuality, and 4) analyzing the impact of variable levels of sex and apomixis on maternal fitness at different geographic scales. For doing so, I use *Paspalum intermedium* Munro ex Morong, a caespitose perennial grass that grows in marshes and wetlands of South America, and has two cytotypes, self-sterile sexual diploids and self-fertile aposporous tetraploids (Norrmann et al. 1989, Ortiz et al. 2013). Both cytotypes co-occur in different combinations (i.e. allopatry, sympatry and parapatry) and are adapted to slightly different ecological settings (Karunarathne et al. 2018). Tetraploids display a wider tolerance to varied ecological conditions whereas diploids occupy only a fraction of the ecological niche of the species, and both cytotypes out-compete each other in their main distribution zones. Since northern tetraploids growing in sympatry to diploids are likely younger than southern allopatric tetraploids (Karunarathne et al. 2018), *P. intermedium* is an ideal model species to study how the environmental heterogeneity influence the expression of sexuality, plant fitness under diverse reproductive modes and ecological setups, and the release of variability that might contribute to the observed adaptive plasticity among apomictic populations.

3.3. MATERIALS AND METHODS

3.3.1. Plant materials and ploidy levels

Paspalum intermedium florets (spikelets) are bisexual consisting of an ovary containing one ovule and two feathery stigmas, and surrounded by three stamens. Florets are exclusively wind pollinated and grouped in racemes and inflorescences. *P. intermedium* plants do not propagate vegetatively, and flowering and fruiting occur from late October till early April.

A total of 39 *P. intermedium* populations were identified in two field trips during the beginning and end of the blooming season of the species (November-December and March, respectively). The distribution area of the populations covers most of the main distribution area of the species in Eastern Gran Chaco, central and Northern Mesopotamia as well as peripheral areas in Northern Pampas and Western Gran Chaco (Supplementary Data. Table S2.1). Around 30 individuals were used to characterize the ploidy level of each population by chromosome counts in mitotic root cells and by flow cytometry with leaf tissues according to Karunarathne et al. (2018). A total of 11 pure diploid, 24 pure tetraploid and four mixed-ploidy populations were identified (Suppl. Table S1; Karunarathne et al. 2018).

3.3.2. Common garden experiments

Three to five individuals per population were transplanted to a common environmental setting in experimental gardens at Instituto de Botánica del Nordeste (IBONE), National University of the Northeast, Argentina. Around 25 plants from 7 different populations were also keep under controlled temperature and humidity inside walk-in climate chambers (York, Pflanzenwuchskammer 1.305, ENGIE Deutscheland, Hamburg, Germany) at the Albrecht-von-Haller Institute for Plant Science, University of Goettingen, Germany; at temperuature range – 18-24 °C, photoperiod – 10-12 h/day, light intensity - 250 mmol/m^2/s, humidity – 80%.

3.3.3. Reproductive pathway analyses

The reproductive mode of three individuals per population was characterized at two developmental stages by using two methodologies, embryology and flow cytometry on seeds. *Embryological analysis*. Inflorescences at meiosis and anthesis of 27 *P. intermedium* populations were collected *in situ* during field explorations, fixed in FAA for 24 hours, transferred to 70% ethanol and storage at 4°C. Individual flowers were dissected under a Stereomicroscope (Leica M125; Leica Microsystems GmbH, Wetzlar, Germany), ovaries were cleared using Methyl Salicylate (Herr 1973) and analyzed under a DIC (Differential Interference Contrast) microscope (Leica DM5500B; Leica Microsystems GmbH, Wetzlar, Germany). A total of 1243 ovules which were fixed during male meiosis from randomly selected samples, were analyzed to check the type of gametophytic apomixis (i.e. diplospory or apospory). For evaluation of reproductive efficiency, distribution of sex and environmental modulation, between 10-20 ovules were examined from each individual and three individuals per population.

Flow cytometry seed analysis. Mature seeds from 20 *P. intermedium* natural populations and 5 populations from common garden experiments were collected under open pollination conditions. At least 30 seeds from each individual (accounting more than 100 seeds per population) were assessed following the protocol described in Karunarathne et al. (2018). Single seed histograms were produced with a Ploidy Analyzer (Sysmex-Partec GmbH, Görlitz, Germany) and were

analyzed with CyView™ data processing software (Sysmex Partec GmbH, Münster, Germany). The relative fluorescence of at least 3000 particles (nuclei) was measured for each sample seed and histogram peaks were assigned to embryo and endosperm tissues following the rationality described in Hojsgaard et al. (2013).

The mean peak values of relative DNA content (C-values) for embryo and endosperm seeds were used to determine their developmental pathways as to sexual or apomictic, where sexual seeds have a diploid embryo (2n; 2C-value: see Figure S2.1) and a triploid endosperm (3n; 3C-value: see Figure S2.1), while apomictic seeds bear a diploid embryo and a pentaploid endosperm (5n; 5C-value see Figure S2.1) (also see Karunarathne et al., 2018). Peak indeces of the embryo (G1) and endosperm (G3 or G5) peaks were also calculated a ratio of the latter to the former; peak index value of 1.32-1.73 represents a sexual seed and a peak index value of 2.28-2.78 represents apomictic seeds (see Supplementary Table S3.1) A maximum coefficient of variation (CV) value of 5% was accepted for each sample peak. The raw data of histograms are stored on the network server of the Department of Systematics, Biodiversity and Evolution of Plants, Albrecht-von-Haller Institute for Plant Sciences, University of Göttingen.

3.3.4. Fecundity (seed set) and fertility (offspring) assessment

Seed set. The number of seeds produced throughout the season was used as a measure of fecundity (Begon et al. 2006, Burns et al. 2013). Thus, fecundity was estimated as the average number of seeds produced per population. During flowering, once all spikelets were in anthesis, three to six inflorescences from each individual were bagged using Sulphite paper crossing bags (Baumann Saatzuchtbedarf GmbH, Waldenburg, Germany), . One month after bagging, the inflorescences were collected and full and empty spikelets (seeds with and without caryopses) were sorted out in two groups using a 757 South Dakota Seed Blower (SeedBuro Equipment Company, Illinois, USA). The total number of full and empty seeds was estimated by weighing three sets of hundred seeds from each inflorescence, averaging and extrapolating that value to the total weight of full and empty seed groups per individual. The total number of inflorescences was recorded throughout the flowering season and used to calculate the number of flowers (ovules) and the number of seeds produced by each individual and population.

Offspring. Fertility, as the capability to produce offspring (Begon et al. 2006), was determined by the number of seedlings produced by diploid and tetraploid individuals after seed germination tests. Seeds from three individuals per population and a total of 30 populations were sown in sterilized soil and kept in a glasshouse under same light, temperature and water regime. Germination ability was checked every second day during 60 days, and indices of germination power and germinability were estimated from the number of seedlings and the total number of caryopses sown for each individual and population.

3.3.5. Reproductive pathway efficiency and maternal fitness

The efficiency of each reproductive pathway (sexual and apomictic) in tetraploid plants was calculated as the ratio between the observed and the expected proportions of spikelets undergoing the meiotic or the apomictic pathway (Hojsgaard et al. 2013). The observed proportion of embryo sacs was estimated as ***nm/(nm+na)*** for the meiotic pathway and ***na/(nm+na)*** for the apomictic pathway, where ***nm*** is the total number of ovules with a meiotic embryo sac (MES), and ***na*** is the total number of ovules with apomictic embryo sacs (AES). A similar formula was used for estimating proportions of sexual and apomictic seeds. The expected proportion were calculated from the data observed in the previous developmental stage, using the formulas ***nm + 0.5 nma/nt*** and ***na + 0.5 nma/nt*** for meiotic and apomictic pathways respectively; where ***nma*** is the number of observed ovules with both meiotic and apomictic pathways and ***nt*** is the total number of ovules analyzed. In our analysis, it was assumed that i) MES and AES occur independent from each other, and ii) they have the same probability of successful development into a seed. A standard Pearson's Chi-squared test was performed on both meiotic and apomictic observed and expected values to check for significant differences. Further, a paired t-test was performed on the mean difference between the observed and expected proportions of sexual and apomictic pathways in both developmental stages.

For the analyses of fitness, I focused on the maternal fitness. The effect of paternal fitness was considered negligible because 1) tetraploid apomicts in *P. intermedium* are self-fertile and self-pollinated, 2) male gametes do not contribute to the formation of parthenogenetic embryos in apomictic seeds although it contributeds to the endosperm formation; endosperm imbalance however, is tolerated in *P.intermedium* (Hojsgaard et al. 2008), and 3) the maternal genotype and maternal environments are both known to affect offspring performance (e.g. maternal plants producing bigger seeds have more resources and hence have advantages in germination and/or establishment; e.g. Primack and Kang 1989). Estimation of maternal fitness potential context-dependent effects on rates of self-fertilization or inbreeding depression are skipped by apomictic progenies, and they might affect only sexual progenies. A cost of self-fertilization in terms of pollen fitness (i.e. pollen discounting; Barrett 2003), is not expected as anthers dehisce at the opening of the floret and each anther in *Paspalum* spp. carries around 800-1,000 pollen grains (Hojsgaard, personal communication), hence pollen availability was considered to be high. Therefore, in *P. intermedium*, fitness estimates based on seed quantity and germinability are expected to effectively reflect plant´s fitness.

Measures of differential reproductive success or maternal fitness were therefore estimated as a product of *fecundity* x *fertility* for each individual and population. These values were used for within and among populations comparisons of relative plant fitness assuming that

individual plants producing more seeds and seedlings contribute more offspring to the next generation than a plant producing few seeds and seedlings does. Although I admit that this approach disregards selective pressure acting upon the establishment of seedlings in natural conditions, an *in situ* test of germinability was not possible due to the extensive distribution of the species.

3.3.6. Ecological, spatial and seasonal effects on reproductive modes

Data for ecological/environmental analysis were downloaded from open source data bases: 19 bioclimatic variables downloaded from WorldClim (1950–2000; version 1.4, Hijmans et al. 2005; www.worldclim.org), UV-B radiation downloaded from glUV (a global UV-B radiation data set for macroecological studies, Beckmann et al. 2014; www.ufz.de/gluv), and photosynthetically active radiation (PAR) data was downloaded from Moderate Resolution Imaging Spectroradiometer (MODIS) database (Myneni et al. 2015; https://lpdaac.usgs.gov), and cloud cover, frost day frequency, and vapor pressure at ground level were downloaded from CGIAR CSI (www.cgiar-csi.org). These data were downloaded as raster grid files either at 2.5 arc minute resolution or (dis)aggregated to match 2.5 arc minute resolution. The environmental data for each population was extracted from these raster layers using the R package *dismo* (Hijmans et al. 2016).

Pearson-Correlation tests were performed between the environmental variables (explanatory variables) and the expression of meiotic and apomictic pathways (response variables) both at blooming (mature embryo sac) and seed stages. A generalized linear model (GLM) was applied to the explanatory variables showing significant correlation to observations (meiotic and apomictic pathways). Since the fitted values of the GLM exhibited a nonlinear pattern of the observed mean values, a nonlinear regression model was used to determine best-fitting parameters and predict responses of reproductive modes. Parameter estimates of the nonlinear regression formula were determined as the parameter values providing the best fit for the mean function in relation to the observations (in this case apomictic/meiotic proportions). The estimation was obtained by minimization of the residual sums of squares (RSS) (Venables and Ripley 2002). Once the estimation of start values was established, a grid search was performed to find the RSS values for a coarse grid based on the (above) estimated ranges of the parameter values. The parameter values that yield the smallest number of RSS in the grid search were used as the starting values for the nonlinear regression analysis (see supplementary information for a detailed explanation). The function *nls2* of the R package nls2 (Grothendieck 2013) was used for the grid search and the nonlinear regression. The function curve in stats R package (R Core Team 2016) was used to add the curve described by the mean function to the plot. A nonparametric bootstrap analysis of 10000

replicates was performed to test the significance of the gradient value obtained for the mean function.

For analysis of seasonal effects on the incidence of reproductive pathways and on reproductive mode efficiency, sampling of flowers and seeds were grouped in two time periods (early season: November-December; and late season: February-March), and a paired t-test and standard Pearson's Chi-squared test were performed on the relevant data.

3.4. RESULTS

3.4.1. Ploidy and reproductive mode evaluation of *P. intermedium* cytotypes

Reproductive pathways were assessed in over 1181 mature ovules of *P. intermedium* from 21 pure tetraploid populations, four pure diploid populations and three mixed-ploidy populations. Ovules analyzed during male meiosis showed that apomixis is initiated from a nucellar cell surrounding the germline. Meiotic and apomictic female gametophytes (MES and AES) were differentiated by their anatomical characteristics. MES consist of an egg cell flanked by two synergids at the micropylar area, a central cell with two polar nuclei, and three to several antipodal cells at the chalazal area (see Figure 3.1). AES contain an egg cell, one or two synergids and a central cell with two (or three) polar nuclei. The absence of antipodal cells is characteristic to AES (Figure 3.1).

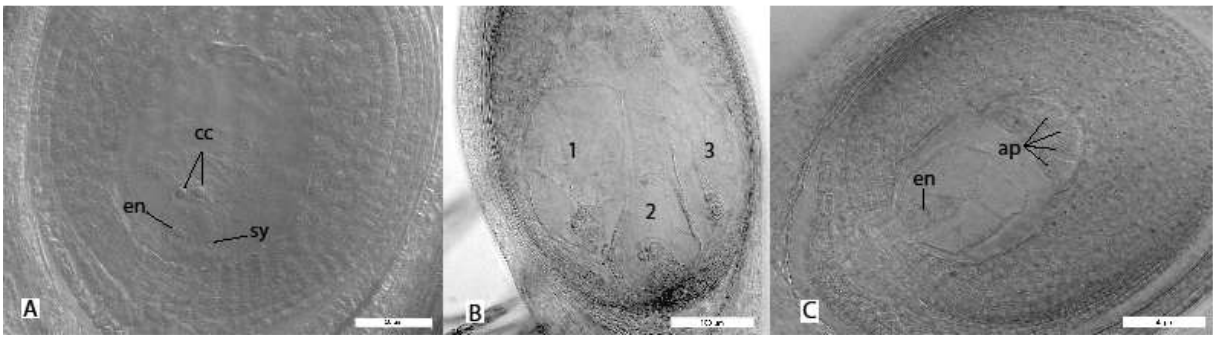

Figure 3.1. Microscopic images of meiotic and apomictic ovules of *P. intermedium* at maturity showing the anatomical differences; A. Apomictic embryo sac of tetraploid, B. Multiple embryo sacs of both meiotic and apomictic origin coexisting in the same ovule (1,3-apomictic, 2-meiotic), C. Diploid meiotic embryo sac. (en – embryo nucleus, cc – central cells, sy – synergids, ap – antipodals)

All ovules from diploid plants were bearing single MES except for one ovule which had two ES carrying antipodal cells. In tetraploid plants, three kinds of ovules were found: i) ovules carrying only MES, ii) ovules carrying only AES, and iii) ovules carrying MES + AES. In ca. 40% of ovules with AES and MES + AES more than one AES were observed. ES aborted or in abortion of

meiotic and apomictic origin were observed, and in a few individual ovules (ca. <1%) no ES was detected. The overall percentages of MES and AES for all tetraploid individuals were 32.7% and 63.6%, respectively. Nevertheless, values of MES and AES varied immensely among all the studied populations (Table 3.1).

Flow cytometry histograms were generated for over 1500 seeds of *P. intermedium* from 14 pure tetraploid populations, four pure diploid populations, and three mixed populations. Histogram analyses revealed all seeds from diploid plants were of sexual origin (having an embryo to endosperm ploidy ratio of 2:3) while among tetraploid plants a broad variation in the proportion of sexual and apomictic (embryo to endosperm ploidy ratio of 2:5) seeds was observed (see details in Table 3.1 and Figure S2.1), with overall average values of ca. 18% sexual and ca. 82% apomictic seeds.

From both embryological assessment and flow cytometric analysis of seeds, it is evident that diploids are exclusively sexual and auto-tetraploids of *P. intermedium* are facultative apomictic. This observation agrees with previous studies on the species itself (Norrmann et al. 1989, Karunarathne et al. 2018).

3.4.1. Efficiency and competition between reproductive pathways

The observed proportions of meiotic (and apomictic) reproductive pathways at ES and seed stages showed a significant difference among all populations (*p-value* for the *paired t-test* = 0.009). Significant differences were also found in comparisons within populations (chi-squared test $\chi^2 <$ 6.11 and *p-value* < 0.013 in all populations) except for two populations (Hojs402 and Hojs478; Table 3.2). The overall proportion of sexual seeds exhibit a significant reduction from the expected 38.2% to the observed 15.3% (*p-value* = 0.0017, $\chi^2 = 9.8478$), while the proportion of apomictic seeds showed a substantial increase from the expected 61.8% to the observed 84.7% (*p-value* = 0.049, $\chi^2 = 3.594$) (Table 3.2). At population level, most differences between expected and observed values were significant (Table 3.2), the highest being 42.4% and 40.2% for apomictic and sexual pathways respectively (Hoj465/2R; Table 3.2), while the lowest were 0.4% for the apomictic and 0.3% for the sexual pathways (not significant; *p-value* > 0.90). In all the studied populations, a reduction in reproductive efficiency of the sexual pathway between proportion in ovules and seeds was observed, ranging from 0.16 to 0.98 (Table 3.2). Conversely, in the apomictic pathway, an increase from 1.0 to 2.07 was observed (Table 3.2: small table with the efficiency analysis). This increase of apomictic pathway efficiency showed a positive correlation (Pearson correlation $r = 0.50$) to the number of ovules with MES + AES, suggesting a competition between reproductive strategies ovules with parallel meiotic and apomictic developments that was realized in seeds toward the apomictic pathway.

Table 3.1. Proportions of meiotic/sexual and apomictic at embryo sac and seed stages in studied populations of *P.intermedium*. Proportions were calculated as a fraction of all the observed ES/seeds. Chi-squared values were calculated for both meiotic/sexual and apomictic proportions with the assumption that the same

Population	Pop. ploidy	ES proportion		Seeds proportion		Chi-squared test for observed values	
		Meiotic	*Apomictic*	*sexual*	*Apomictic*	χ^2	*p-value*
Hoj402/1C	4	0.161	0.839	0.169	0.831	0.0530	0.817
Hoj403/1D	4	0.318	0.591	0.068	0.932	34.906	<0.001
Hoj404/1E	4	0.333	0.667	0.076	0.924	51.020	<0.001
Hoj405/1F	4	0.385	0.615	0.100	0.900	34.305	<0.001
Hoj409/1H	4	0.267	0.733	0.061	0.939	21.767	<0.001
Hoj410/1I	4	0.327	0.673	0.263	0.737	1.8500	0.174
Hoj414/1J	4	0.050	0.950	0.067	0.933	0.1210	0.780
Hoj415/1K	4	0.250	0.750	0.143	0.857	6.1180	0.013
Hoj424/1S	4	0.333	0.667	0.129	0.871	18.810	<0.001
Hoj445/2H	4	0.433	0.567	0.096	0.904	46.148	<0.001
Hoj453/2Ñ	4	0.466	0.534	0.167	0.833	31.260	<0.001
Hoj455/2P	4	0.383	0.617	0.225	0.775	10.511	0.001
Hoj456/2Q	2,4	0.444	0.556	0.235	0.765	17.644	<0.001
Hoj465/2R	4	0.500	0.500	0.076	0.924	71.978	<0.001
Hoj470/2T	2,3,4	0.725	0.275	0.333	0.667	76.955	<0.001
Hoj475/2U	4	0.396	0.604	0.184	0.816	18.844	<0.001
Hoj478/2V	4	0.063	0.938	0.037	0.963	1.1410	0.286
Hoj471/2X	4	0.360	0.640	0.172	0.828	15.357	<0.001
Hoj468/2S	2	0.980	0.000	1.000	0.000		
Hoj422/1Q	2	0.990	0.000	1.000	0.000		
M26/1X	2	0.990	0.000	1.000	0.000		
M31/1W	2	0.990	0.000	1.000	0.000		

proportion of each reproductive pathway is expected at the seed stage as in the ES stage

In mixed ploidy populations where both diploid and tetraploid individuals of *P. intermedium* grow together, tetraploids showed considerably different proportions of sexual and apomictic pathways compared to those of tetraploids in pure populations. While in mixed populations the average of ovules with MES was 45% (ranging from 33% to 51%), in pure tetraploid populations it was 34.1% (ranging from 7% to 41%). At the seed stage, the same mixed populations showed a proportion of sexual seeds (21%) similar to that of pure tetraploid populations (ca. 18%).

3.4.2. Fitness variation in diploids and tetraploids cytotypes

Fecundity assessments show that the average number of spikelets produced per inflorescence is similar between diploid and tetraploid populations (4805.18 florets in diploids and 4348.87 in tetraploids; Table 3.3). However, the percentage of full seeds in diploids is twice as high as in tetraploids (32.61% in diploids versus 15.83% in tetraploids; Table 3.3). *Fertility* measured by germinability tests also showed similar values in both diploid and tetraploid individuals and populations. As a result, relative fitness between diploid and tetraploid individuals and populations reflects approximately the relative proportions of seed set, with diploids having a two folds higher reproductive fitness (f_{2x}=0.276) compared to that of tetraploids (f_{4x}=0.135).

The regional evaluation of fitness values of different populations (Table 3.3) indicates that tetraploids have lower fecundity in Southern areas (14.43%) compared to the Northern and Central areas, while diploids in these areas maintain similar values (Table 3.3). Interestingly, the pure populations of both cytotypes in the Central region show the highest values of fecundity (37.04% for diploids and 19.99% for tetraploids) and fitness (0.435 in diploids and 0.174 in 4xtetraploids). In mixed-ploidy populations, diploid individuals showed the lowest fitness values (0.031) among all diploid populations evaluated, while local tetraploids surpassed the fitness of diploids having almost a 10 fold higher fitness (0.293) (Table 3.3). The only triploid found in one of the mixed-ploidy populations surprisingly showed the highest fecundity value (39.55%) observed among all cytotypes although its fitness was very low (0.039), mainly due to a reduced number of inflorescences and low germinability (Table 3.3).

3.4.3. Climatic variation and incidence of reproductive pathways

Initial scatter plots of each environmental variable verses proportions of apomictic and sexual pathways showed no visible patterns in all but one variable. A Pearson correlation test confirmed a moderate correlation between Mean Diurnal Range (MDR; Mean difference of monthly maximum and minimum temperatures) and reproductive pathways at ovule stages (r=0.69 for sex; r= -0.67 for apomictic) (Figure 3.2; Supplementary Table S3.2: All climatic-reproductive correlations must be in a table). This correlation is inversed between reproductive

pathways, meaning that sexual gametophytes proportions increased proportionally to MDR while apomictic gametophytes decreased.

Accordingly, proportions of sexual and apomictic seeds showed a similar behavior and sexual seeds increased with MDR (r=55 for sex) and apomictic seeds decreased with MDR (r = -0.56 for apomictic) (Figure 3.2; Table S3.2). Two other bioclimatic variables (Bio5 and Bio8) which showed strong association to MDR (r = 0.81 and r = 0.78, respectively; Supplementary Table S3.2) were weakly correlated (r < 0.41 in all combinations) to reproductive proportions at both developmental stages. Therefore, these two variables were not considered for further analysis.

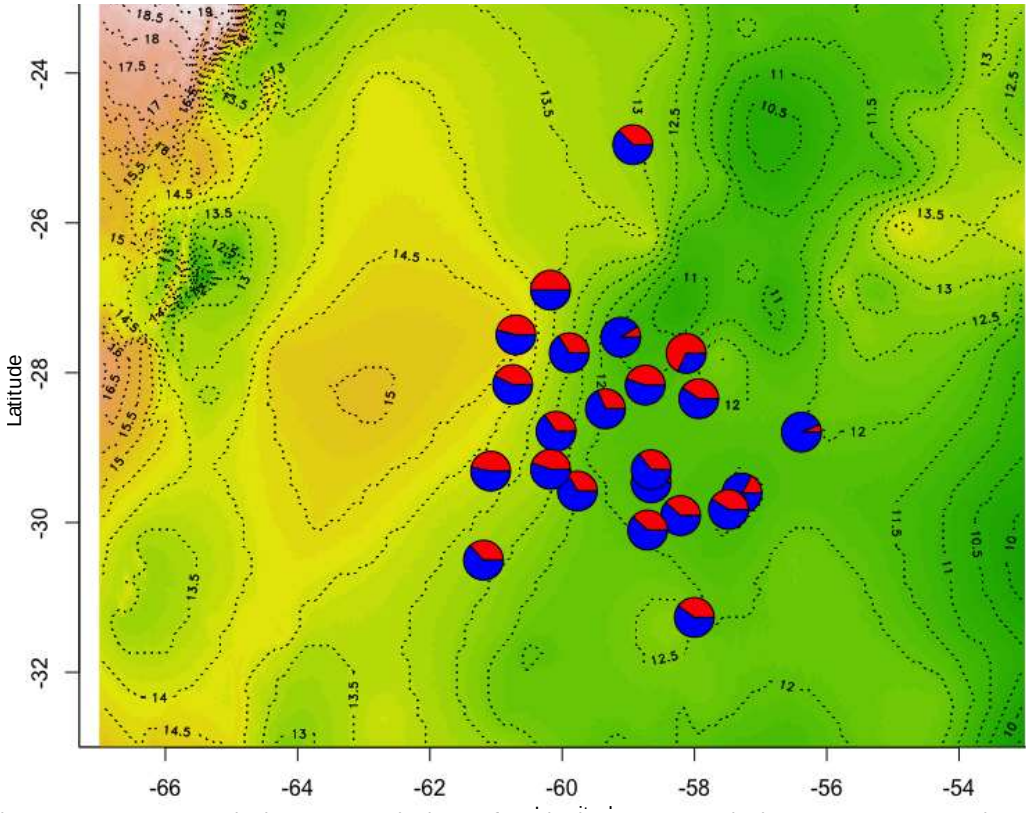

Figure 3.2. Map depicting the variation of meiotic and apomictic ES percentages in the studied apomictic tetraploid populations of *P.intermedium* with the mean diurnal range (MDR); Contours demarcate the MDR variation zones: temperature changes in Celsius; pies: blue – apomictic, red – meiotic.

We performed a Generalized Linear Model (GLM) with Gaussian inverse link on the diurnal variation data and the proportions of apomictic and meiotic reproductive pathways at the two developmental stages. The overall values show a negative relationship between MDR and the occurrence of AES (t = 4.18, *p-value* = 0.0006) and apomictic seeds (t = 2.324, *p-value* = 0.03) and a positive influence between MDR and the meiotic pathway (t = -4.18, *p-value* = 0.0006). Within-population analysis showed a significant influence of MDR on the proportion of AES and apomictic seeds in all cases with a stronger effect at mid MDR values (*t-test* for GLM, *p-value* = 0.013) and low at high MDR values (Figure 3.3). This indicates that reproductive modes are sensitive to and modulated by the environment.

Table 3.2. Reproductive pathway efficiency analysis of studied populations of *P.intermedium*. The expected and observed proportion values were calculated using the formulae described in Hojsgaard et al. (2013); observed values are the observed proportions of sexual and apomictic seeds and expected values were calculated using the observed proportions of meiotic and apomictic pathways at ES stage (also see the materials and methods). Chi-squared values were calculated for these observed and expected proportions. Reproductive mode efficiency was calculated as a ratio of observed to expected values for both pathways

Population	Population ploidy	Reproductive Mode proportions				Chi-squared for ex. vs obs. values		Reprod. Mode efficiency (obs. /ex.)	
		Exp. Sex	*Obs. Sex*	*exp. Ap.*	*obs. Ap.*	χ^2	*p-value*	*Sex*	*Ap.*
Hoj402/1C	4	0.173	0.169	0.827	0.831	0.0110	0.916	0.981	1.004
Hoj403/1D	4	0.415	0.068	0.585	0.932	49.597	<0.001	0.164	1.594
Hoj404/1E	4	0.387	0.076	0.613	0.924	38.126	<0.001	0.196	1.507
Hoj405/1F	4	0.401	0.100	0.599	0.900	37.719	<0.001	0.249	1.503
Hoj409/1H	4	0.307	0.061	0.693	0.939	28.445	<0.001	0.198	1.355
Hoj410/1I	4	0.356	0.263	0.644	0.737	3.7730	0.042	0.740	1.144
Hoj414/1J	4	0.093	0.067	0.907	0.933	3.0140	0.031	0.719	1.029
Hoj415/1K	4	0.318	0.143	0.682	0.857	14.121	<0.001	0.450	1.256
Hoj424/1S	4	0.376	0.129	0.624	0.871	26.003	<0.001	0.342	1.397
Hoj445/2H	4	0.448	0.096	0.552	0.904	50.104	<0.001	0.215	1.636
Hoj453/2Ñ	4	0.469	0.167	0.531	0.833	44.751	<0.001	0.355	1.569
Hoj455/2P	4	0.429	0.225	0.571	0.775	16.989	<0.001	0.525	1.358
Hoj456/2Q	2,4	0.463	0.235	0.537	0.765	20.908	<0.001	0.509	1.423
Hoj465/2R	4	0.500	0.076	0.500	0.924	71.910	<0.001	0.152	1.848
Hoj470/2T	2,3,4	0.678	0.333	0.322	0.667	54.520	<0.001	0.491	2.072
Hoj475/2U	4	0.400	0.184	0.600	0.816	19.440	<0.001	0.459	1.361
Hoj478/2V	4	0.063	0.037	0.938	0.963	1.0750	0.300	0.593	1.027
Hoj471/2X	4	0.407	0.172	0.593	0.828	22.882	<0.001	0.423	1.396

Table 3.3. Analysis of reproductive mode fitness in all the studied populations of *P.intermedium*. The table presents i) the overall fitness of each cytotype from all the locations as average values and ii) relative fitness of the two cytotypes separated by the region of occurrence; the regions separation is according to Karunarathne et al. (2018).

	Seed set (full seed %)		No. of spikelets /inflorescence		No. of inflorescence /individual		Germinability (fertility)		Relative fitness	Relative fitness /population
OVERALL FITNESS		*SE*		*SE*		*SE*		*SE*		
2x	32.6	3.15	4805.2	360.6	39.2	5.13	0.739	0.025	0.276	0.275
4x	15.8	1.51	4348.9	296.8	31.9	2.70	0.791	0.017	0.135	0.129
REGIONAL FITNESS										
Northern										
2x	31.2	14.97	5261.3	436.6	35.8	6.49	0.702	0.041	0.255	0.267
4x	18.7	8.32	4349.3	412.4	34.2	5.99	0.760	0.029	0.137	0.128
Central										
2x	29.1	4.74	4381.7	556.2	42.4	7.81	0.774	0.026	0.296	0.281
4x	18.1	3.25	4555.9	537.5	33.9	4.67	0.742	0.028	0.154	0.146
Southern										
2x	-	-	-	-	-	-	-	-	-	-
4x	14.4	2.05	4219.8	461.5	32.6	4.59	0.829	0.028	0.135	0.152
Mix pop. in central										
2x	10.2	1.26	3934.4	691.1	14.7	2.91	0.829	0.087	0.031	0.031
3x	39.5	12.65	2590.0	1586.0	8.0	2.00	0.710	0.000	0.039	0.039
4x	15.0	7.62	4640.4	745.1	27.6	10.81	0.704	0.063	0.293	0.197
Pure pop. in central										
2x	37.0	5.69	4026.0	599.8	58.7	8.22	0.787	0.020	0.435	0.435
3x	-	-	-	-	-	-	-	-	-	-
4x	20.0	3.39	4433.7	590.5	36.9	4.77	0.768	0.024	0.174	0.174

SE – standard error

In order to better understand the ecological influence on reproductive modes and to model its possible responses, a nonlinear function for the observed expression of meiotic and apomictic pathways and the MDR was formulated. The nonlinear equation best explaining our results takes the form of an exponential increase/decay with a horizontal asymptote. For apomixis ES proportion, it can be written as

$$A_{(d)} = A_m + A_0 \bullet e\,(-k^{(d-\,d0)})$$

Where, A_m is the minimum AES%, A_0 is the maximum AES%, k is the gradient constant, d is diurnal variation (MDR) and the d_0 is the temperature at which AES% is 100%. The values obtained from the grid search (see Materials and Methods) were, A_m = 30%, k = 0.21 and d_0 = 7.8; A_0 was assumed to be 100 as it is the maximum theoretical AES% possible. Therefore, the equation can be written as

$$A_{(d)} = 30 + 100 \bullet e\,(-0.21^{(d-7.8)}) \quad \ldots\ldots\ldots\ldots\ldots\ldots \text{ (see Figure 3.4)}$$

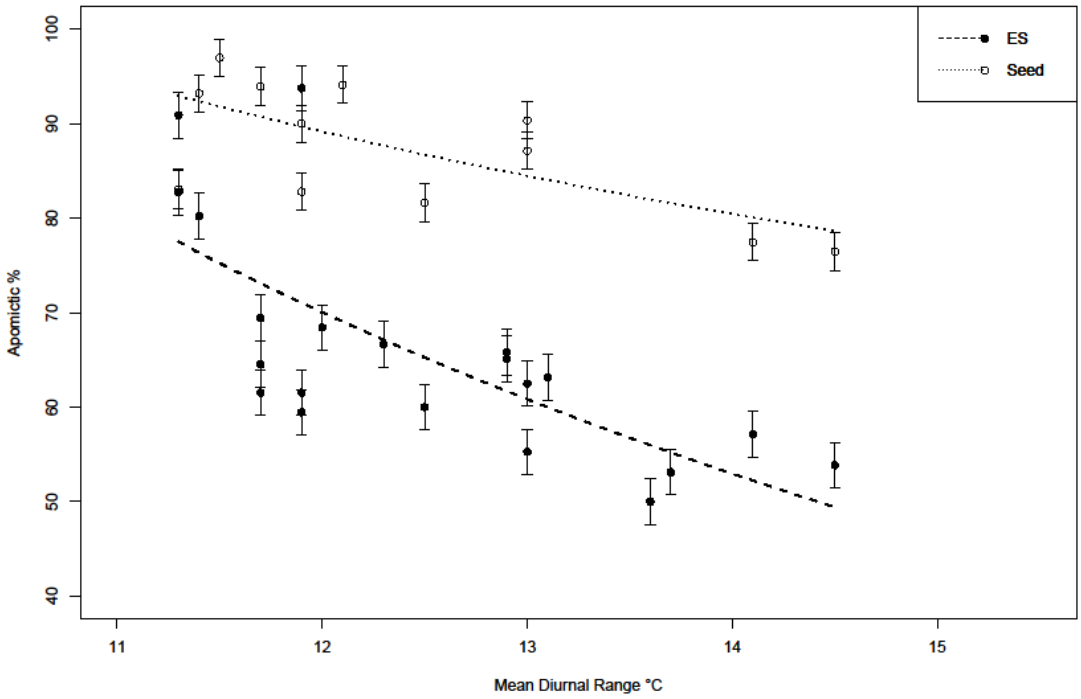

Figure 3.3. GLM plot of the fitted values for apomictic proportions at both embryo sacs and seed stages in all the studied *P. intermedium* populations; whiskers indicate the standard error.

3.4.4. Seasonal changes in meiotic and apomictic incidence

A paired *t-Test* between the mean proportion of MES (and AES) for each population collected at two seasons (see materials and methods) showed a significant difference (Apomictic: t = 2.99, df = 16.591, *p-value* = 0.008; Meiotic: t = -2.4566, df = 16.296, *p-value* = 0.0256) indicating a seasonal variation in the expression of apomictic and meiotic pathways, likely associated to climatic variation. However, the proportion of sexual and apomictic seeds showed no significant variations (t = 1.0655, df = 12.022, *p-value* = 0.3076). The changes observed in reproductive efficiency of each apomictic and meiotic pathways at ovule and seed stages were not significant between seasons (early season: χ^2 = 42, df = 36, *p-value* = 0.227; late season: χ^2 = 56, df = 49, *p-value* = 0.2289). This indicates that reproductive modes are affected by the seasonal changes only at the ovule development stage.

3.5. DISCUSSION

Asexuality is usually assumed to have a two-fold fitness advantage compare to sexuals (e.g. silvertown 2008). Differences in the reproductive mode and the degree of their expression is highly variable at both within and among populations in the studied *Paspalum intermedium* populations. Facultative apomixis in these populations are highly influenced by the environmental factors leading to the differences in the expression of apomixis from the embryo sac stage to the seeds.

3.5.1. Reproductive variability in *Paspalum intermedium*

The genus *Paspalum* is characterized by a large variation in reproductive systems categorized by their ploidy levels and reproductive modes (Ortiz et al. 2013). As in many *Paspalum* species and most studied apomictic plant systems in angiosperms, *P. intermedium* shows a reproductive dimorphism linked to different chromosomal races. Diploid cytotypes are self-sterile obligate sexuals and derived tetraploid cytotypes are self-fertile apomicts (Norrmann et al. 1989, Karunarathne et al. 2018). The type of apomixis found in *P. intermedium* is aposporous, implying that the asexual pathway is independent from the meiotic one as it develops from a somatic nucellar cell surrounding the embryo sac. In agreement with various reproductive studies on individual plants from different species (Quarin 1992, Urbani et al. 2002, Hojsgaard et al. 2008, Siena et al. 2008, Sartor et al. 2011, Cosendai et al. 2013), my population level analysis covering most of species distribution show diploids had a highly stable sexual reproductive mode while polyploids show variable incidence of both sexuality and apomixis along the distribution area. I found levels of sexuality and apomixis in ovules ranging from 6-68% and 32-94% respectively, while at seeds variation was 3-33% for sex and 67-96% for apomixis. The analysis

of reproductive modes revealed the expression of sexuality and apomixis in tetraploid facultative apomictic *P. intermedium* plants is geographically structured to a certain extent (see details below). So far, all studies on facultative apomictic plants have suggested the allocation to sexual or asexual seeds is determined by genotype-by-environment interactions (e.g. Aliyu et al. 2010, Hojsgaard et al. 2013, Molins et al. 2014, Schinkel et al. 2016a).

3.5.2. Apomictic pathway efficacy excels at a cost of depleted fitness

Seeds coming from common garden experiments

Despite the large variation observed among *P. intermedium* populations at the levels of facultativeness between the two developmental stages, the proportion of apomixis significantly increased in most cases at the expenses of sexuality, as indicated by the reproductive mode efficiency values for both pathways. Many spikelets (63% of the total) presented two or more apomictic embryo sacs inside the same ovule, suggesting a strong penetrance of the trait which may explain its higher reproductive efficiency. Another factor that might provide an advantage for the apomictic pathway against the sexual one is the higher ploidy of the former. Selection is more effective eliminating deleterious recessive mutations in haploid organisms than diploids because of masking effects (Otto and Gerstein 2008). While there is no chromosomal reduction in apomictically derived gametophytes, meiotic gametophytes are haploid and more likely to expose deleterious mutations and developmental problems. Yet, since plants are tetraploids, even meiotically reduced gametophytes have at least two copies for each locus thus masking effects are expected.

Another relevant factor likely influencing reproductive pathway efficiency relates to the embryo sac competition for space within the ovule. The orientation of the embryo sacs within the ovule is not random (e.g. Willemse and van Went 1984, Hojsgaard et al. 2013) and while dislocated aposporous embryo sacs toward the chalazal zone and closer to the funiculus might have a more direct access to resources from the sporophytic tissue, most meiotic embryo sacs had a well-developed egg-apparatus and synergid cells with a filiform apparatus well inserted in the micropylar end of the ovule, and therefore they are conveniently positioned for pollen tube access.

The development of meiotic female gametophytes into sexual seeds was drastically reduced. Accordingly, the observation of a strict association between apomixis efficiency and the number of ovules with both meiotic and apomictic embryo sacs indicates the sexual pathway in ovules of *P. intermedium* is handicapped and likely failed to form seeds in most cases. A similar observation was reported in five *Paspalum malacophyllum* genotypes showing a complete depletion of sexuality from ovules to adult progenies (Hojsgaard et al. 2014a). The most probable explanation is likely linked to the genetic nature of the trait. Apomixis is known to be caused by a

de-regulation of the sexual program upon which apomixis is superimposed (Hand and Koltunow 2014). Massive up- and down-regulation of genes during megaspore and embryo sac formations characterize apomictic ovules of all studied species in different plant genera, including *Boechera* spp (Sharbel et al. 2010), *Hieracium* spp. (Rabiger et al. 2016), *Ranunculus* spp. (Pellino et al. 2013), *Hypericum* spp. (Galla et al. 2015), *Pennisetum* spp. (Akiyama et al. 2004), and *Paspalum* spp. (Polegri et al. 2010, Ortiz et al. 2017).

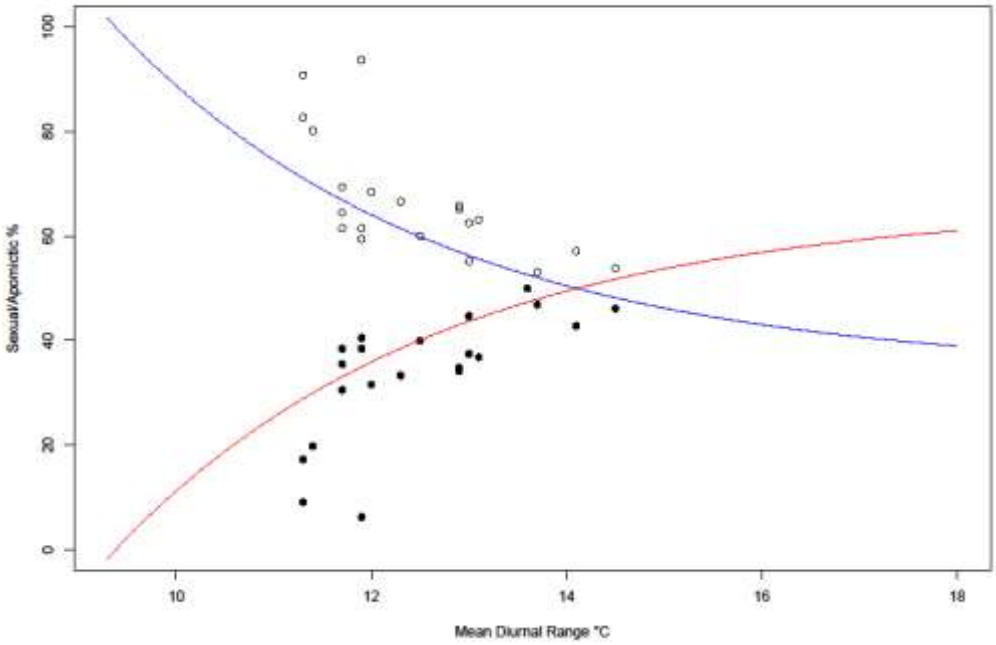

Figure 3.4. Nonlinear curve fitting of the mean functions ($A_{(d)} = 30 + 100 \bullet e^{(-0.21^{(d-7.8)})}$) for meiotic and apomictic embryo sac proportions of *P.intermedium*, observed under different mean diurnal range values in the studied area.

Even though the type of apomixis in *P. intermedium* is aposporous implying that both meiotic and apomictic pathways are initiated from independent cell types inside the ovule, the inherent genetic changes causing apomixis are likely destabilizing sexual development. This seems to be also the reason of the observed upsurge in the number of aborted ovules that fail to produce seeds (whether sexual and/or asexual) and cause a sharp reduction in fitness of tetraploid apomicts compared to sexual diploids in pure populations along the species distribution. Likewise, Schinkel et al. (2016) reported that obligate sexual individuals of *Ranunculus kuepferi* produce higher number of achenes compared to the apomictic individuals. In *P. intermedium*, I found a direct link between the incidence of apomixis, ovule abortion and reduced seed set in most natural populations. The only exception is in those cases were diploids growing in sympatry with polyploids show a remarkable depletion of fitness. Since *P. intermedium* is wind-pollinated, it is

not expect to have pre-mating barriers to crossing in nature. Like in other sexual-apomictic complexes, apomictic embryo sacs are recalcitrant to hybridization because the unreduced egg-cell develops parthenogenetically. In addition, maternal-to-paternal genome contribution to the development of the endosperm are relaxed in apomicts (e.g. Talent 2009), including *Paspalum* spp. (Quarin 1999). *P. intermedium* is a pseudogamous apomict, i.e. central cell fertilization is needed for developing a functional seed. Therefore, in mixed-ploidy populations, introgression of pollen from diploids into ovules of tetraploids will be avoided by parthenogenesis without affecting perceptively the development of the endosperm and a functional seed (endosperm imbalance is tolarated in the genus *Paspalum*, Sartor et al. 2011). In contraposition, introgression of pollen from tetraploids into diploids will create a triploid zygote and an endosperm with incompatible maternal-to-paternal contribution (i.e. triploid block) to sexual seed development (Köhler et al. 2010). Thus, in mixed-ploidy populations of *P. intermedium*, unidirectional introgression from polyploid apomicts to diploid sexuals is expected to increase number of ineffective matings and non-viable progeny with dramatic consequences on plant fitness. Experimental crossings will help us to test this hypothesis.

3.5.3. A reproductive *TUG OF WAR*: environmental stimuli versus reproductive efficacy/genetic background

Flowering is controlled by environmental conditions and an intricate network of regulatory pathways that play important roles in flowering-time control, like photoreception, circadian clock regulation, growth regulator synthesis and response, chromatin structure, and response to low temperatures (Mouradov et al. 2002). Adaptive responses to cold seasonal climates (including cold acclimation, freezing tolerance, endodormancy, and vernalization) point to an evolutionary lability of such traits and a potential role for local adaptation in response to climate change (Preston and Sandve 2013). The relevance of such lability can be exemplified with the observed niche transition that enabled the evolution of seasonal cold tolerance within the Pooideae grass family and supported its extensive radiation within temperate regions (Zhong et al. 2018). Sexuality is intrinsically associated to flowering in most angiosperms: the sexual development of ovules and anthers and the formation of a sexual seed and offspring. However, in facultative apomictic plants, apomixis emerges as a parallel alternative to sexuality, where both sexual (meiotic) and apomictic developmental programs can be activated simultaneously within a single ovule and compete to produce a seed (Hojsgaard et al. 2013). Hence, the environmental conditions that affect flowering will also influence the molecular interaction between meiotic and apomictic developmental programs, and the output will depend on how such conditions will favor one pathway against the other, determining the incidence of sexuality versus apomixis.

Apomixis behaves as a dominant trait against sexuality. In *Paspalum*, the genetic factors responsible for apomixis are located in a large chromosomal region recalcitrant to recombination that is inherited as a block (e.g. Pupilli et al. 2004, Hojsgaard et al. 2011). A similar situation is observed in other apomictic grasses (e.g. Akiyama et al., 2011). Genetic analysis of DNA segments within the apomixis locus in *Paspalum* shows highly interrupted genes sequences (Calderini et al. 2006), and gene expression studies revealed apomixis involves a genetic reprograming that affects the expression of a variety of genes including meiotic genes, transcription factors, stress-associated genes (Polegri et al. 2010, Okada et al. 2013, Shah et al. 2016, Ortiz et al. 2017) and genes needed for the emergence of apomixis during ovule development (Mancini et al. 2018). Therefore, in facultative apomictic plants, apomixis is a leading developmental mechanism that is superimposed over, and drastically distress the regular sexual program. Nevertheless, despite its genetic dominance, apomixis shows incomplete penetrance (Matzk et al. 2005).

Thus, the interaction of apomixis factors with the genomic background of each asexual clone and the environmental conditions determine variable levels of expression of the character as well as the level of realized sexuality in facultative apomicts. As mentioned above, previous studies using different experimental setups had shown the proportion of sexuality in facultative apomicts increases under stressors, including temperature (Šarhanová et al. 2012, Mateo De Arias 2015, Klatt et al. 2018), water availability (Rodrigo et al. 2017) and photoperiod (Quarin 1986, Rebozzio et al. 2011, Klatt et al. 2016).

In my analysis, the first one using in situ population level data, beside the observed occurrence of sexual (diploid) and apomictic (tetraploid) cytotypes strictly delineated by climatic variables like Mean temperature of coldest quarter, frost day frequency and photosynthetically active radiation (Karunarathne et al. 2018), within facultative polyploids, a significant correlation between the occurrence of sexual ovules and seeds and the seasonal variation of daily temperature was observed. A change in mean diurnal range can induce a stress response and alterations in physiology and biosynthesis pathways during flower development (Gent and Ma 1998, Cohen et al. 2012). Adaptive evolution of low-temperature-induced stress responses is relevant for adaptation to cold habitats in grasses (Vigeland et al. 2013). In asexual plants with reduced genetic and genotype variability, higher frequencies of sex in apomictic populations exposed to colder and wider temperature range may have an important role in facilitating local adaptation of clonal populations as well as enhancing further geographical expansions.

Combined with the results obtained from the common garden experiments, the data suggest that sex is environmental modulated locally in *P. intermedium* populations, and point towards a developmental *tug of war* between meiotic and apomictic programs to make the most

out of reproductive season. Maternal investment is expected to allocate resources among offspring maximizing plant fitness (e.g. Povilus et al. 2018). However, in *P. intermedium* the conflict between genetic factors promoting the expression of apomixis and the environmental stressors stimulating sexuality, is likely the basis for the drastic reduction of fitness in facultative apomictic polyploids compared to sexual diploids. Alternatively, apomictic polyploids can better tolerate environmental variability than sexual diploid counterparts (Karunarathne et al. 2018), and among tetraploid populations, sexuality is higher in areas of greater environmental stress. Accordingly, seasonal variation shows a significant increase in the formation of meiotic female gametophytes among apomictic populations (*paired t-test p-value* = 0.01) associated to the drier and warmer season (December – March). Our modelling of population level data further indicates that total sexuality will never be reached (fixed) in these polyploids, a situation observed in natural populations of *P. intermedium*, and in all other studied apomictic species where homopolyploids individuals and populations are always apomictic and no single case of natural obligate sexuals has been yet recorded (Asker and Jerling 1992, Carman 1997).

Temporal and spatial changes in the incidence of sexuality may be a consequence of the inherent nature of apomixis expression and deregulation of ovule gene networks; alternatively, modulation of residual sexuality in facultative apomicts may have evolved as a response to adaptive pressure, allowing clonal lineages to maintain adapted genotypes keeping backed up genetic variability until environmental changes allow for the creation of new gene combinations able to address novel ecological challenges.

4. Population genetic structure analysis echoes the distribution, coexistence and niche divergence of cytotypes in the polyploid grass species *Paspalum intermedium* (Poaceae)

This chapter presents the results of population structure analysis of the studied *Paspalum intermedium* populations in this thesis. The findings have been compiled and the manuscript for publication is under preparation.

4.1. ABSTRACT

Polyploidization is a recurrent process in plants and provides greater potential for diversification. Neopolyploids in natural populations should go under substantial structural changes in their genetics, reproductive mode (e.g. apomixis), and ecological preferences to ensure their successful establishment. These processes are well reflected by their genetic variation. However, there is a lack of non-model systems that exhibit successful changes with pronounced reflection for studies. *Paspalum intermedium* is a polyploid complex with different ploidy levels and different reproductive modes (i.e. obligate sexual diploids and facultative apomictic tetraploids), with both niche divergence and cytotype coexistence, hence provides an ideal situation to study genetic variation in polyploid complexes. Flow cytometry, genetic markers amplified fragment length polymorphism (AFLPs), and geographical data were used to assess the genetic variation between cytotypes, within cytotypes, among populations and within populations. To get insights into the genetic structure variation depending on the reproductive mode and how it explains the niche variation between cytotypes, the results were compared with the distribution patterns and different ecological preferences of the cytotypes. My findings show that apomictic autotetraploids are of multiple independent origins. Although diploids show higher genetic variation, within and among population genetic variation equally make up the observed variation in all cytotypes. All individuals fall into three genetic clusters with substantial genetic admixture. Together with reproductive pathway analysis, results of genetic variation analyses suggest that the contact zone of the two cytotypes is primary in origin where tetraploids frequently occur in mix ploidy populations. Genetic cluster maps point to a distribution of genetic variation in accordance with niche differentiation. Polyploidization in *P. intermedium* is a recurring phenomenon and the newly arisen polyploids successfully establish themselves by acquiring enough genetic variation that allows them to adapt to new environments. Genetic variation analysis points to a slight deviation from the known *General Purpose Genotype* and the *Frozen Niche Variation* concepts as there is neither a common genotype nor are the diploids occupying a part of diploid sexuals' niche. The present study provides important insights into the mechanisms that aid neopolyploids to survive, coexist, expand and establish successfully after polyploidization.

4.2. INTRODUCTION

Polyploidization in plants is a recurring and a pivotal evolutionary phenomenon that brings benefits for plant diversification both in the short term as well as in the long run (Werth et al. 1985b, Soltis et al. 2010, Symonds et al. 2010). Comparative genomic studies show that approximately 15% of plant speciation events resulted from polyploidy (Wood et al. 2009) and that polyploidy is substantially associated to higher plant diversity (Symonds et al. 2010, Jiao et al. 2011). Moreover, a crucial step of polyploidization, unreduced gamete formation, was found to be occurring at a high rate of approximately 0.5% per gamete (Ramsey and Schemske 1998, Wood et al. 2009). As a result, the occurrence of new polyploids in natural populations is unequivocally higher than expected. However, polyploidization *per se* can act as a double-edged sword. On the one hand, it can act as an instantaneous mechanism for speciation because of reproductive isolation (see Soltis et al. 2009). On the other hand, due to competitive exclusion by the majority cytotype (i.e. minority cytotype disadvantage, Levin 1975), continuously occurring new polyploids will most likely go extinct because they arise among their diploid progenitors (Parisod et al. 2010). Therefore, mechanisms that help newly arisen polyploids to overcome competition, survive and establish themselves devoid of reproductive incapability, are key to the success of neopolyploids. Even though there are studies focusing on different mechanisms explaining the ecological consequences of polyploidization, there is a lack of non-model plant systems with pronounced population structural changes where genetic structure can explain and reaffirm the observed patterns of polyploid coexistence.

Although there have been a few opposite views (e.g. polyploids are common in nature but not significantly differentiated from their diploid relatives; Arrigo and Barker 2012), ecological and niche variation in neopolyploids is crucial to avoid competition with their already established diploid parents and escape minority cytotype disadvantage (Hegarty and Hiscock 2008, Zozomová-Lihová et al. 2015), one of the main demographic obstacles faced by emerging polyploid individuals. Furthermore, studies show that higher polyploid establishment is associated with higher environmental stochasticity (Oswald and Nuismer 2011), resulting in polyploids colonizing and establishing in newer and harsher environments (Baack 2005). This represents a win-win situation for the polyploids as well as for the species itself as polyploid establishment and range expansion of the species is eventually achieved. Coexistence of different cytotypes has also been reported frequently in plants when there is higher self-compatibility in the cytotype with frequency disadvantage (Fowler and Levin 1984, Kao 2007). Mechanisms on how different cytotypes achieve niche differentiation and/or coexistence and the advantages and disadvantages of these two processes are still not clear.

Apomixis (asexual reproduction via seeds) coupled with polyploidy, not only provides reproductive assurance to neopolyploids by aiding them overcome minority cytotype exclusion (Levin 1975), but also enhances dispersal ability and colonizing new habitats and reinforce founder events (Baker 1955). Due to the avoidance of meiosis, apomixis counteracts against genetic drift and maintain higher heterozygosity (Paun et al. 2006, Cosendai et al. 2013). Furthermore, processes such as, mutation accumulation, genetic restructuring, and residual sexuality introduce additional genetic variation to apomictic populations (Hörandl and Paun 2007, Hojsgaard and Hörandl 2015), further diversifying the apomictic taxa. This provides new polyploids with novel traits for increased tolerance to harsher environmental conditions thus resulting in wider distribution (e.g. geographical parthenogenesis) (Suda et al. 2004, Brochmann et al. 2004, Hörandl 2006).

The General purpose genotype hypothesis (Baker 1967, Lynch 1984) explains that one fit genotype with higher tolerance to a broader ecological setting may colonize different habitats, while the frozen niche variation hypothesis assumes that specialized multiple polyploids carrying a portion of the genetic variation arising from genetically varying sexual progenitors will efficiently partition underutilized resources by the ancestors. This will allow them to occur in sympatry or completely eliminate sexual ancestors (Vrijenhoek 1984, 1994). Although these two concepts seem mutually exclusive, recent views on the two hypotheses explain that they are complex syllogisms with shared common assumptions presenting possible processes of interclonal selection through which the asexual populations acquire their ecological breadth clonal divergence (see Vrijenboek and Parker Jr. 2009). On the other hand, coexistence (e.g. sympatry) of different ploidy levels allow gene flow resulting in genetic admixture (Petit et al. 1999, Paun et al. 2006, Zozomová-Lihová et al. 2015). This mainly takes place from diploids to higher ploidy levels although the opposite is also present (Bretagnolle and Thompson 1996, Van Dijk and Bakx-Schotman 1997, Ramsey and Schemske 1998). Mixing of these newly arisen genotypes with various origins and reproductive pathways will introduce novel restructuring of the genetic material resulting in larger population level structural changes (Baack 2005, Sabara et al. 2013). This may further enhance trait variation (e.g. reversal to sex) and allopatric divergence (Hojsgaard and Hörandl 2015).

Study of population structure of polyploids in comparison to their diploid progenitors in plant systems of different temporal stages, has shown significant importance in terms of understanding the evolutionary history, and can generate a glimpse of the diploid ancestors' evolutionary course and provide a fine scale resolution of the origin of different ploidy levels (Symonds et al. 2010), as well as providing clues as to what the fate of the neopolyploids would be (Soltis and Soltis 2000). Furthermore, it can also provide information on various factors that

shape the distribution patterns observed in plants; for example, the influence of environmental factors and spatial separation on the coexistence and establishment of the polyploid complexes (Burton and Husband 1999, Lo et al. 2009). In this regard, studies on newly established polyploids are of increased interest as they can provide crucial insights into the genetic processes that take place soon after and/or during the process of establishment of polyploids.

Paspalum intermedium Munro ex Morong is a grass species of the plant sub-family Panicoideae with two major cytotypes: diploids (2n = 2x = 20) and tetraploids (2n = 4x = 40); and two reproductive modes (self-sterile sexual diploids and self-fertile apomictic tetraploids). Tetraploids are autopolyploids and the two cytotypes occur in different ecological settings (i.e. allopatry, sympatry and parapatry) (Norrmann et al. 1989). Furthermore, the species occurs in a wide range of ecological and climatic gradients (e.g. latitudinal gradient) in the region where it has diversified (i.e. Sub-tropical Argentina, Brazil, Paraguay and Bolivia) (Zuloaga et al. 2012). Most importantly, in a recent study, (Karunarathne et al. 2018) showed an existence of niche divergence between diploids and tetraploids owning to an optimal ecological/climatic preference by the differing cytotypes (Figure 4.1). The study also presented interesting dynamics of cytotype displacement and the existence of a contact zone of 2x and 4x including mix ploidy populations, apart from the north-south latitudinal separation of the two cytotype populations. Divergent genetic systems in the species, a common characteristic of species within *Paspalum* L., is known to provide the necessary genetic diversity and ecological capacity to overcome environmental hardship (Tilman and Lehman 2001, Allan et al. 2015). There is also indirect evidence suggesting the relatively recent establishment of the polyploid (Hojsgaard et al. 2009, Karunarathne et al. 2018). Therefore, *P.intermedium* makes an ideal non-model plant system to examine population structure variation and patterns of genetic variation with regards to niche divergence, cytotype coexistence and recurrence of polyploidy as well as to decipher the backstage role of apomixis in plant evolution.

In the present study, using flow cytometry, genetic marker AFLPs, and ecological and geographical data, I aim i) to assess the genetic structure of populations in two *P. intermedium* cytotypes showing niche divergence, ii) to determine the origin and genetic variability within and among autotetraploid populations, iii) to examine the genetic composition of mixed-ploidy and contact zone populations, and finally iv) to draw inferences on the distribution patterns and ecological amplitude of the two cytotypes based on the genetic variability.

4.3. MATERIALS AND METHODS

4.3.1. Sampling

Sampling was done covering the core and peripheral distribution areas of the species (i.e. Pampas, Mesopotamia, Gran Chaco of Argentina). Leaf materials were collected in silica gel from 35 populations consisting of 24 pure tetraploid populations, nine pure diploid populations, and four mix ploidy populations (Figure 4.1). From all the sampling populations, leaf materials were collected from at least 20 individuals from each population. In the first part of our study, I analyzed close to 1200 plants for ploidy levels by flow cytometry (see Karunarathne et al. 2018). From this, 867 individuals come from the populations analyzed here for the population genetic structure (see Table 4.1). Genetic marker analysis by AFLP fingerprinting was performed on all these selected individuals from the studied populations.

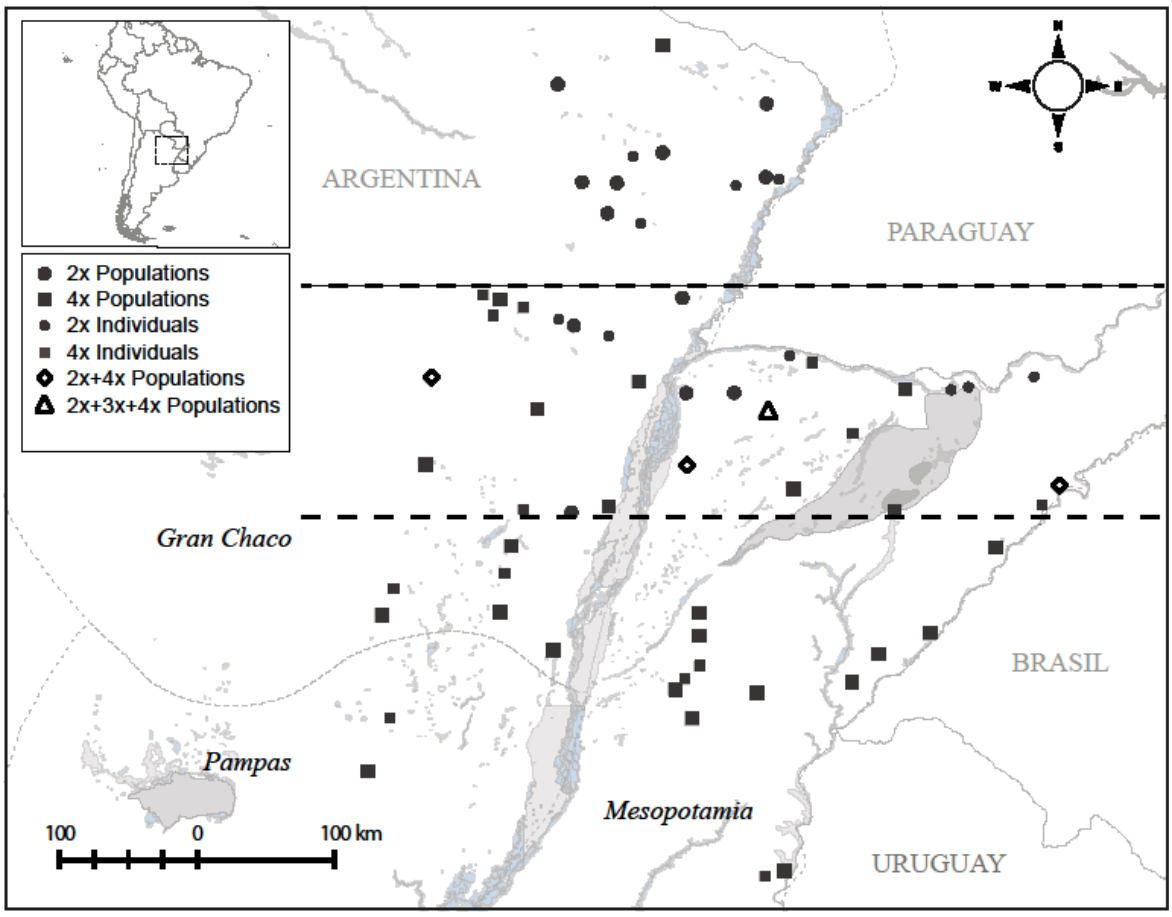

Figure 4.1. Map depicting the collection location of studies populations and their ploidy levels (adopted from Karunarathne et al. 2018). The dashed lines demarcate the contact zone of cytotypes in the middle separating the north and south diploid and tetraploid distribution zones respectively.

4.3.2. Amplified Fragment Length Polymorphism (AFLPs)

For AFLP, we followed the methodology described by Vos et al. 1995) with a modification skipping the pre-selective amplification of the digested fragments. The reason for this modification was a result of failure of selective amplification yielding unexplainable large size DNA clumps. Therefore, here, after the adapter ligation to the digested fragments using a frequent cutter (*Mse I*) and a rare cutter (*EcoR I*) restriction enzymes, a direct selective amplification PCR was performed instead of the original pre-selective PCR. Further, to avoid the amplification of bulk selective-amplified fragments, I used selective primer combinations with four additional bases in one of the primers, though not in all combinations. The three primer combinations used were *EcoR I*- ACA-5′ FAM/*Mse I*- GAAC, *EcoR I* - AATG/ *Mse I* -AAC-5′ HEX, *EcoR I* - AGA/ *Mse I* - ACA-5′ TAMRA. The reproducibility of PCRs was checked with 10 duplicate samples with each primer pair.

For restriction digestion and ligation, approximately 500 ng of genome DNA of each sample was digested overnight with *EcoR I* (5 Units) and *Mse I* (1 Unit) (New England Biolabs, Frankfurt, Germany), and T4 DNA ligase (Promega Corporation, Mannheim, Germany) (1 Unit) with *EcoR* adapter pair (5 *p*mol) and *Mse* adapter pair (50 *p*mol) with the presence of NaCl (0.05 M) and BSA (0.05 mg/ml) in 1X Ligase buffer (Promega Corporation, Mannheim, Germany). Direct selective amplification reaction mixture consisted of 1X PCR buffer (10x NH_4 Reaction buffer: Bioline GmbH, Germany), 2.5 mM $MgCl_2$ (Bioline GmbH, Germany), 0.2 mM dNTPs (Promega Corporation, Mannheim, Germany), 4 pmol of each *EcoR* and *Mse* primers, 1 Unit Taq polymerase (BioTaq – Bioline GmbH, Germany), ca. 80 ng of digested DNA in 25 µl final volume. The PCR was done in a Thermal Cycler (BioRad T100: Bio-Rad Laboratories GmbH, Munich, Germany) with the following program. Denatured at 94 °C, 2 min, 9 x (94 °C, 1 sec; 65 °C, 30 sec, -1 °C/cycle; 72 °C, 2 min), 23 x (94 °C, 1 sec; 56 °C, 30 sec; 72 °C, 2 min), 60 °C, 30 min. Amplified fragments were analyzed in an ABI 3130xl Genetic Analyzer (Applied Biosystems inc., Foster City, CA, USA) with the 500 ROX (Applied Biosystems Inc.) size standard. A total of 887 individual fingerprints were retained after the initial analysis of all the individuals (48 atypical fingerprints were removed). Genotyping and binary presence-absence matrices were assembled in GeneMarker 2.6.0 (Softgenetics, PA, USA), with a threshold of 75 RFU for scoring bands of size range of 100–510 bp (small fragments between 50-100 were not considered due to the possibility of non-homologous fragments: Vekemans et al. 2002). All the peaks were checked in the panel editor eliminating non-reproducible bands by comparing to replicated samples where the reproducibility of the data was checked using the error rate with 30 duplicate samples, where the similarity of the scoring (i.e. presence-absence of fragments) was cross checked. The error rate was less than 0.1% indicating high reliability of the data.

4.3.3. Statistical Analyses

The combined binary matrix (stored in the network server of Department of Systematics, Biodiversity and Evolution of Plants Albrecht-von-Haller Institute for Plant Sciences University of Goettingen) of the three primer combinations was analyzed with the R-script AFLPDAT (Ehrich 2007) to calculate diversity indices (i.e. Masatoshi Nei 1987) gene diversity for each population), and genotype diversity (using Nei's (1987) formula for haplotype diversity). The binary matrix was assembled into an individual genotype data object with the R package *ADEGENET* (includes a method that can hadle clonal data and allows for analyses of mixed-ploidy data sets with a correction for allele copy-number ambiguity in polyploids) (Jombart 2008), which was used in the rest of the genetic analyses in the R environment (R Core Team 2016). A Neighbor-Joining tree was constructed using the Prevosti's Distance Coefficient (a measurement over all loci of the proportion of unshared alleles) with a bootstrap analysis of 1000 sample size. The R package *POPPR* 2.7.1 (Kamvar et al. 2014) was used for the distance matrix and the bootstrapping. Principal Coordinate Analysis (PCoA) was computed based on pairwise Euclidian distance used in the DAPC (Discriminant Analysis of Principal Component) function of the *ADEGENET* R package. Analysis of Molecular Variance (AMOVA) was calculated on the discrete dissimilarity matrix with 1000 permutations also using *POPPR* R package. For AMOVA both ploidy and populations were used as different strata to calculate both between ploidy and within and among population molecular variance.

Bayesian model-based clustering implemented in the "find.clusters" function of R package *ADEGENET was* used to determine the potential number of clusters that can describe the data best. Here, a BIC (Bayesian Information Criteria) is calculated using *k-means* algorithm (also Ripley's K-function, where sum of squares from points to the assigned cluster centers is minimized: Baddeley and Turner 2005) and the resulting BIC values are plotted against increasing number of *k* (clusters). Ideally, the number of clusters where the BIC value starts to increase is taken as the best cluster solution. In the present case, the BIC value did not increase (supplementary Figure S4.1). Therefore, according to the plot any number of clusters more than two and less than 15 will describe the data. Considering the number of groups observed in the NJ tree, k=3 was taken as the number of clusters, thus it is a biologically meaningful number of clusters. This was also tested with ad hoc statistic ΔK based on the rate of change in the log probability of data between successive K values described by Evanno et al. (2005) (Figure S4.1b). Plotting the genetic clusters was performed using the R package *LEA* (Frichot and Francois 2014).

A Mantel test was performed using pairwise Euclidean distance (R package *VEGAN*: Oksanen et al. 2016) to calculate the geographical isolation of each cytotype and population based on genetic data. The R package *MPMCORRELOGRAM* (Matesanz et al. 2011) was used to

visualize the geographic isolation based on distance intervals (Supplementary Figure S4.2). The R package *LEA* (Frichot and Francois 2014) was used to plot the genetic admixture on the map (figure 4).

4.4. RESULTS

In the combined binary matrix, a total of 189 fragments were scored; 84 from the *EcoR-Mse* (ACA- GAAC), 58 from *EcoR-Mse* (AATG- AAC) and 44 from *EcoR-Mse* (AGA- ACA). Out of this, 66.2 % were polymorphic. The number of bands per individual ranged from 84 to 91. Cytotype specific fragments were higher in tetraploids (41) than in diploids (34). Therefore, a significantly higher number of bands ($p = 0.02$) were present in tetraploids.

Diversity analysis (Nei's gene diversity) showed that all diploids have significantly higher values (paired t-test *p-value* < 0.001) in both genotype and gene diversity (Table 4.1) and thus the effective number of genotypes were 100% in diploid populations while it ranged from 1 to 100 % in tetraploids. Interestingly, in mixed-ploidy populations, this was relatively high (30-100%) and was significantly correlated ($r^2 = 0.91$, $p < 0.01$) to the number of 2x individuals in the population; one of these populations (Hojs 481/2W: 24-2x, 6-4x) harbored five non-clonal 4x individuals, indicative of independent origin. The highest number of effective genotypes observed in 4x populations was 13 (46.4 %) while six populations were pure clonal populations (Table 4.1). AMOVA revealed that half of the genetic variation (50.18 %) is observed within populations, the rest accounting for among populations. When cytotype was assigned as preferred hierarchy, within cytotype genetic variation was 36 %, dividing the rest equally between within and among population variations. Among population variation increased to 64.8% in tetraploids when cytotypes were analyzed separately, while values for diploids did not change noticeably.

Three major clusters were observed in the unrooted NJ tree (Figure 4.2) with strong bootstrap support (>90%) although altogether seven clusters resolved in the tree. These sub clusters were not well supported by the bootstrap values (<70%). All the pure 2x and 4x populations were grouped in all three clusters indicating the independent multiple origins of tetraploids. Individuals of several 2x populations (Hojs420, Hojs422, Hojs423, Hojs425 and M31) were grouped in two or all clusters indicating high genetic variation in those populations as well as supporting the three clusters. Interestingly, mixed ploidy populations were also grouped in all three major clusters indicating high within population genetic variation.

Bayesian clustering revealed three major clusters (k=3) where majority of the pure 4x and 2x populations made each one separate cluster while the rest with mix populations made the third cluster. However, the PCoA (Figure 4.3A) showed a continuous genetic variation in 4x populations except for a few populations (Figure 4.3B) while all the diploids were clustered close to each other

than tetraploids were to themselves. Interestingly, the tetraploid population (Hojs 424) far in the north of the distribution clustered with diploids. Two of the mixed-ploidy populations clustered close to diploids while the other two clustered closer to tetraploids. The triploid individual clustered with diploids. Cluster analysis also shows substantial admixture among populations (Figure 4.4), especially in the populations in and close to the contact zone. This can be seen quite clearly when the genetic clusters were plotted as pies on the map (Figure 4.4, also see Karunarathne et al. 2018). Most interestingly, two mixed-ploidy populations (Hojs456/2Q and Hojs487/2Y) harbor greater amounts of admixture compared to all the other populations. Further, the isolated 4x populations (Hojs451/2M, Hojs453/2Ñ(EN), Hojs475/2U) show very low or no admixture suggesting that they are recent in origin.

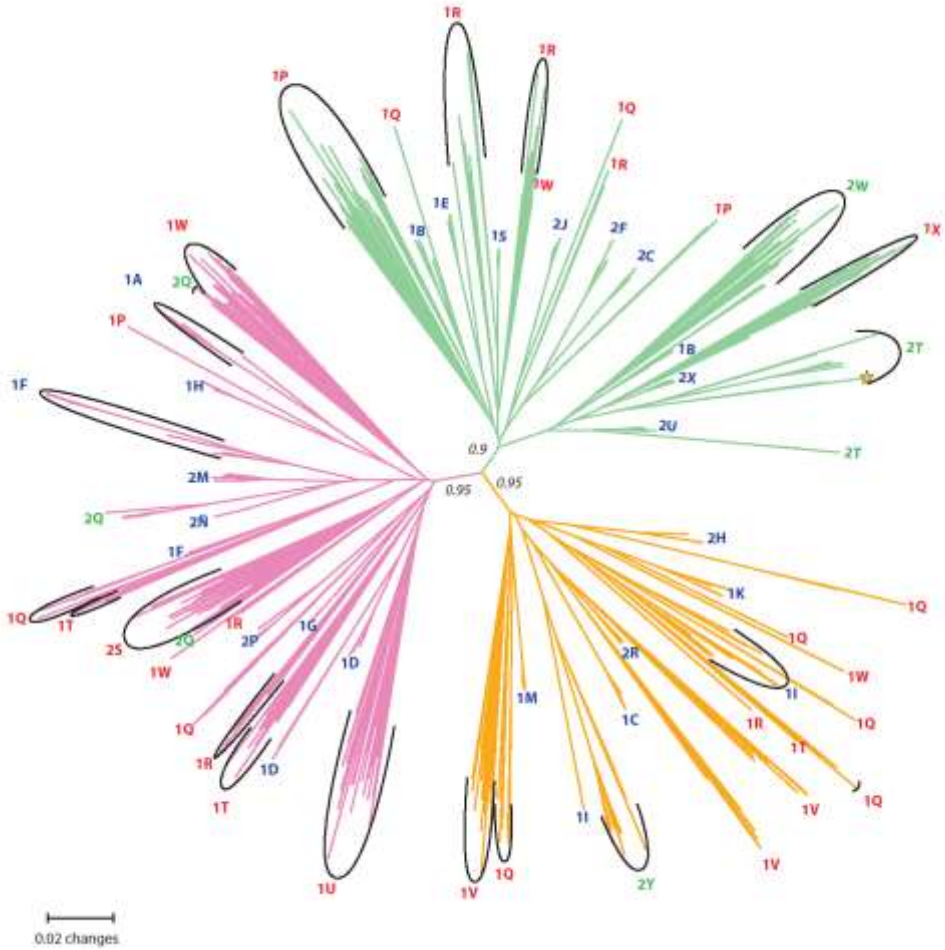

Figure 4.2. The unrooted Neighbor-Joining (NJ) tree constructed using the Prevosti's Distance Coefficient among amplified fragment polymorphism (AFLP) of all the studied individuals of *P. intermedium* with a bootstrap analysis of 1000 sample size. The bootstrap values are shown only for the main branches. The tip labels show the respective population (red – diploid populations, blue – tetraploid populations, green – mixed-ploidy populations, strar* - triploid individual).

Table 4. 1All the studied populations of *Paspalum intermedium* in the present study. (population codes are as in Karunarathne et al. 2018), ploidy levels of the population, number of individuals retained in the genetic marker analysis (AFLPs), genotype diversity, effective number of genotypes and Nei's gene diversity calculated from AFLPs.

Population	Population Ploidy	No. of individuals	Genotype diversity	No. of effective genotypes	cytotype gene diversity	Nei's gene diversity
Hojs420/1P	2	26	1.000	26.000		0.165
Hojs422/1Q	2	26	1.000	26.000		0.208
Hojs423/1R	2	24	1.000	24.000		0.176
Hojs425/1T	2	17	1.000	17.000		0.174
Hojs429/1U	2	22	1.000	22.000	0.169	0.157
Hojs432/1V	2	26	0.997	24.143		0.188
M31/1W	2	21	1.000	21.000		0.173
M26/1X	2	11	1.000	11.000		0.146
Hojs468/2S	2	25	1.000	25.000		0.135
Hojs401/1A	4	23	0.249	1.313		0.098
M29/1B	4	26	0.772	3.885		0.067
Hojs402/1C	4	26	0.000	1.000		0.000
Hojs403/1D	4	30	0.480	1.867		0.024
Hojs404/1E	4	20	0.195	1.227		0.026
Hojs405/1F	4	26	0.895	7.191		0.075
Hojs406/1G	4	11	0.000	1.000		0.000
Hojs409/1H	4	23	0.000	1.000		0.000
Hojs410/1I	4	28	0.958	13.067		0.107
Hojs414/1J	4	27	0.000	1.000		0.000
Hojs415/1K	4	27	0.359	1.528	0.032	0.034
Hojs416/1M	4	24	0.000	1.000		0.000
Hojs424/1S	4	25	0.000	1.000		0.000
Hojs440/2C	4	24	0.228	1.280		0.015
Hojs443/2F	4	29	0.567	2.207		0.023
Hojs445/2H	4	29	0.488	1.890		0.039
Hojs451/2M	4	28	0.198	1.237		0.034
Hojs453/2Ñ	4	11	0.182	1.198		0.015
Hojs455/2P	4	19	0.105	1.111		0.054
Hojs465/2R	4	17	0.485	1.841		0.024
Hojs475/2U	4	27	0.501	1.934		0.024
Hojs471/2X	4	19	0.731	3.252		0.043
Hojs470/2T	2,3,4	25	0.810	4.496		0.134
Hojs456/2Q	2,4	25	0.367	1.543	0.123	0.137
Hojs481/2W	2,4	23	1.000	23.000		0.147
Hojs487/2Y	2,4	29	0.820	4.806		0.075

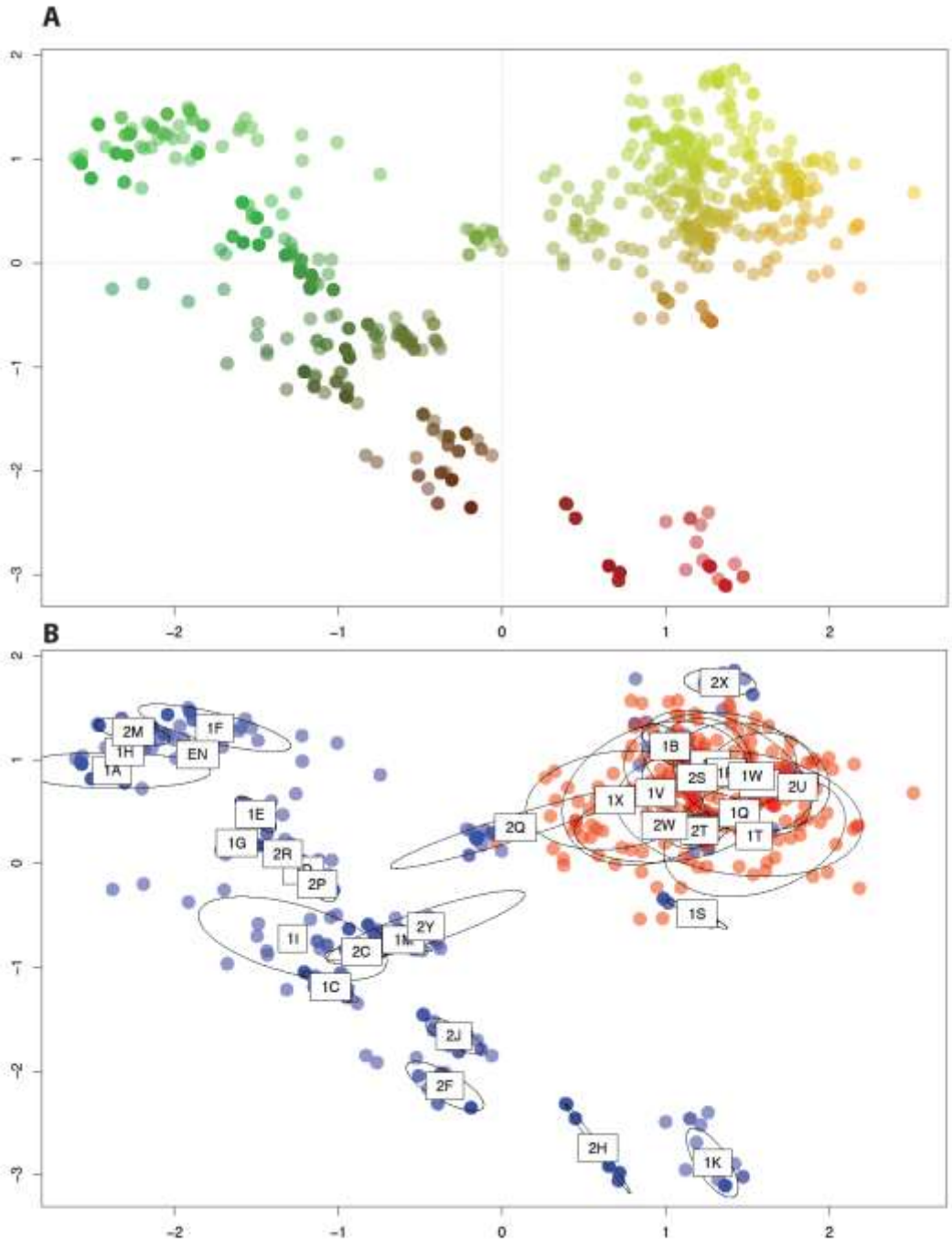

Figure 4.3. Principle coordinate analysis of the studied populations of *Paspalum intermedium* based on pairwise Euclidian distance used in the DAPC (Discriminant Analysis of Principal Component) function of the *ADEGENET* R package; The first two axes represent 37 and 21 % of total variation. A. genetic similarity among all the individuals depicted by RGY (i.e. Red, Green, Yellow) color scheme, B. genetic variation between cytotypes from all the populations – red: diploids, blue: tetraploids, labels show the population codes, ellipses represent the 95% dispersion of each indicated population

Mantel test for the geographic isolation of the population (test statistic = 0.360, p = 0.001) demonstrated genetic variation strongly correlated to distance. Furthermore, when the overall distance found among populations and genetic clusters of the studied individuals were plotted against distance intervals for all the geographical distance intervals (Mantel correlogram: Matesanz et al. 2011), almost all groups (except for two) indicated significant isolation (Supplementary Figure S4.2: filled points are significant; i.e. $p < 0.05$). This is expected especially in apomictic species as there is substantial reproductive isolation among individuals (see discussion for details).

4.5. DISCUSSION

Studying the genetic composition and the population structure in apomictic populations is crucial for understanding the evolutionary history, establishment and evolutionary fate of polyploid complexes and associated geographical parthenogenesis. The present genetic analysis of the *P.intermedium* populations and indirect evidence from species distribution modeling (Karunarathne et al. 2018) and chromosome counts (e.g. Caponio and Quarin 1993, Hojsgaard et al. 2009) suggest that the polyploid is recently established in terms of species' evolutionary course. Despite having multiple independent origin, apomictic tetraploids do not show drastic genetic differentiation from the diploid progenitors. Higher amount of genetic differentiation with varying population structures are expected in older polyploid systems as processes such as accumulation of mutations, interploid hybridization, and residual sexuality are mechanisms increasing genetic variation in apomictic populations (e.g. Smith 1989, Paun et al. 2006, Hörandl and Paun 2007, Cosendai et al. 2013) and references therein).

4.5.1. Genetic variation and genotype composition in different cytotypes

Despite the noticeable number of cytotype-specific bands in the AFLP genotypes, considerable proportion of bands (33.8%) are shared between the main two cytotypes; this is in accordance with the previously observed tetrasomic inheritance and autopolyploid origin of the tetraploids (Norrmann et al. 1989, Hojsgaard et al. 2008). Unique dominant fragments in apomicts are likely a consequence of post polyploidization rearrangements of the genomes as has been observed in many other systems (e.g. Ainouche et al. 2003, Hegarty and Hiscock 2005, Paun et al. 2006). Nevertheless, most tetraploid populations show a very low genotype diversity (Table 4.1) indicating rather recent founder events and that most 4x populations are isolated population with a few clonal genotypes. Though not in its entirety, this observation has not been often reported in facultative apomictic populations (e.g. Martens et al. 2009, Vrijenhoek and Parker-Jr. 2009, Cosendai et al. 2013), where strict clonality is extremely rare in nature, except perhaps for

a few obligate apomicts (e.g. *Taraxacum officinale* Van der Hulst et al. 2000). Most interestingly, when the genetic and genotype diversity in apomictic populations was analyzed in relation to the proportion of residual sexuality (reproductive pathway efficiency: chapter 3) and they show a strong positive correlation to the proportion of residual sexuality ($r^2 = 0.71$) except in mixed ploidy populations. This further reinforces the hypothesis that residual sexuality plays a major role in the causality of genetic diversity in apomictic populations (Hojsgaard and Hörandl 2015). Despite the low within population genetic variation in tetraploids, we observed a significant among population diversity (AMOVA test statics = 0.4102, $p = 0.001$), which was also observed in the PCoA (discussed below). Nevertheless, mixed-ploidy populations maintain a relatively high genetic variation (Table 4.1) even among tetraploid individuals presumably due to various mechanisms that favor assortative mating and decrease in cytotype reproductive interactions (Petit et al. 1999, Sabara et al. 2013) (discussed below). Alternatively, the possibility of higher amount of gene flow introducing genetic variability to higher ploidies via new polyploid from the primary genepool of diploids can result in the same observation.

4.5.2. Population structure and genetic clusters

Genetic clusters can be biologically meaningful when they best explain the genetic variation observed in the data. However, since all clusters are models, there is no true *k* (number of clusters) (Kalinowski 2011 and references therein). In my cluster analysis, the test for best *k* did not point to a clear number of clusters (see materials and methods). This is either an indication of higher genetic admixture (discussed below) through gene flow (Paun et al. 2006, Cosendai et al. 2013) and/or tetraploid populations are of multiple independent origin (e.g. Zozomová-Lihová et al. 2015) or of high number of clusters with undetectable variations. My observations support the latter as the genetic variation within 4x populations are relatively low. However, the assignment of three clusters explained the variation optimally and the assignment of $k = 2$ did not change the admixture values in tetraploids noticeably. Clusters were unstable upon the increase of k (>3) with admixture in almost all individuals (see Figure S4.3). Further, when the two cytotypes were analyzed separately (see materials and methods), both showed three optimal clusters (data not shown). This was also evident in the NJ tree by the presence of 4x populations placed in three clusters closely related to diploids; and the branching of 4x within clusters is variable indicating low genetic variation. This observation is expected in newer systems as within and among population variability in closely related taxa are related to the different ages and histories of the respective systems (Fehrer et al. 2005, Lo et al. 2009).

Diploid individuals from all populations made a compact cluster in the PCoA indicating a higher similarity among diploids irrelevant of the population of origin, despite having a higher genetic diversity within 2x populations, unlike among tetraploids. Constant gene flow

among populations, given the self-incompatibility and geographically restricted distribution of diploids is a possible explanation for this observation. Naturally, frequent gene flow and seed dispersal to nearby diploids populations, given the proximity, results in stretching of the genetic variability of diploid populations while once a 4x individual (an outlier) arises and found a population, it rather captures and fixes a fraction of diploids' genetic variability and can only differentiate further from ancestors due to post polyploidization rearrangements of the genome. Furthermore, selection pressure may also be a factor in these populations as we discussed in (Karunarathne et al. 2018), the diploids are highly specialized for their current niche and thus only most fitting genotypes are retained; a similar observation was reported in *Taraxacum* and *Chondrilla* (Van Dijk 2003). In contrast, several pure tetraploid populations (M29/1B, Hojs474/2U, Hojs471/2X, and Hojs424/1S) and three mixed-ploidy populations (Hojs470/2T, Hojs456/2Q, and Hojs481/2W) clustered with diploids. This points toward a low level of genetic differentiation in these populations from their (diploid) progenitors. There are multiple studies showing the recurrence of polyploidization and hybridization in nature (Lo et al. 2009, Soltis et al. 2009, Arrigo and Barker 2012). However, their differentiation, establishment and expansion depend on several factors such as rate of formation and the interplay between genetic and ecological factors promoting their survival (Soltis et al. 2014). Genetic differentiation of new polyploids, although not always, is key for successful establishment (Cosendai and Hörandl 2010), especially when colonizing new environments (Zozomová-Lihová et al. 2015). Hence, the lower level of differentiation in these pure 4x populations may indicate that they are of recent origin and without enough time to accumulate independent mutations and differentiate from their progenitor populations/genotypes.

4.5.3. Genetic admixture and ecological niche divergence

Exchange of genetic material among individuals of the same or different ploidies has been commonly reported in polyploid and apomictic plants (Parisod et al. 2010, Cosendai et al. 2013), thus resulting in genetic admixture. In this regard, residual sexuality, hybridization, and outcrossing act as mediators of genetic admixture thereby promise for beneficial genetic variation (Paun et al. 2006, Hörandl and Paun 2007, Hojsgaard and Hörandl 2015). In *P. intermedium,* however hybridization is not known as of now, except in few single cases (Honfi 2003). The present results show that there is a considerable level of genetic admixture among and within both cytotypes (Figure 4.4), especially in diploids. Although admixture in tetraploid apomictic populations is relatively low, several closely occurring populations show mixed genetic structure, indicating some form of gene flow. This seems to be correlated to the distribution and isolation by distance (see below) among these populations. Moreover, the likely recent origin of apomictic

tetraploid populations in peripheral areas may be a factor limiting historical likelihood of genetic exchange between populations and therefore the observed levels of admixture.

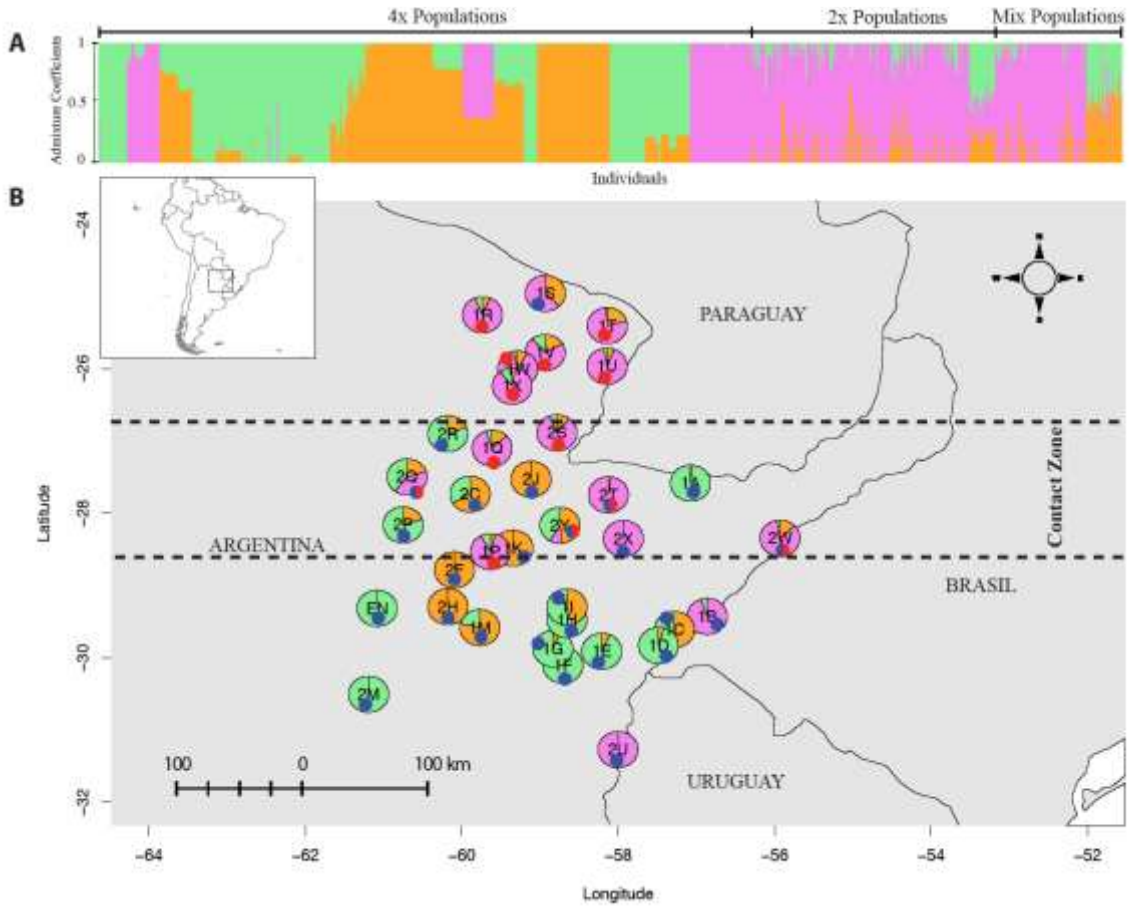

Figure 4.4. Genetic cluster in *Paspalum intermedium* inferred from AFLPs; A. Bayesian clustering of all the individuals at *K* = 3. Vertical bars represent the individuals with the proportion of the admixture (i.e. admixture coefficients) in different colors. B. Admixture coefficients of populations plotted on the map indicating the collection location. Different clusters are represented by the same colors and in A. Small colored circles indicate the ploidy level of each population (red – diploids, blue – tetraploid, red and blue – mix populations). The dash line on the map shows the contact zone between the diploids and tetraploids

In contrast, the geographic distribution of different genetic clusters of the studied species (studied populations) partly supports the hypothesis that assumes the partitioning of genetic structure so that resources in the broader niche are shared between the cytotypes as well as among different clonal polyploids (Frozen Niche: Vrijenhoek and Parker-Jr. 2009). However, since there is less between-cytotype competition in the southern distribution range of the species, my observation is that on the one hand, genotypes with different tolerant levels to harsher environmental conditions (e.g. colder and drier climate; especially the conditions that the diploid ancestors do not occur in) have been successful in different areas rather than freezing a certain part of niche partitioning. This becomes clear when the genetic clusters obtained from Bayesian

analysis plotted on the map (Figure 4.4). On the other hand, diploids with specialized genotypes to its restricted ecological settings dominate the core area, thus resulting in ecological niche divergence and range expansion (Levin 2003, Zozomová-Lihová et al. 2015, Karunarathne et al. 2018). Several studies have shown that increased environmental stress increases residual sexuality in apomictic plants (e.g. Knox 1967, Nogler 1984, Quarin 1986, Mateo De Arias 2015, Rodrigo et al. 2017, Karunarathne et al. under review) and may act as a mechanism for creating new genotypes better adapted to new environments and climate. This further supports the observations that for successful establishment of autopolyploids, ecological divergence and/or stochasticity favoring autopolyploids in small populations is unequivocally imperative (Petit et al. 1999, Rausch and Morgan 2005, Parisod et al. 2010). Overall, geographical distribution and genetic variation observed in *P. intermedium* does not fit either of the afore mentioned two hypotheses of geographical parthenogenesis (discussed further in chapter 5) Therefore, I propose *Casting genotype model* where different genotypes with variable tolerance to the changing and harsher environmental conditions spread away from the progenitor populations and genotypes bearing genetic variations that allow them to succed in new environments will survive and expand, which in turn results in niche divergence and expansion.

4.5.4. Spatial distribution and isolation by distance

The spatial arrangement of populations is an important factor that determines specific genetic structure within and among populations (Cruse-Sanders and Hamrick 2004, Matesanz et al. 2011) and can provide useful information on key life history such as dispersal, genetic drift, and selection processes (Ng et al. 2004). All the studied populations in the present study show isolation by distance and significant spatial dissociation ($p = 0.001$) for almost all the distances (Supplementary Figure S4.2). However, this isolation mainly comes from between cytotype isolation indicating that dispersal of the apomictic polyploids plays a major role in shaping the genetic structure of the species. This further reinforces the observation of niche divergence reported previously (Karunarathne et al. 2018). Moreover, strong isolation in apomicts is an indication of less interaction among populations and possibly leading to directional selection for local adaptations (Carson 1968), this however cannot be attributed being useful for niche expansion and wider distribution without the help of applied studies such as transplantation experiments. Part of this may also have been a result of anthropogenic activities in the region as most grasslands are used for cattle grazing. More insights on the influence on the genetic structure by isolation can be gained through studies focusing on the mix ploidy populations and the contact zone where the different cytotypes interact (e.g. Cosendai et al. 2011, 2013; Zozomová-Lihová et al. 2015). Nevertheless, the present study provides useful preliminary information on the genetic structure variation in relation to coexistence and niche divergence in apomictic complexes.

4.5.5. Mixed-ploidy populations and the contact zone

Geographical or distributional ranges where different ploidy levels occur in sympatry (contact zones) and populations with mixed ploidy levels are of increased interest as they provide ideal platforms to study characteristic conditions of the early stage poplyploid establishment and to test hypotheses concerning dynamics and evolution of polyploid complexes (Petit et al. 1999, Cosendai et al. 2013, Sabara et al. 2013, Zozomová-Lihová et al. 2015). Three major important processes observed in two cytotype contact zones have been discussed: a) reproductive restrains between cytotypes by sterile intermediate cytotypes (e.g. triploid block), b) regenerate conditions that occur in the establishment of polyploids, c) enhance the dynamics and further evolution of polyploid complexes (see Petit et al. 1999). Depending on the origin of the contact zone they are categorized into two: i) primary – emergence of neopolyploids within a diploid population, ii) secondary – formerly allopatric diploids and polyploids coming into contact. The present results show that the contact zone between the diploid and tetraploids of *P. intermedium* is primary in origin. Individuals of mixed-ploidy populations clustering together and with diploids, especially the triploid individual clustering with diploids, and genetic and genotypic variation among tetraploid individuals in mixed-ploidy populations where all tetraploids are non-clonal, support this assumption. Furthermore, no seeds with embryo to endosperm ploidy ratios that originate from tetraploid as pollen doners were found in the flow cytometric seed screening. This observation implies the absence of backcrosses from tetraploid to diploid usually observed in secondary contact zones (e.g. Zozomová-Lihová et al. 2015). I assume that the initial tetraploid populations originated at the contact zone and spread to the south of the distribution range. Therefore, the contact zone acts as a *perenial source* of neotetraploids of *P. intermedium*. In accordance, we reported that there is a tendency of tetraploids displacing the diploid populations in the contact zone, however, depending on the climatic and niche preferences of each cytotype (see Karunarathne et al. 2018). Nevertheless, with the current data, I cannot explain the presence of mixed-ploidy populations only on the south of the diploids' range. Hypothetically, even though rare, all diploid populations should give rise to autotetraploids in *P. intermedium,* but we only observe the generation of new tetraploids in the south border of the diploid distribution, despite our thorough sampling in the area. This may be a consequence of environmental influence on the production of tetraploids in diploids as this has shown to affect the rate of polyploidization (see Soltis and Soltis 2009). A detailed look to the populations in the contact zone with thorough sampling covering as much of geographical distribution of the contact zone as possible, exclusively covering the spatial arrangement of the two ploidies accompanied by a genetic analysis will provide better insight into the dynamics of the occurrence and the establishment of polyploids. On the other hand, the observation of a pure tetraploid population (Hojs424/1S) with

higher genetic variation and close affinity to the diploids, far north of the distribution may be an indication of the occurrence of tetraploids at the edge of the diploid distribution.

4.6. CONCLUSIONS

The present results show that apomictic autotetraploids are of multiple independent origins. This indicates that the polyploidization in *P. intermedium* is recurrent. The placement of tetraploids in different genetic clusters in both NJ tree and the cluster analysis indicates that tetraploid populations show substantial among-population genetic variation confirming that higher genetic variation in apomictic polyploid populations aid them in niche divergence and expansion. The scattered distribution of genetic variation along the distribution range reinforces this observation. The contact zone of the diploids and tetraploids is a primary contact zone where tetraploids frequently occur in mixed-ploidy populations. Therefore, the contact zone acts as a *perenial source* of genetic variation and tetraploid generation, especially at the periphery of the diploid distribution where they may be constantly under selective pressure from varying climatic conditions. Therefore, this observation does not necessarily agree with the known hypotheses General purpose genotypes and Frozen Niche Variation by not having a clear definition as to what governs the geographical parthenogenesis observed in the species. There is significant isolation of genetic variation by distance among populations. However, I cannot elaborate on these variations as distance among the observed genetic clusters are inherently high due to the broad distribution range. Therefore, a closer look to the mixed-ploidy populations and the contact zone will provide greater insights into genetic variation in sympatry. Overall, the present study provides important insights into the mechanisms that aid newly arisen polyploid to survive, coexist, expand and establish themselves after polyploidization.

5. GENERAL DISCUSSION

In this thesis, I have addressed several important questions pertaining to plant polyploidy, apomixis, geographical parthenogenesis, species range expansion, and population structure of polyploids and apomicts, all leading to a broader understanding of plant evolution and diversification. The model system, *Paspalum intermedium* is a noteworthy in such studies as it provided not only an ideal platform but also valuable insights to our understanding of ecological and niche divergence of different cytotypes, cytotype coexistence, population structure variation in comparison to reproductive modes and distribution, and environmental influence on the expression of apomixis. Major reviews on polyploidy and apomixis in plants have frequently mentioned the significance of studies using different approaches and model systems to address relevant questions as it has been clearly evident that polyploidy and apomixis are not only linked together but also are recurrent and have multiple independent origins (e.g. Soltis and Soltis 1999, Bicknell and Koltunow 2004, Soltis et al. 2004, 2009, 2010, Hörandl 2006, 2008, Tucker and Koltunow 2009, Jiao et al. 2011, Koltunow et al. 2013, Hojsgaard et al. 2014b). Even though the present study is not complete in any way for answering all the research questions I have brought up here, it was immensely useful in getting further insights into all the concepts in concern and contributed to our collective understanding of plant evolution.

5.1. Cytotype Composition in *Paspalum intermedium*

From all the populations studied in this thesis, the majority was pure tetraploid populations (24 populations) and tetraploids made 64.8% of all the studied individuals (presented in chapter 2). Previous studies on the species had suggested that autotetraploids occur in abundance (e.g. Norrmann et al., 1989; Hojsgaard et al., 2009) but had not examined the relative abundance of each cytotype. Increased abundance of higher ploidies however has been observed in other systems of autopolyploidy (e.g. Parisod and Joost 2010, Cosendai et al. 2011, Oswald and Nuismer 2011, Zozomová-Lihová et al. 2015) although a common causality is not observed in all systems. Superior colonizing ability and higher climatic tolerances can be seen listed as two important factors for the observed cytotype occurrence variability in *P.intermedium*. The observation of mixed-ploidy populations in a contact zone of the two ploidy levels is a crucial finding towards understanding the variation of cytotype composition throughout the geographical distribution of the species. As a highlight, observation of a rare triploid may be immensely usefull

in understading processes through which the polyploids are fromed in *P. intermedium* as study of triploid formation in natural poupulations have provided an opportunity to study the role of intermediate or rare ploidy in polyploid formation and population dynamics (Ramsey and Schemske 1998, Husband 2004).

Interactions of different forms between cytotypes, e.g. genetically, physiologically and ecologically, may shape the composition of populations both locally and regionally (e.g. Halverson et al. 2008, Sonnleitner et al. 2010). Inter-cytotype competition for resources and mating partners is another vital component of cytotype coexistence (Cosendai et al. 2011, Schinkel et al. 2016b, Kirchheimer et al. 2018). In accordance, the ecological specialization and local environmental conditions influence the distribution of the two cytotypes substantially; this is reflected by the displacement of one cytotype making a directional turnover as a result of unstable temporal coexistence of the two cytotypes. The regional and spatial separation of the diploids and tetraploids with their scatterred replacement and biased recruitment of new polyploid individuals in mixed ploidy populations provide support for the observed cytotype displacement. A study probing on fine scale ecological and spatial variations in mixed-ploidy populations of *P.intermedium* will provide valuable insights to the factors underlying cytotype co-existence and ploidy establishment.

5.2. Spatial and Geographical Distribution of Cytotypes and Their Ecological Importance

Studies on traits associated with environmental differntiation and reproductive mode variation and cytotype distribution patterns provides valuable insights into diploid–polyploid dynamics and factors responsible for contraction–expansion cycles (e.g. Cosendai and Hörandl 2010, Caperta et al. 2016, Sonnleitner et al. 2016). In this thesis, I attempted to recognize the environmental factors and biological traits in *P. intermedium* (presented in chapter 2) that may affect cytotype coexistence, population dynamics and ecological adaptation in both macro and micro scale, during the early events of polyploidization and speciation in plants.

The observed geographical distribution range of *P. intermedium* is centered in northern and eastern Argentina with few records from neighboring regions (Zuloaga et al. 2012). The study revealed a North–South spatial segregation of the two cytotypes with a narrow East–West overlapping zone in the center. Even though tetraploids are evolutionarily younger than diploids, it has become the most common cytotype, occupying two-thirds of the species' geographic range. The latitudinal tailing of the species' distribution may represent a glimpse of the progression of past cytotype displacement (see chapter 2) and/or the influence of other external factors such as anthropogenic activities. Nevertheless, the presence of a pure tetraploid population

in the far north of the distribution provides us an invaluable opportunity to probe into to the factors that affect cytotype distribution with genetic data (discussed below).

In this study, I observed that the ecological preferences of the two cytotypes exhibit significant differentiation owning to a change of niche optima. The tetraploids have acquired a larger range of climatic preferences by becoming generalists while diploids exhibit a highly specialized narrow niche. Spoelhof et al. (2017) compiled findings of studies showing the broadening of the niche and adapting to extreme environmental conditions by autotetraploids. The findings of my thesis are also in congruence with this observation. Therefore, although the findings show substantial similarities to the *Frozen Niche Variation* concept, tetraploids occupying new niches and evident broadening of the ecological preferences with substantial genetic variation (see below and chapter 4) points to an exception of both General Purpose Genotype and Frozen Niche Variation concepts. Along with the reproductive assurance by apomixis for the autotetraploids, this represents an example of species niche expansion and ecological exploitation. Furthermore, if at all reversal to sex is observed in *Paspalum*, it is highly likely that isolated populations at the periphery will diversify further broadening the genetic variation.

Prediction of past distribution range of *P.intermedium* indicated a dynamic range shift in accordance with the climate change. The presence of a "macro-scale" cytotype turnover implies that the polyploid complex is highly sensitive to the climate and the preferred niche, especially by the diploids. *P. intermedium*, therefore, is a highly dynamic polyploid system with pronounced polyploid establishment and potential cytotype diversification. Nevertheless, transplantation experiments addressing the ecological preferences of the two cytotypes will immensely provide a clearer conclusion on this observation.

5.3. Reproductive Modes, Competition and Reproductive Fitness

While apomixis in plants acts as a shield for genomic instability, unbalanced chromosomal segregation, and frequency-dependent reproductive disadvantage caused by hybridization or polyploidy (reviewed in Hojsgaard 2018), residual sexuality in apomictic plant populations may contribute to genotypic variation, avoid lethal mutation accumulation, and favor resilient genetic variation (see Hörandl and Paun 2007, Hojsgaard and Hörandl 2015). Different apomictic plant species exhibit varied levels of residual sexuality (e.g. Naumova et al. 1999, Espinoza et al. 2002, Bicknell and Koltunow 2004, Hojsgaard et al. 2013) with possible underlying genetic control as well as environmental influence. However, studies addressing the causality of differed levels of reproductive modes in natural populations remain limited. In this study (chapter 3), I observed a similar variation of levels of sexual and apomictic reproductive modes in both ovules (6-68% and 32-94% in sex and apomixis respectively) and seeds (3-33% for sex and 67-96% for apomixis). Genetic and population structure aside (see chapter 4), this variation follows

an environmental stress alternation (mean diurnal range: see chapter 3). As addressed by several studies (e.g. Garcia-Aguilar et al. 2010, Armenta-Medina et al. 2011, Grimanelli 2012, Podio et al. 2014), epigenetic responses to environmental factors may play a considerable role in reproductive mode determination in *P.intermedium*. Nevertheless, a detailed epigenetic profiling of these apomictic populations will provide a clear conclusion on this aspect.

Diploids of *P.intermedium* are obligate sexuals as it has been observed in this study as well as previous studies (e.g Quarin 1992, Hojsgaard et al. 2008). Although abnormalities of embryo sac formation has been observed in diploids (Honfi et al. 1990, Hojsgaard et al. 2009), no apomictic embryo sacs were observed and the turnover rate of sexsual embryo sacs was 99.7% (0.3% aborted). In contrast, apomictic plants indicated an active competition of meiotic and apomictic reproductive pathways favoring the apomictic embryo sac and seed development. The penetrance of apomixis has been put to question as it has been observed to be a crucial factor for apomixis turnover in plants (e.g. Matzk et al. 2005, Aliyu et al. 2010). In my study, *P.intermedium* exhibited a strong penetrance of apomixis by having a high percentage of ovules (63%) with more than one apomictic embryo sacs and a substantial reduction of meiotic embryo sacs in these individuals (0 – 10% meiotic embryo sacs in individuals with multiple AES, the average meiotic embryo sac percentage of all the populations was 32.7%). However, this high percentage of apomixis comes at the cost of a reduction in the reproductive pathway fitness (see chapter 3) compared to sexual diploids. The fecundity and thus fitness of diploid populations were, in almost in all cases, twice that of apomictic populations (fertility and germinability did not show noticeable difference between cytotypes). This suggests that despite the reproductive assurance, apomicts have reduced fitness in natural populations while diploids have capitalized on the sexual reproductive fitness. However, genomic instabilities, pollination variation, and apomictic pathway regulation variation cannot be ruled out and hence a comprehensive look into genomics in apomictic embryo sac development will provide a clearer understanding of such fitness differences.

5.4. Population Structure and Genetic Composition

Obligate apomicts where strict clonality can exist, are rare in natural populations although it has been reported in plants (e.g. Martens et al., 2009). Whereas in most natural apomictic populations, a considerable genetic and genotypic diversity is observed with higher clonal diversity (Cosendai et al. 2011, 2013, Zozomová-Lihová et al. 2015). *Paspalum intermedium*, in the contrary, harbors a higher percentage of populations with a single genotype (25% of all pure 4x populations) (presented in chapter 4). This indicates considerable reproductive isolation and isolation by dispersal. Obligate diploid sexuals maintain a higher genetic and genotypic diversity although the among-population genetic variation is relatively lower than that

of tetraploids, while mixed-ploidy populations show intermediate genetic variations between pure 2x and 4x populations. The independent multiple of origin of autotetraploids explains the observation of higher genetic variation among tetraploid populations, where the 4x individuals captured a portion of genetic variation from 2x progenitors and acquiring further variations with time as it has been observed in other systems (see Soltis et al. 2016, Mandáková and Lysak 2018). The geographical distribution of genetic clusters observed within the species (see Chapter 4, Figure 4.4) provides another clue about the origin of genetic variation in 4x population from 2x sexuals, where tetraploids share varying levels of genetic variations with multiple diploid populations.

Apart from the genetic variation between cytotypes and among populations, genetic clusters observed and their spatial distribution may explain the inevitable ecological and niche divergence between the cytotypes in *P.intermedium* (Karunarathne et al. 2018). The broad genetic distance present among 4x populations (see PCoA Chapter 4, Figure 4.3) may have helped them to acquire traits that favor wider tolerance to environmental changes (Bolnick et al. 2011, Sonnleitner et al. 2016), thus achieving broader distribution (geographical parthenogenesis), unlike diploids where constant gene flow may have prevented them from adapting to new environments. Nevertheless, fine scale analysis of both ecological and genetic variations in contact zones where both cytotypes occur in sympatry as well as parapatry will help us to gain clear insights to the underlying forces of ecological differentiation observed between diploids and tetraploids of *P.intermedium* as they are the *perenial source* of early stage polyploidization.

Overall, the findings of this thesis elucidate that genetic and ecological trait changes as a result of polyploidy, accompanied by superior colonizing abilities of apomixis have enabled the observed niche divergence and range expansion in *Paspalum intermedium*, giving rise to a scenario of geographical parthenogenesis. Adapting to harsher environmental conditions with a shift in niche optima have been observed to be crusial in apomictic plants (e.g. Schinkel et al. 2016b, Karunarathne et al. 2018, Kirchheimer et al. 2018), which is clearly observed also in autotetraploids of *P. intermedium*. Furthermore, phenological differentiation and reproductive mode modulation by environment in facultative apomicts may be playing a crucial role in introducing new genetic variation critical for adapting to changing environments, especially in peripheral populations. Therefore, *Paspalum intermedium* provides a unique opportunity to study the evolutionary fate of polyploids and ecological consequences of apomixis.

6. LITERATURE CITED

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SUPPLEMENTARY DATA

Table S2.1. Details on collection sites, collection codes, number of individuals and ploidy of *P. intermedium* materials collected in the present study.

Col._code	Longitude Latitude	Location and vouchers	Elevation (m)	No._ind	Ploidy (x=10)
Hojs401	WO057.0760 S27.5831	Ctes, NR12, b/ Ituzaingó and Itá Ibaté *HojsHonMar401* (CTES, MNES)	81	30	4x#
M29	WO57.5069 S29.8516	Ctes, Dpt Gral San Martín, NR14, 4km South-western PR126 intersection *MarSched29* (CTES)	48	30	4x#
Hojs402	WO057.28145 S29.60798	Ctes, PR123, b/ access to NR14 and Aguapey creek *HojsHonMar402* (CTES, MNES)	80	30	4x
Hojs403	WO057.48440 S29.82671	Ctes, PR126, b/ Curuzú Cuatiá and Paso de los Libres *HojsHonMar403* (CTES, MNES)	74	32	4x
Hojs404	WO058.20937 S29.90815	Ctes, PR126, b/ Curuzú Cuatiá and Sauce *HojsHonMar404* (CTES, MNES)	81	30	4x
Hojs405	WO058.70831 S30.10079	Ctes, Dpt Sauce, PR126, 9 km of Sauce, b/ C.Cuatiá and Sauce *HojsHonMar405* (CTES, MNES)	81	31	4x
Hojs406	WO058.83574 S29.87327	Ctes, Dpt Sauce, PR23, b/ Barrancas´creek and Perugorría *HojsHonMar406* (CTES, MNES)	64	20	4x
Hojs407	WO058.76494 S29.80516	Ctes, Dpt Sauce, PR 23, 59 km from Perugorría	76	1	4x
Hojs408	WO058.74647 S29.78708	Ctes, Dpt Sauce, PR 23, 57 km from Perugorría	68	1	4x
Hojs409	WO058.65584 S29.47009	Ctes, Dpt Curuzú Cuatiá, PR23, 16 km from Perugorría *HojsHonMar409* (CTES, MNES)	66	30	4x
Hojs410	WO058.65475 S29.29530	Ctes, PR24, b/ Perugorría and intersection to NR12 *HojsHonMar410*	45	31	4x#
Hojs411	WO057.47974 S27.92195	Ctes, Dpt Concepción ó San Miguel, NR118, 16 Km North-eastern San Miguel	78	1	4x
Hojs412	WO056.59378 S27.56915	Ctes, NR12, 9 Km North-eastern Ituzaingó		1	2x
Hojs413	WO057.96355 S27.32959	Ctes, NR12, 88 Km North-eastern Corrientes´ city		1	2x
Hojs414	WO059.11377 S27.52680	Chaco, NR11, near Resistencia *HojsSchedMar414* (CTES, MNES)	61	30	4x
Hojs415	WO059.34552 S28.48242	Sta Fe, Villa Ocampo, NR11 and access to slaughterhouse *HojsSchedMar415* (CTES, MNES)	55	30	4x
Hojs416	WO059.77002 S29.58014	Sta Fe, PR1, 3 km Southern Romang (1 km before El Gusano creek) *HojsSchedMar416* (CTES, MNES)	35		4x
Hojs417	WO059.79140 S29.73126	Sta Fe, PR1, 10 km South El Gusano creek	31	3	
Hojs420	WO059.63136 S28.52924	Sta Fe, PR32, 5 Km Southern Villa Ana	61	30	2x
Hojs421	WO059.34472 S27.17748	Chaco, NR16, 47 Km North-eastern Resistencia	71	1	2x
Hojs422	WO059.61130 S27.09917	Chaco, NR16, 73 km North-eastern from Resistencia *HojsSchedMar422* (CTES, MNES)	73	30	2x

Hojs423	WO059.73522 S25.25398	Formosa, NR95, 13 km Eastern Ibarreta	106	31	2x
Hojs424	WO058.93208 S24.95655	Formosa, NR86, 41 km North-western Espinillo *HojsSchedMar424* (CTES, MNES)	88	30	4x
Hojs425	WO058.13835 S25.40336	Formosa, PR6, 15 km Eastern Riacho He He	75	30	2x
Hojs426	WO057.92860 S25.48052	Formosa, PR6, 37 km Eastern Riacho He He	66	2	na
Hojs427	WO057.86054 S25.54320	Formosa, NR11, 32 km South-eastern Clorinda	66	1	na
Hojs428	WO058.04519 S25.981791	Formosa, NR11, 67 km South-eastern Clorinda	60	1	2x
Hojs429	WO058.14276 S25.96397	Formosa, NR11, 25 km Northern Formosa´s city	60	30	2x
Hojs430	WO058.37255 S26.02715	Formosa, NR81, 26 km North-western Formosa´s city	79	1	2x
Hojs431	WO058.75047 S25.85316	Formosa, NR81, 68 km North-western Formosa´s city	75	1	na
Hojs432	WO058.93347 S25.77588	Formosa, NR81, 89 km North-western Formosa´s city	92	30	2x
M31	WO59.5572 S26.0305	Formosa, PR3 *MarSched31* (CTES)	89	30	2x
M26	WO59.3546 S26.2404	Formosa, PR3, 9 km Northern El Colorado town *MarSched26* (CTES)	82	30	2x
Hojs433	WO59.15915 S25.80561	Formosa, PR3, 10 km Southern Pirané	89	1	2x
Hojs434	WO59.09938 S26.31699	Formosa, PR9, 27 km Eastern El Colorado	72	1	2x
Hojs435	WO58.73112 S26.58489	Formosa, PR9, 71 km South-eastern El Colorado	66	1	na
Hojs436	WO58.64436 S26.67490	Chaco, NR11, border with Formosa, 2,6 km Southern Mansilla	57	1	na
Hojs437	WO58.78233 S26.88637	Chaco, NR11, 30 km South-eastern Mansilla	56	1	na
Hojs440	WO59.8931 S27.73475	Chaco, PR7, towards La Sabana settlement	62	30	4x
Hojs441	WO59.98304 S28.37057	Santa Fe, PR3, after Cañada Ombú settlement *HojsKaruSchedMar441* (CTES, MNES)	59	1	na
Hojs442	WO59.99956 S28.50595	Sta Fe, b/Los Tábanos and Golondrina´s settlements	50	1	4x
Hojs443	WO60.09288 S28.78237	Sta Fe, PR3, Southern La Colmena settlement *HojsKaruSchedMar443* (CTES, MNES)	58	30	4x
Hojs444	WO60.14272 S28.99336	Sta Fe, PR3, 12 km before PR40, 5 km South Garabato settlement	59	1	4x
Hojs445	WO60.092 S28.7822	Sta Fe, PR3, 2,5 Km after Toba asttlement, 21 km Northern Vera *HojsKaruSchedMar445* (CTES, MNES)	55	30	4x#

Hojs448	WO61.0186 S30.09820	Sta Fe, 16 km Western intersection b/RP2 and PR13, back way on PR13	48	1	4x
Hojs451	WO61.19000 S30.50303	Sta Fe, PR4, 21 km Southern San Cristobar *HojsKaruSchedMar451* (CTES, MNES)	64	30	4x
Hojs453	WO61.08156 S29.31401	Sta Fe, PR13, 3-5 km before intersect NR98 towards Chaco *HojsKaruSchedMar453* (CTES, MNES)	59	16	4x
Hojs454	WO60.99241 S29.1106	Sta Fe, PR13, 20 km North of NR98 intersection	61	1	4x
Hojs455	WO60.74791 S28.15866	Sta Fe, PR13, 18 km before the border with Chaco *HojsKaruSchedMar455* (CTES, MNES)	61	30	4x#
Hojs456	WO60.69983 S27.49539	Chaco, NR95, 10 km after Villa Angela, towards R.S. Peña *HojsKaruSchedMar456* (CTES, MNES)	85	30	2x, 4x
Hojs460	WO60.19020 S26.89304	Chaco, NR16, 6 km Eastern intersection PR4, towards Resistencia *HojsKaruSchedMar460* (CTES, MNES)	85	1	4x
Hojs465	WO60.17674 S26.89774	Chaco, NR16 and intersection to Colonia Aborigen, from R.S. Peña towards Resistencia *HojsKaruSchedMar465* (CTES, MNES)	86	30	4x
Hojs466	WO59.99973 S26.95990	Chaco, NR16, 15 km Western intersection PR7	84	1	4x
Hojs467	WO59.7264 S27.05106	Chaco, NR16, 15 km Eastern intersection PR7	72	1	2x
Hojs468	WO58.78228 S26.88631	Chaco, NR11, 66 km Northern Resistencia *HojsKaruSchedMar468* (CTES, MNES)	57	30	2x
Hojs470	WO58.12484 S27.74285	Ctes, PR5, 53 km from San Luis del Palmar towards Caá Catí *HojsKaruHonMar470* (CTES, MNES)	59	31	2x, 3x, 4x
Hojs471	WO57.93462 S28.34835	Corrientes, PR6, 9 km North-western Concepción *HojsKaruHonMar471* (CTES, MNES)	64	30	4x
Hojs474	WO58.01721 S31.28989	Entre Ríos, 6 km Eastern NR14, access to Salto Grande International Bridge *HojsKaruHonMar474* (CTES, MNES)	25	1	4x
Hojs475	WO58.00263 S31.26702	Entre Rios, dirt road on Monseñor Ricardo Rösch Avenue *HojsKaruHonMar475* (CTES, MNES)	21	30	4x
Hojs477	WO56.80818 S29.35417	Ctes, NR14, b/ Yapeyú and La Cruz settlements	53	2	na

		HojsKaruHonMar477 (CTES, MNES)			
Hojs478	WO56.38324 S28.79345	Ctes,NR14, 37 km Northern Alvear town *HojsKaruHonMar478* (CTES, MNES)	82	30	4x
Hojs479	WO56.10051 S28.60016	Corrientes, Dpt Santo Tomé, NR14, milestone km 679 *HojsKaruHonMar479* (CTES, MNES)	66	1	na
Hojs480	WO56.02895 S28.47094	Corrientes, Dpt Santo Tomé, PR94, 8.8 km before Sto Tomé´s city *HojsKaruHonMar480*	61	1	4x
Hojs481	WO55.93099 S28.34389	Ctes, PR94, 27 km Northern Santo Tome´s city, after PR174 intersection *HojsKaruMar481* (CTES, MNES)	79	30	2x, 4x
Hojs482	WO56.09195 S27.49069	Ctes, NR12, 24 km South-eastern Posadas´ city, after PR34 intersection	156	1	2x
Hojs483	WO56.64299 S27.58813	Ctes, NR12, milestone km 1250, 1 km Eastern NR120 intersection, bridge on the route *HojsKaruMar483* (CTES, MNES)	78	2	2x
Hojs485	WO57.48130 S27.92212	Ctes, NR118, 27 km South-Eastern Loreto´s village *HojsKaruMar485* (CTES, MNES)	72	1	4x
Hojs486	WO58.74216 S28.17197	Ctes, NR12, 15 km North-western Saladas´ city *HojsKaruMar486* (CTES, MNES)	76	1	na
Hojs487	WO58.74547 S28.16563	Ctes, NR12, 16 km North-western Saladas, milestone km 951	51	31	2x, 4x
M9	WO58.385400 S 27.613250	Corrientes, PR5, 21 km from San Luis del Palmar towards Caá Catí	67	10	2x
M15	WO57.2640 S28.8716	Corrientes, PR114, Estancia Cerro Tuna, 5km before Miriñay´s swamp *MarSched15*	72	14	4x
M28	WO58.753983 S27.615017	Corrientes, 3 km Southern Riachuelo, back way along railways	63	17	2x#
M35	WO57.78926 S27.38394	Corrientes, NR12, 45 km Eastern Itá Ibaté	77	2	4x#

Collector abreviations: Hojs – Hojsgaard, Mar - Martinez, Karu – Karunarathne, Sched – Schedler

Table S2.2. Collections sites of *P. intermedium* plant materials with known ploidy level from previous studies

Col_code	Longitude Latitude	Location	Ploidy (x=10)	Reference	Date
Q3758	WO59.2904 S27.8573	Argentina, Chaco, Basail	2x	Norrmann et al. 1989	25/10/1982
Q3749	WO58.9741 S27.002	Argentina, Chaco, 50 km Northern Resistencia	2x	Norrmann et al. 1989	14/10/1982
Q3752	WO58.9712 S27.0171	Argentina, Chaco, 48 km Northern Resistencia	2x	Norrmann et al. 1989	14/10/1982
Sch22857	WO59.3825 S26.997	Argentina, Chaco, 13 km Northern La Verde, estancia Dos Tranqueras	2x	Norrmann et al. 1989	15/10/1982
Q3757	WO56.6817 S27.5936	Argentina, Corrientes, Ituzaingó	2x	Norrmann et al. 1989	25/10/1982
Q3790	WO56.0408 S28.6108	Argentina, Corrientes, 17 km south of Santo Tomé	2x	Norrmann et al. 1989	06/05/1983
N96	WO60.2882 S26.8599	Argentina, Chaco, 5 km west of Quitilipi	2x	Norrmann et al. 1989	15/12/1983
N97	WO60.109 S26.9265	Argentina, Chaco, 9 km west of Machagai	2x	Norrmann et al. 1989	15/12/1983
N98	WO59.6962 S27.0102	Argentina, Chaco, 15 km east of Presidencia de la Plaza	2x	Norrmann et al. 1989	15/12/1983
PI 404654	WO57.9788 S24.6896	Paraguay, Presidente Hayes, Route 9, 65 km northwest of Paraguay River	2x	Norrmann et al. 1989	22/01/1975
V12220	WO54.6499 S28.4018	Brazil, Rio Grande do Sul, 30 km E de Sao Luis Gonzaga	2x	Honfi et al. 1990	03/12/1989
Q4020	WO54.194 S23.1286	Brazil, Mato Grosso do Sul, 8 km S de Naviraí, junto ao Rio Amambai.	2x	Honfi et al. 1990	30/11/1988
Q4019	WO54.2985 S23.0245	Brazil, Mato Grosso do Sul, 10 km WNW de Naviraí.	2x	Honfi et al. 1990	20/12/1988
V11801	WO54.7937 S22.4001	Brazil, Mato Grosso do Sul, 18 km S de Dourados.	2x	Honfi et al. 1990	16/06/1988
Q4034	WO58.1961 S25.5452	Argentina, 70 km N de Formosa	2x	Honfi et al. 1990	10/1988
Rua35	WO56.5139 S22.2642	Paraguay, Amambay, 15 km S de Bella Vista. Rua35 (BAA)	2x	Hojsgaard et al. 2009	07/01/1994
V11802	WO54.8038 S22.4297	Brasil, MGS, 18 km S Dourados	2x	Vaio et al. 2005	unknown
Q3846	WO58.7361 S28.1527	Argentina, Corrientes, 76 km east-southeast of Corrientes city	4x	Norrmann et al. 1989	05/01/1984

Q3850	WO43.9885 S19.8456	Brazil, Minas Gerais, Belo Horizonte, by the lake of Pampulha dam	4x	Norrmann et al. 1989	09/01/1984
Q3784	WO57.0669 S29.6266	Argentina, Corrientes, 39 km north of Paso de los Ubres	4x	Norrmann et al. 1989	17/05/1983
Q3856	WO59.4775 S28.8543	Argentina, Santa Fé, Las Garzas	4x	Norrmann et al. 1989	26/10/1984
Q3848	WO60.4071 S30.412	Argentina, Santa Fé, 5 km southern Gobernador Crespo	4x	Norrmann et al. 1989	09/01/1984
Q3842	WO58.7428 S28.0838	Argentina, Corrientes, 67 km South-eastern Corrientes´ city	4x	Norrmann et al. 1989	05/01/1984
Q3859	WO55.5308 S27.2872	Argentina, Misiones, between San Ignacio and Santa Ana, near Yabebiry stream	4x	Norrmann et al. 1989	11/12/1984
Q3855	WO56.5963 S29.0969	Argentina, Corrientes, Alvear, near Aguapey River	4x	Norrmann et al. 1989	26/10/1984
Q3753	WO59.365 S26.3035	Argentina; Formosa, El Colorado	4x	Norrmann et al. 1989	14/10/1982
Q3865	WO58.8308 S27.4833	Argentina, Corrientes, Corrientes city	4x	Norrmann et al. 1989	13/05/1985
Q3867	WO59.2151 S28.0949	Argentina, Santa Fé, 5 km south of Florencia	4x	Norrmann et al. 1989	26/11/1985
K2258	WO62.0000 S16.1333	Bolivia, Dpt Santa Cruz , Prov. Ñuflo Chávez , Estancia La Pachanga, 52 km S of Concepción, route to Lomerío (ISC, LPB, F, US, MO, NY)	4x	Norrmann et al. 1994	28/11/1986
K1631	WO62.0833 S16.1333	Bolivia, Dpt Santa Cruz , Prov. Ñuflo Chávez , Estancia Viera, 2 km S of Concepción, route to Lomerío (ISC, LPB, F, US, MO, NY)	4x	Norrmann et al. 1994	20/01/1986
K1673		Bolivia, Dpt Santa Cruz, Prov. Velasco, 5 km E of Santa Cruz de la Frontera (ISC, LPB, F, US, MO, NY)	4x	Norrmann et al. 1994	27/01/1986
V11920	WO55.2658 S22.3549	Brazil, Mato Grosso do Sul, 50 km WSW de Dourados, rio Dourados.	4x	Honfi et al. 1990	20/11/1989
Q4036	WO58.4853 S29.4385	Argentina, Corrientes, 16 km SE de Perugorria	4x	Honfi et al. 1990	28/10/1988
M5327	WO58.7884 S27.948	Argentina, Corrientes, Empedrado, Rta. Nac. 12, Km 958, Ayo. San Lorenzo, Morrone et al. 5327 (MO, SI)	4x	Sede et al. 2010	05/04/2005

Table S3.1. Flow cytometric seed analysis (FCSS) peak indices

Population	Ind. No.	Mother Ploidy	G_1	G_3 (G_5)	C2	C3 (C5)	Peak Index (G3(G5)/G2)
Hojs401	12	4	2	2.82	2	3	1.41
Hojs401	12	4	2	4.58	2	5	2.29
Hojs401	12	4	2	4.80	2	5	2.40
Hojs401	12	4	2	4.81	2	5	2.41
Hojs401	12	4	2	4.86	2	5	2.43
Hojs401	12	4	2	4.86	2	5	2.43
Hojs401	12	4	2	4.86	2	5	2.43
Hojs401	12	4	2	4.87	2	5	2.44
Hojs401	12	4	2	4.88	2	5	2.44
Hojs401	12	4	2	4.89	2	5	2.44
Hojs401	12	4	2	4.89	2	5	2.45
Hojs401	12	4	2	4.90	2	5	2.45
Hojs401	12	4	2	4.91	2	5	2.45
Hojs401	12	4	2	4.93	2	5	2.47
Hojs401	12	4	2	4.93	2	5	2.47
Hojs401	12	4	2	4.93	2	5	2.47
Hojs401	12	4	2	4.99	2	5	2.50
Hojs401	12	4	2	5.10	2	5	2.55
Hojs401	16	4	2	4.72	2	5	2.36
Hojs401	16	4	2	4.73	2	5	2.36
Hojs401	16	4	2	4.76	2	5	2.38
Hojs401	16	4	2	4.77	2	5	2.38
Hojs401	16	4	2	4.77	2	5	2.39
Hojs401	16	4	2	4.78	2	5	2.39
Hojs401	16	4	2	4.80	2	5	2.40
Hojs401	16	4	2	4.81	2	5	2.41
Hojs401	16	4	2	4.81	2	5	2.41
Hojs401	16	4	2	4.83	2	5	2.41
Hojs401	16	4	2	4.83	2	5	2.42
Hojs401	16	4	2	4.84	2	5	2.42
Hojs401	16	4	2	4.85	2	5	2.42
Hojs401	16	4	2	4.85	2	5	2.43
Hojs401	16	4	2	4.86	2	5	2.43
Hojs401	16	4	2	4.87	2	5	2.43
Hojs401	16	4	2	4.87	2	5	2.43
Hojs401	16	4	2	4.91	2	5	2.46
Hojs401	16	4	2	4.92	2	5	2.46
Hojs401	16	4	2	5.10	2	5	2.55
Hojs401	21	4	2	2.89	2	3	1.44
Hojs401	21	4	2	4.61	2	5	2.31

Hojs401	21	4	2	4.62	2	5	2.31
Hojs401	21	4	2	4.71	2	5	2.35
Hojs401	21	4	2	4.73	2	5	2.37
Hojs401	21	4	2	4.77	2	5	2.38
Hojs401	21	4	2	4.78	2	5	2.39
Hojs401	21	4	2	4.78	2	5	2.39
Hojs401	21	4	2	4.79	2	5	2.40
Hojs401	21	4	2	4.81	2	5	2.41
Hojs401	21	4	2	4.82	2	5	2.41
Hojs401	21	4	2	4.82	2	5	2.41
Hojs401	21	4	2	4.83	2	5	2.42
Hojs401	21	4	2	4.83	2	5	2.42
Hojs401	21	4	2	4.84	2	5	2.42
Hojs401	21	4	2	4.84	2	5	2.42
Hojs401	21	4	2	4.85	2	5	2.42
Hojs401	21	4	2	4.85	2	5	2.42
Hojs401	21	4	2	4.85	2	5	2.42
Hojs401	21	4	2	4.89	2	5	2.45
Hojs401	21	4	2	5.00	2	5	2.50
Hojs401	21	4	2	5.00	2	5	2.50
Hojs401	21	4	2	5.03	2	5	2.52
Hojs401	21	4	2	5.18	2	5	2.59
Hojs403	18	4	2	4.75	2	5	2.38
Hojs403	18	4	2	4.85	2	5	2.43
Hojs403	18	4	2	4.85	2	5	2.43
Hojs403	18	4	2	4.87	2	5	2.43
Hojs403	18	4	2	4.90	2	5	2.45
Hojs403	19	4	2	4.79	2	5	2.40
Hojs403	19	4	2	4.83	2	5	2.41
Hojs403	19	4	2	4.84	2	5	2.42
Hojs403	19	4	2	4.89	2	5	2.44
Hojs403	19	4	2	4.89	2	5	2.44
Hojs403	19	4	2	4.90	2	5	2.45
Hojs403	19	4	2	4.92	2	5	2.46
Hojs403	20	4	2	2.93	2	3	1.47
Hojs403	20	4	2	3.26	2	3	1.63
Hojs403	20	4	2	4.71	2	5	2.36
Hojs403	20	4	2	4.78	2	5	2.39
Hojs403	20	4	2	4.79	2	5	2.39
Hojs403	20	4	2	4.81	2	5	2.40
Hojs403	20	4	2	4.83	2	5	2.41
Hojs403	20	4	2	4.90	2	5	2.45
Hojs403	20	4	2	4.91	2	5	2.45
Hojs403	20	4	2	4.91	2	5	2.46

Hojs403	20	4	2	4.94	2	5	2.47
Hojs403	20	4	2	4.99	2	5	2.49
Hojs403	20	4	2	5.00	2	5	2.50
Hojs403	20	4	2	5.04	2	5	2.52
Hojs405	4	4	2	4.75	2	5	2.37
Hojs405	4	4	2	4.76	2	5	2.38
Hojs405	4	4	2	4.82	2	5	2.41
Hojs405	4	4	2	4.85	2	5	2.42
Hojs405	4	4	2	4.87	2	5	2.43
Hojs405	12	4	2	2.85	2	3	1.42
Hojs405	12	4	2	4.80	2	5	2.40
Hojs405	12	4	2	4.86	2	5	2.43
Hojs405	12	4	2	5.46	2	5	2.73
Hojs409	6	4	2	4.61	2	5	2.30
Hojs409	6	4	2	4.66	2	5	2.33
Hojs409	6	4	2	4.68	2	5	2.34
Hojs409	6	4	2	4.69	2	5	2.34
Hojs409	6	4	2	4.76	2	5	2.38
Hojs409	6	4	2	4.83	2	5	2.42
Hojs409	6	4	2	4.87	2	5	2.44
Hojs409	6	4	2	5.02	2	5	2.51
Hojs409	6	4	2	5.09	2	5	2.55
Hojs409	10	4	2	2.93	2	3	1.47
Hojs409	10	4	2	4.59	2	5	2.29
Hojs409	17	4	2	3.10	2	3	1.55
Hojs409	17	4	2	4.65	2	5	2.33
Hojs409	17	4	2	4.72	2	5	2.36
Hojs409	17	4	2	4.75	2	5	2.37
Hojs409	17	4	2	4.79	2	5	2.40
Hojs409	17	4	2	4.80	2	5	2.40
Hojs409	17	4	2	4.82	2	5	2.41
Hojs409	17	4	2	4.86	2	5	2.43
Hojs409	17	4	2	4.87	2	5	2.44
Hojs409	17	4	2	4.88	2	5	2.44
Hojs409	17	4	2	4.92	2	5	2.46
Hojs409	17	4	2	4.95	2	5	2.48
Hojs409	17	4	2	4.99	2	5	2.49
Hojs410	13	4	2	4.88	2	5	2.44
Hojs410	17	4	2	2.99	2	3	1.50
Hojs410	17	4	2	3.13	2	3	1.57
Hojs410	17	4	2	3.19	2	3	1.60
Hojs410	17	4	2	3.24	2	3	1.62
Hojs410	17	4	2	4.59	2	5	2.30
Hojs410	17	4	2	4.67	2	5	2.34

Hojs410	17	4	2	4.69	2	5	2.34
Hojs410	17	4	2	4.72	2	5	2.36
Hojs410	17	4	2	4.74	2	5	2.37
Hojs410	17	4	2	4.75	2	5	2.37
Hojs410	17	4	2	4.76	2	5	2.38
Hojs410	17	4	2	4.78	2	5	2.39
Hojs410	17	4	2	4.85	2	5	2.42
Hojs410	17	4	2	4.92	2	5	2.46
Hojs415	2	4	2	4.56	2	5	2.28
Hojs415	2	4	2	4.65	2	5	2.32
Hojs415	2	4	2	4.67	2	5	2.33
Hojs415	2	4	2	4.68	2	5	2.34
Hojs415	2	4	2	4.71	2	5	2.35
Hojs415	2	4	2	4.75	2	5	2.38
Hojs415	2	4	2	4.76	2	5	2.38
Hojs415	2	4	2	4.78	2	5	2.39
Hojs415	2	4	2	4.79	2	5	2.39
Hojs415	2	4	2	4.81	2	5	2.41
Hojs415	3	4	2	3.05	2	3	1.53
Hojs415	3	4	2	4.66	2	5	2.33
Hojs415	3	4	2	4.68	2	5	2.34
Hojs415	3	4	2	4.71	2	5	2.36
Hojs415	3	4	2	4.75	2	5	2.37
Hojs415	3	4	2	4.78	2	5	2.39
Hojs415	3	4	2	4.81	2	5	2.40
Hojs415	3	4	2	4.84	2	5	2.42
Hojs415	3	4	2	4.85	2	5	2.43
Hojs415	3	4	2	4.85	2	5	2.43
Hojs415	3	4	2	4.87	2	5	2.44
Hojs415	3	4	2	4.88	2	5	2.44
Hojs415	3	4	2	4.89	2	5	2.45
Hojs415	5	4	2	2.71	2	3	1.35
Hojs415	5	4	2	3.08	2	3	1.54
Hojs415	5	4	2	4.70	2	5	2.35
Hojs415	5	4	2	4.74	2	5	2.37
Hojs415	5	4	2	4.74	2	5	2.37
Hojs415	5	4	2	4.74	2	5	2.37
Hojs415	5	4	2	4.75	2	5	2.37
Hojs415	5	4	2	4.75	2	5	2.38
Hojs415	5	4	2	4.76	2	5	2.38
Hojs415	5	4	2	4.76	2	5	2.38
Hojs415	5	4	2	4.77	2	5	2.39
Hojs415	5	4	2	4.78	2	5	2.39
Hojs415	5	4	2	4.82	2	5	2.41

Hojs415	5	4	2	4.85	2	5	2.42
Hojs415	5	4	2	4.85	2	5	2.42
Hojs415	5	4	2	4.86	2	5	2.43
Hojs415	5	4	2	4.87	2	5	2.43
Hojs415	5	4	2	4.92	2	5	2.46
Hojs415	5	4	2	4.96	2	5	2.48
Hojs415	5	4	2	4.97	2	5	2.48
Hojs445	10	4	2	2.99	2	3	1.49
Hojs445	10	4	2	2.99	2	3	1.49
Hojs445	10	4	2	4.58	2	5	2.29
Hojs445	10	4	2	4.69	2	5	2.35
Hojs445	10	4	2	4.79	2	5	2.39
Hojs445	10	4	2	4.80	2	5	2.40
Hojs445	10	4	2	4.83	2	5	2.42
Hojs445	10	4	2	4.85	2	5	2.43
Hojs445	10	4	2	4.86	2	5	2.43
Hojs445	10	4	2	4.87	2	5	2.43
Hojs445	10	4	2	4.87	2	5	2.43
Hojs445	10	4	2	4.91	2	5	2.46
Hojs445	10	4	2	4.91	2	5	2.46
Hojs445	10	4	2	4.92	2	5	2.46
Hojs445	10	4	2	4.93	2	5	2.46
Hojs445	10	4	2	4.94	2	5	2.47
Hojs445	10	4	2	4.94	2	5	2.47
Hojs445	10	4	2	4.96	2	5	2.48
Hojs445	10	4	2	5.50	2	5	2.75
Hojs445	17	4	2	2.80	2	3	1.40
Hojs445	17	4	2	4.73	2	5	2.36
Hojs445	17	4	2	4.77	2	5	2.39
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Hojs445	17	4	2	4.79	2	5	2.39
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Hojs445	17	4	2	4.81	2	5	2.41
Hojs445	17	4	2	4.81	2	5	2.41
Hojs445	17	4	2	4.81	2	5	2.41
Hojs445	17	4	2	4.83	2	5	2.41
Hojs445	17	4	2	4.85	2	5	2.43
Hojs445	17	4	2	4.89	2	5	2.45
Hojs445	17	4	2	4.92	2	5	2.46
Hojs445	17	4	2	4.98	2	5	2.49
Hojs445	17	4	2	4.99	2	5	2.49
Hojs445	24	4	2	4.79	2	5	2.39
Hojs445	24	4	2	4.82	2	5	2.41
Hojs445	24	4	2	4.83	2	5	2.41

Hojs445	24	4	2	4.85	2	5	2.42
Hojs445	24	4	2	4.86	2	5	2.43
Hojs445	24	4	2	4.87	2	5	2.44
Hojs445	24	4	2	4.88	2	5	2.44
Hojs445	24	4	2	4.98	2	5	2.49
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Hojs455	1	4	2	2.83	2	3	1.42
Hojs455	1	4	2	2.84	2	3	1.42
Hojs455	1	4	2	3.21	2	3	1.61
Hojs455	1	4	2	3.33	2	3	1.67
Hojs455	1	4	2	4.70	2	5	2.35
Hojs455	1	4	2	4.75	2	5	2.37
Hojs455	1	4	2	4.82	2	5	2.41
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Hojs455	1	4	2	4.88	2	5	2.44
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Hojs455	1	4	2	4.91	2	5	2.46
Hojs455	1	4	2	4.96	2	5	2.48
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Hojs455	7	4	2	2.96	2	3	1.48
Hojs455	7	4	2	2.97	2	3	1.48
Hojs455	7	4	2	2.98	2	3	1.49
Hojs455	7	4	2	3.02	2	3	1.51
Hojs455	7	4	2	4.73	2	5	2.36
Hojs455	7	4	2	4.76	2	5	2.38
Hojs455	7	4	2	4.82	2	5	2.41
Hojs455	26	4	2	3.02	2	3	1.51
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Hojs455	26	4	2	4.83	2	5	2.41
Hojs455	26	4	2	4.84	2	5	2.42
Hojs455	26	4	2	4.85	2	5	2.43
Hojs455	26	4	2	4.86	2	5	2.43
Hojs455	26	4	2	5.01	2	5	2.50
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Hojs456	1	2	2	2.85	2	3	1.42
Hojs456	1	2	2	2.94	2	3	1.47
Hojs456	1	2	2	2.98	2	3	1.49
Hojs456	1	2	2	3.01	2	3	1.50

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Hojs456	a	4	2	4.86	2	5	2.43
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Hojs456	b	4	2	4.82	2	5	2.41
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Hojs456	b	4	2	4.89	2	5	2.44
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Hojs465	1	4	2	5.05	2	5	2.53
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Hojs465	13	4	2	4.90	2	5	2.45
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Hojs465	13	4	2	5.04	2	5	2.52
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Hojs465	21	4	2	4.83	2	5	2.41
Hojs465	21	4	2	4.85	2	5	2.42
Hojs465	21	4	2	4.88	2	5	2.44
Hojs465	21	4	2	4.92	2	5	2.46
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Hojs468	24	2	2	2.94	2	3	1.47
Hojs468	24	2	2	2.95	2	3	1.48
Hojs468	24	2	2	3.01	2	3	1.50
Hojs468	24	2	2	3.01	2	3	1.51
Hojs468	27	2	2	2.69	2	3	1.34
Hojs468	27	2	2	2.76	2	3	1.38
Hojs468	27	2	2	2.79	2	3	1.40
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Hojs468	27	2	2	2.84	2	3	1.42
Hojs468	27	2	2	2.86	2	3	1.43
Hojs468	27	2	2	2.89	2	3	1.45
Hojs468	27	2	2	2.92	2	3	1.46
Hojs470	1	2	2	2.77	2	3	1.39
Hojs470	1	2	2	3.04	2	3	1.52
Hojs470	2	3	2	2.72	2	3	1.36
Hojs470	2	3	2	2.75	2	3	1.38
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Hojs470	2	3	2	2.93	2	3	1.47
Hojs470	2	3	2	2.94	2	3	1.47
Hojs470	2	3	2	3.47	2	3	1.73

Hojs470	2	3	2	4.70	2	5	2.35
Hojs470	2	3	2	4.74	2	5	2.37
Hojs470	2	3	2	4.83	2	5	2.41
Hojs470	2	3	2	4.85	2	5	2.42
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Hojs470	2	3	2	4.85	2	5	2.43
Hojs470	2	3	2	4.85	2	5	2.43
Hojs470	2	3	2	4.87	2	5	2.43
Hojs470	2	3	2	4.89	2	5	2.44
Hojs470	2	3	2	4.89	2	5	2.45
Hojs470	2	3	2	4.92	2	5	2.46
Hojs470	2	3	2	4.93	2	5	2.46
Hojs470	2	3	2	4.94	2	5	2.47
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Hojs471	5	4	2	4.87	2	5	2.44
Hojs471	5	4	2	4.89	2	5	2.44
Hojs471	5	4	2	5.06	2	5	2.53
Hojs471	16	4	2	2.91	2	3	1.45
Hojs471	16	4	2	3.01	2	3	1.51
Hojs471	16	4	2	3.17	2	3	1.58
Hojs471	16	4	2	4.77	2	5	2.38
Hojs471	16	4	2	4.81	2	5	2.41
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Hojs471	16	4	2	4.86	2	5	2.43
Hojs471	16	4	2	4.91	2	5	2.46
Hojs471	16	4	2	4.97	2	5	2.48
Hojs471	16	4	2	5.01	2	5	2.50
Hojs471	26	4	2	4.78	2	5	2.39
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Hojs471	26	4	2	4.85	2	5	2.42
Hojs471	26	4	2	4.91	2	5	2.45
Hojs471	26	4	2	4.95	2	5	2.47
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Hojs478	21	4	2	4.86	2	5	2.43
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Hojs478	21	4	2	4.88	2	5	2.44
Hojs478	21	4	2	4.88	2	5	2.44
Hojs478	28	4	2	3.04	2	3	1.52
Hojs478	28	4	2	4.75	2	5	2.38
Hojs478	28	4	2	4.78	2	5	2.39
Hojs478	28	4	2	4.84	2	5	2.42

Hojs478	28	4	2	4.86	2	5	2.43
Hojs478	28	4	2	4.88	2	5	2.44
Hojs478	28	4	2	4.93	2	5	2.47
Hojs478	28	4	2	5.04	2	5	2.52
Hojs478	28	4	2	5.13	2	5	2.57
Hojs478	29	4	2	4.74	2	5	2.37
Hojs478	29	4	2	4.82	2	5	2.41
Hojs478	29	4	2	4.83	2	5	2.41
Hojs478	29	4	2	4.88	2	5	2.44
Hojs478	29	4	2	4.89	2	5	2.44
Hojs478	29	4	2	5.03	2	5	2.51
Hojs478	29	4	2	5.05	2	5	2.52
Hojs481	21	4	2	2.97	2	3	1.48
Hojs481	21	4	2	4.73	2	5	2.37
Hojs481	21	4	2	4.75	2	5	2.37
Hojs481	21	4	2	4.85	2	5	2.43
Hojs481	21	4	2	4.87	2	5	2.43
Hojs481	21	4	2	4.89	2	5	2.44
Hojs481	21	4	2	4.89	2	5	2.44
Hojs481	21	4	2	4.90	2	5	2.45
Hojs481	21	4	2	4.90	2	5	2.45
Hojs481	21	4	2	4.91	2	5	2.45
Hojs481	21	4	2	4.94	2	5	2.47
Hojs481	21	4	2	4.97	2	5	2.48
Hojs481	21	4	2	5.03	2	5	2.52
Hojs481	21	4	2	5.04	2	5	2.52
Hojs481	21	4	2	5.10	2	5	2.55

G1: embryo peak, G3/5: endosperm peak, C1: relative DNA content of the embryo nuclie, C3/5 relative DNA content of the endosperm nuclie; seeds of sexual origin are shaded in grey

Table S3.2. Correlation of meiotic/sexual and apomictic pathways in seeds and embryo sacs to environmental variables

Climatic Variable	Embryo Sac		Seeds	
	p.value	Pearson correlation	*p.value*	Pearson correlation
BIO1 = Annual Mean Temperature	0.446	-0.181	0.989	-0.004
BIO2 = Mean Diurnal Range	0.019	-0.69	0.030	-0.559
BIO3 = Isothermality (BIO2/BIO7) (* 100)	0.050	-0.362	0.691	-0.112
BIO4 = Temperature Seasonality	0.881	0.036	0.223	-0.334
BIO5 = Max Temperature of Warmest Month	0.066	-0.419	0.083	-0.462
BIO6 = Min Temperature of Coldest Month	0.511	0.160	0.174	0.371
BIO7 = Temperature Annual Range	0.045	-0.452	0.055	-0.379
BIO8 = Mean Temperature of Wettest Quarter	0.190	-0.306	0.051	-0.465
BIO9 = Mean Temperature of Driest Quarter	0.530	-0.149	0.766	0.084
BIO10 = Mean Temperature of Warmest Quarter	0.361	-0.216	0.619	-0.140
BIO11 = Mean Temperature of Coldest Quarter	0.530	-0.149	0.766	0.084
BIO12 = Annual Precipitation	0.059	0.429	0.115	0.424
BIO13 = Precipitation of Wettest Month	0.338	0.226	0.796	-0.073
BIO14 = Precipitation of Driest Month	0.104	0.375	0.058	0.518
BIO15 = Precipitation Seasonality	0.082	-0.398	0.051	-0.432
BIO16 = Precipitation of Wettest Quarter	0.401	0.199	0.698	0.109
BIO17 = Precipitation of Driest Quarter	0.100	0.378	0.077	0.370
BIO18 = Precipitation of Warmest Quarter	0.687	-0.096	0.295	-0.289
BIO19 = Precipitation of Coldest Quarter	0.100	0.378	0.077	0.370
UV-B radiation	0.300	-0.244	0.567	-0.161
Elevation	0.549	-0.143	0.414	0.228
Photosynthetically Active Radiation (PAR)	0.523	-0.152	0.708	0.106
Average Cloud Cover	0.456	0.177	0.233	0.328
Frost Day frequency	0.616	-0.119	0.536	-0.174
Vapor pressure at ground level	0.833	-0.050	0.475	0.200

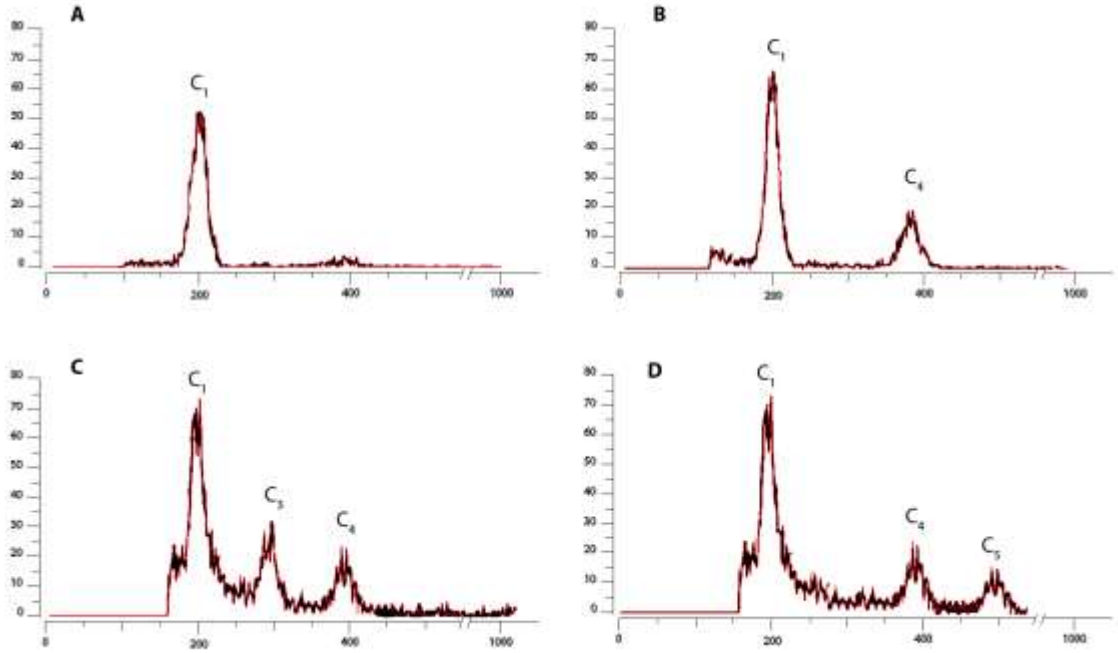

Figure S2.1. Flow cytometry histograms from different tissues and individuals of *P. intermedium*. A. Leaf tissue of diploid standard peak 2x (C_1) peak positioned at 200. B. Leaf tissue of tetraploid 4x (C_4) peak using diploid as an internal standard (C_1), C. A tetraploid seed of sexual origin showing embryo peak (C_1), endosperm peak (C_3) and G_2 phase peak of the embryo nuclie (C_4), D. a tetraploid seed of apomictic origin showing endosperm peak (C_1) G2 phase of the embryo nuclei (C_4) and the endosperm peak (C_5).

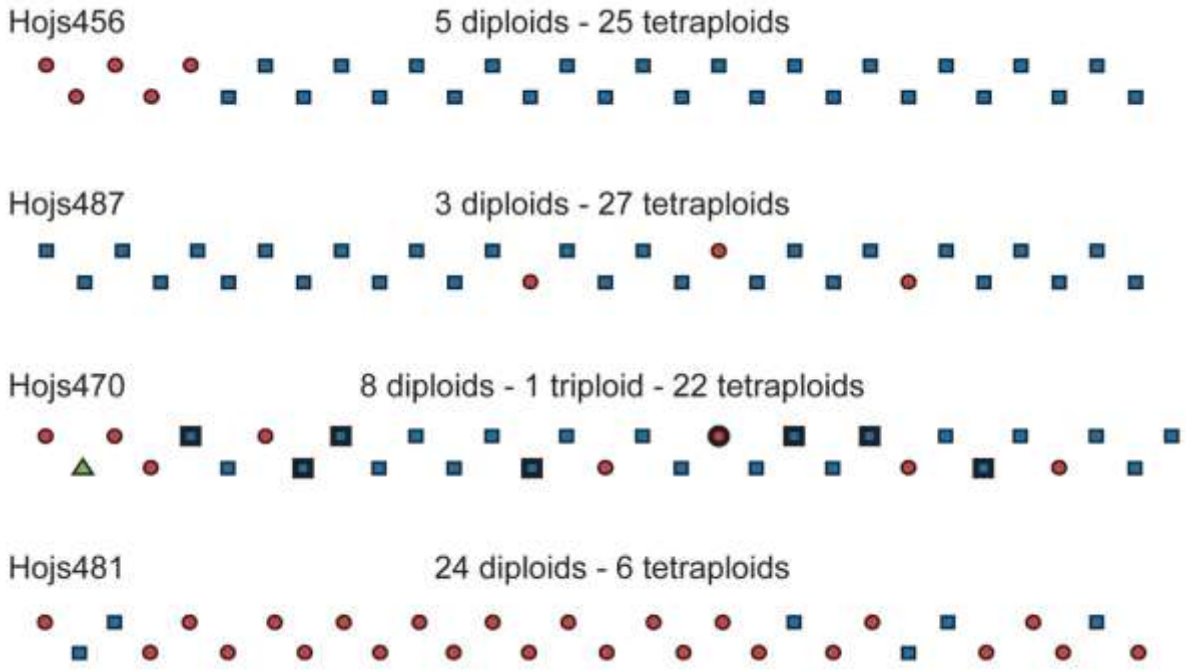

Figure S2.2. Composition and placement of individual cytotypes within mixed-ploidy populations

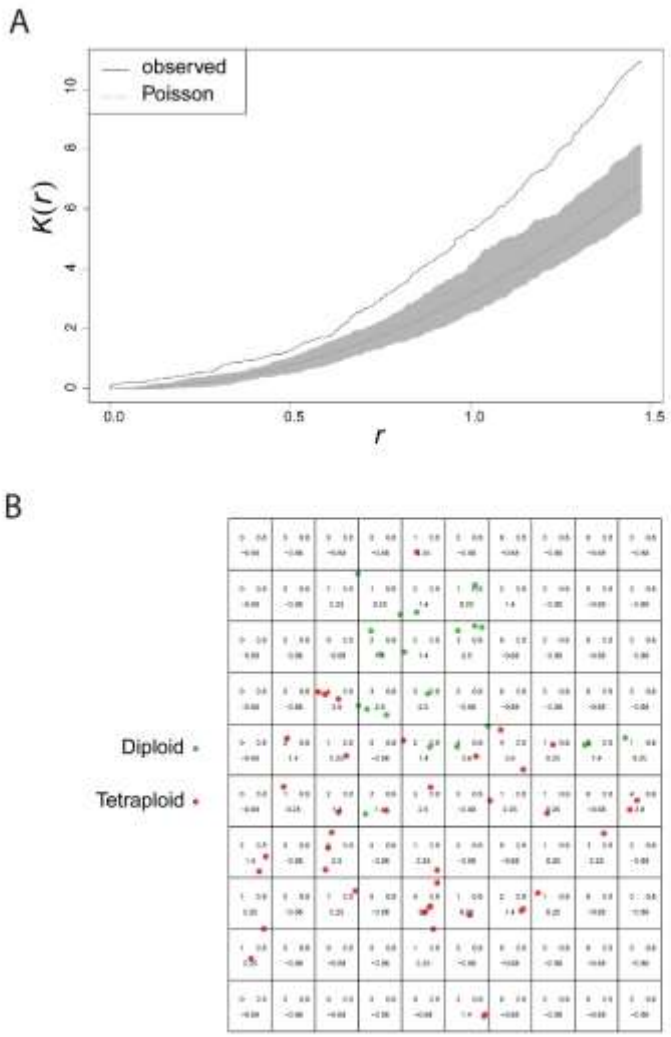

Figure S2.3. Spatial analysis of cytotype distribution of *P. intermedium*.

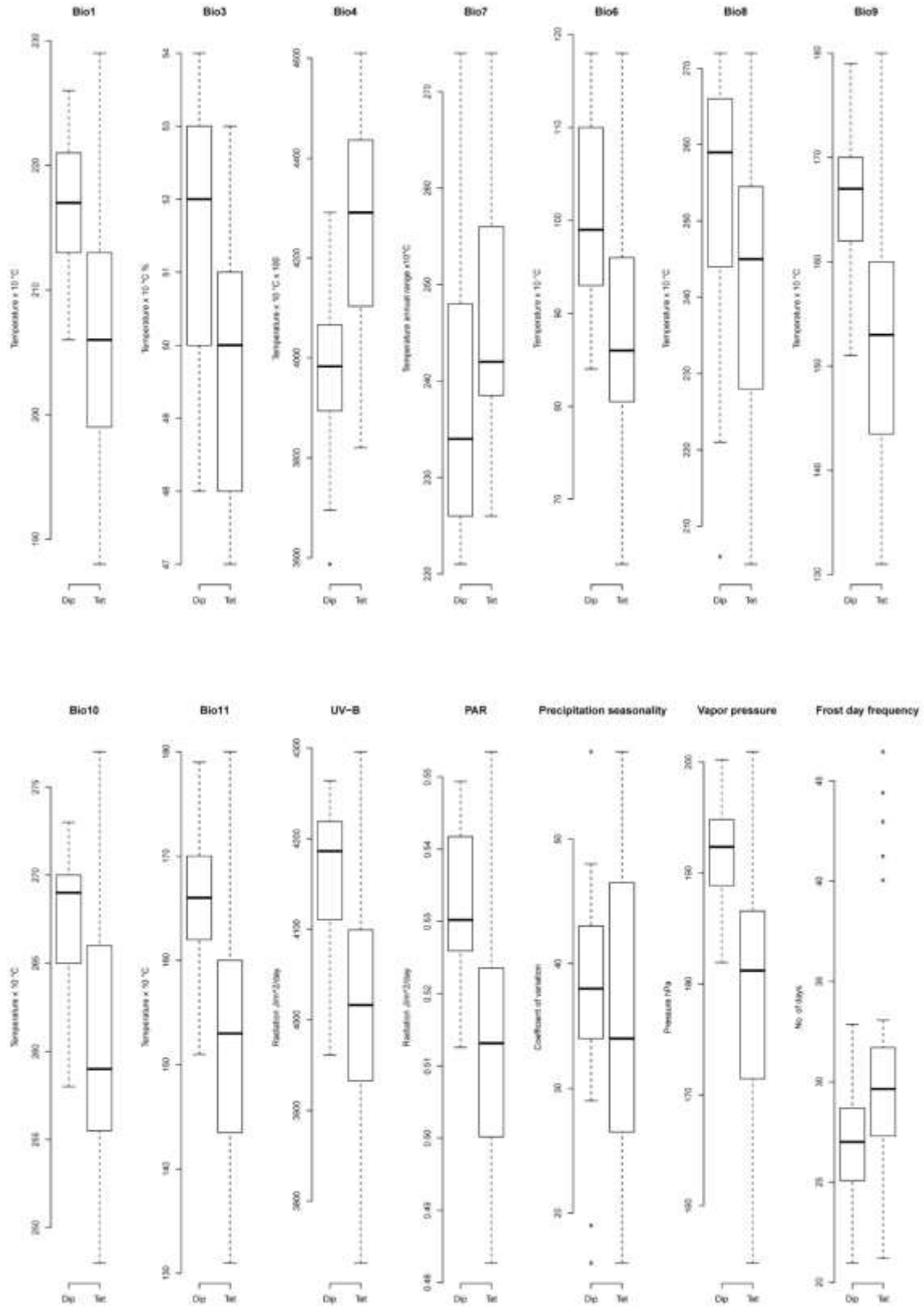

Figure S2.4. Boxplots depicting *P. intermedium* cytotype ecological preferences and niche differentiation for most significant bioclimatic and environmental variables. Whiskers represent minimum (lower) and maximum (upper) values falling out of the Inter-Quartile ranges. Dots represent minor and major outliers. Bio1 through Bio11 is as in WorldClim dataset (Hijmans *et al.* 2005).

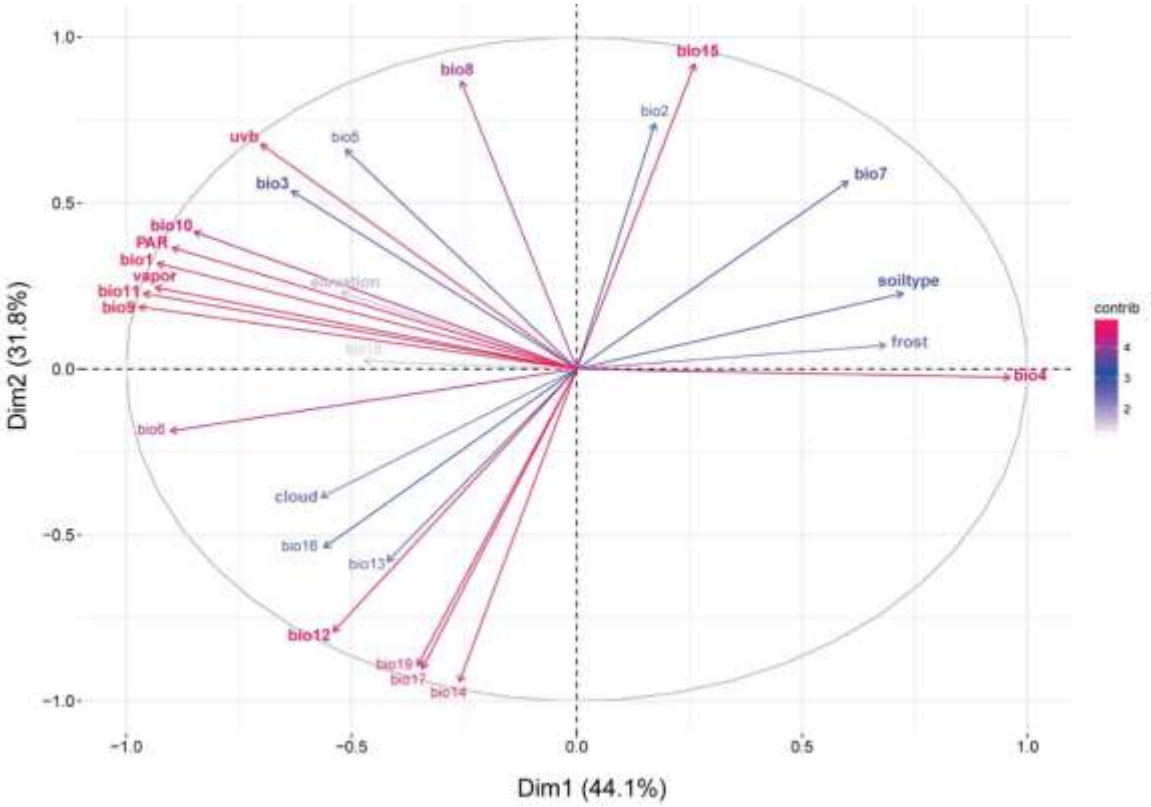

Figure S2.5. Ordination scaling plot for environmental variables and their dimensional contribution to the distribution of diploid and tetraploid *P. intermedium* cytotypes.

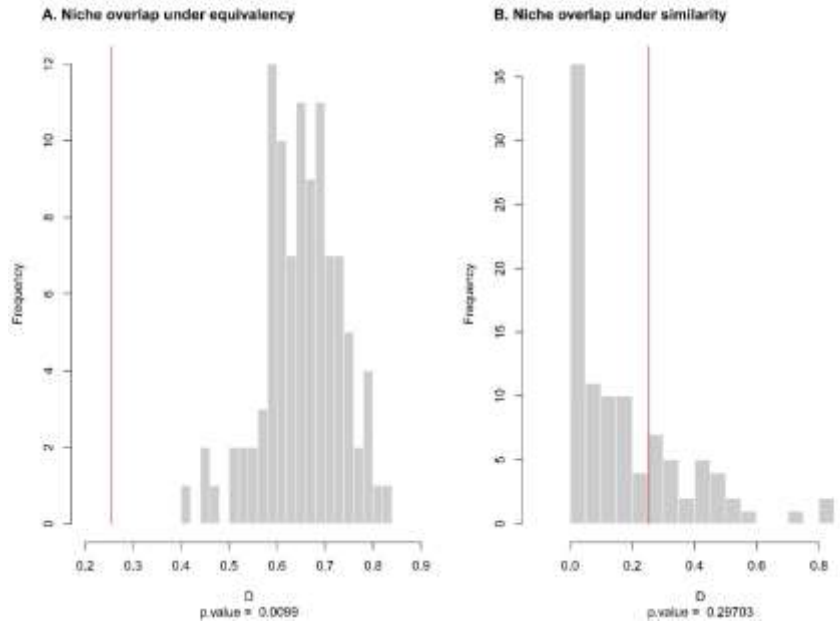

Figure S2.6. Histograms displaying the distribution of 100 randomly simulated niche overlap scores contrasted to the observed niche overlap (red line – 0.25). Schoener´s D index is plotted under the assumption of **A**. niche equivalency **B**. niche similarity between cytotypes.

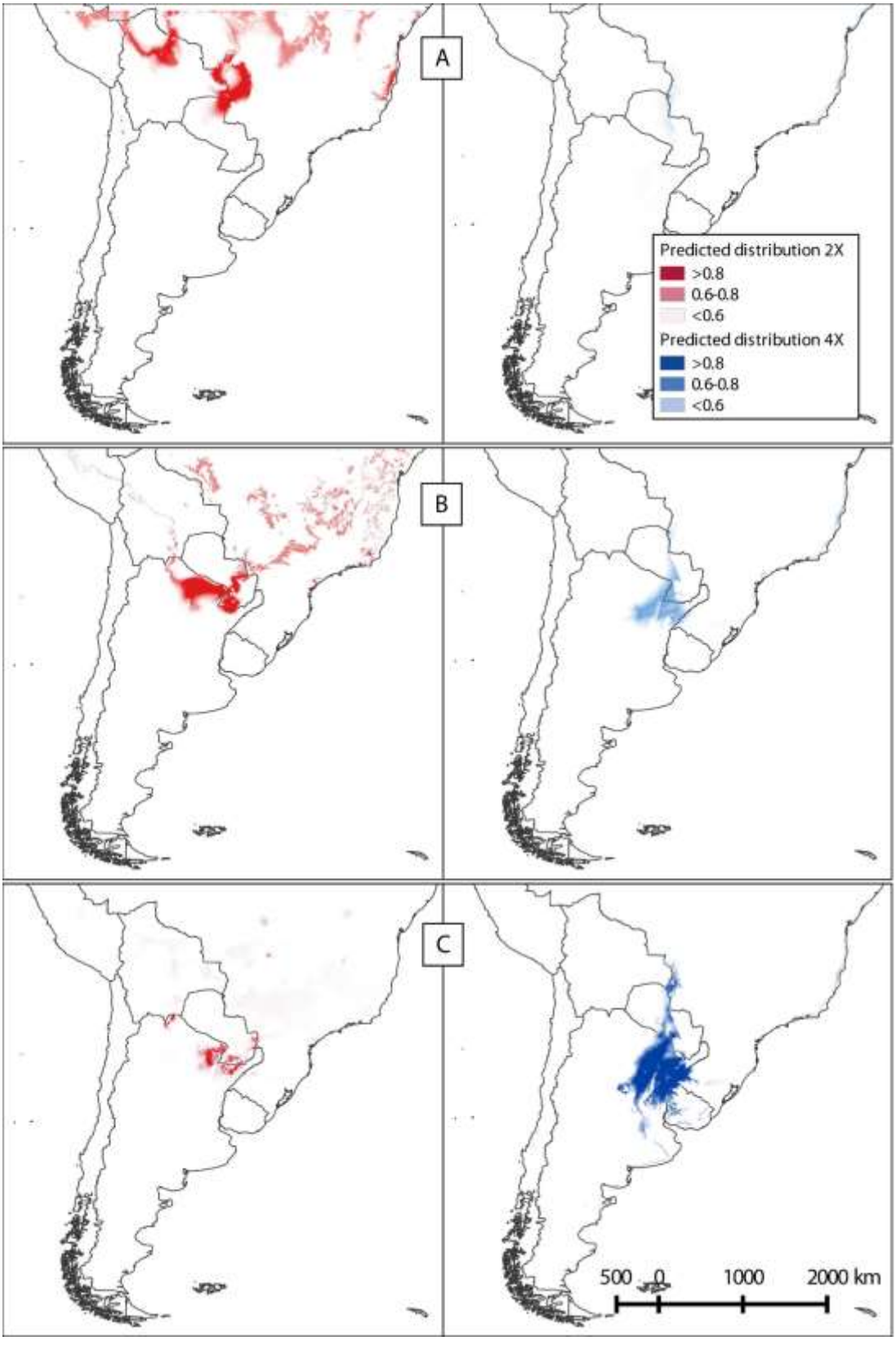

Figure S2.7. Reconstruction of past environmental niches showing suitable habitats available for diploid and tetraploid cytotypes of *P. intermedium*. A. Habitat availability for the two cytotypes during Last Glacial Maximum (ca. 21000 y.a), **B**. Habitat availability for the two cytotypes during Mid-Holocene (ca. 6000 y.a), **C**. Current niches for the two cytotypes.

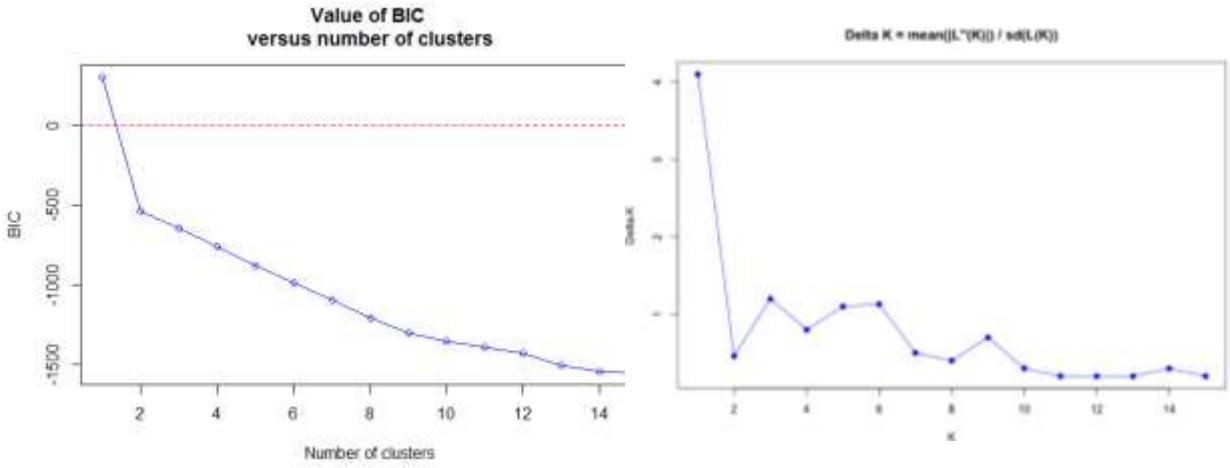

Figure S4.1. A. Bayesian model-based determination of potential number of clusters implemented in the "find.clusters" function of R package *ADEGENET*. A BIC (Bayesian Information Criteria) is calculated using *k-means* algorithm (also Ripley's K-function: Baddeley and Turner 2005) and the resulting BIC values are plotted against increasing number of *k* (clusters). B. Evanno plot (ΔK vs. K) of cluster determination of the AFLP marker data using the method described by Evanno et al. (2005).

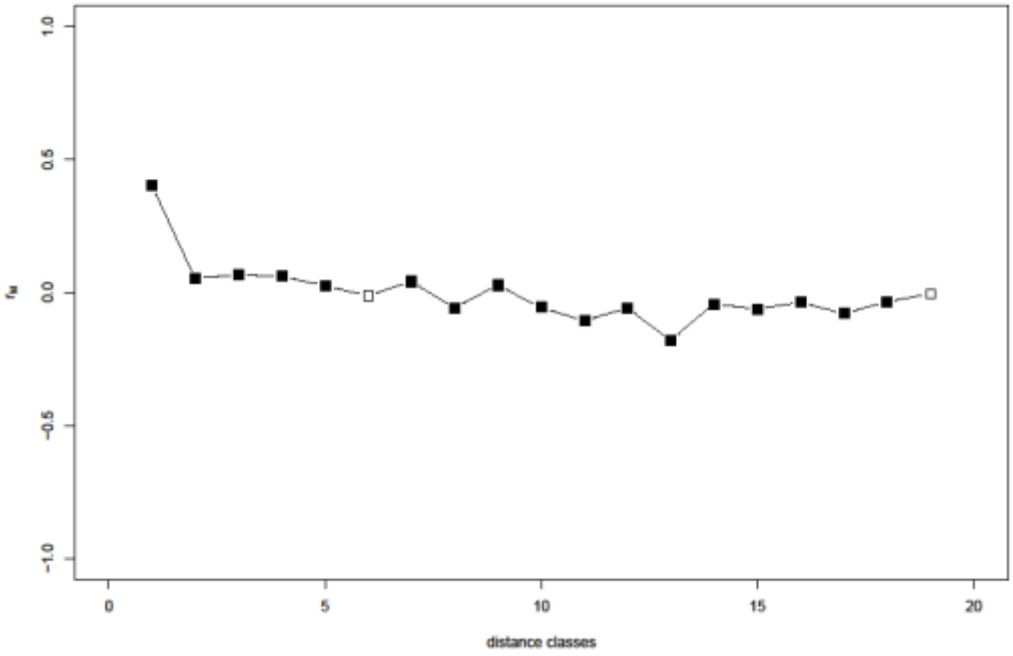

Figure S4.2. Mantel test correlogram showing geographical isolation of *P.intermedium* cytotypes and population based on genetic data

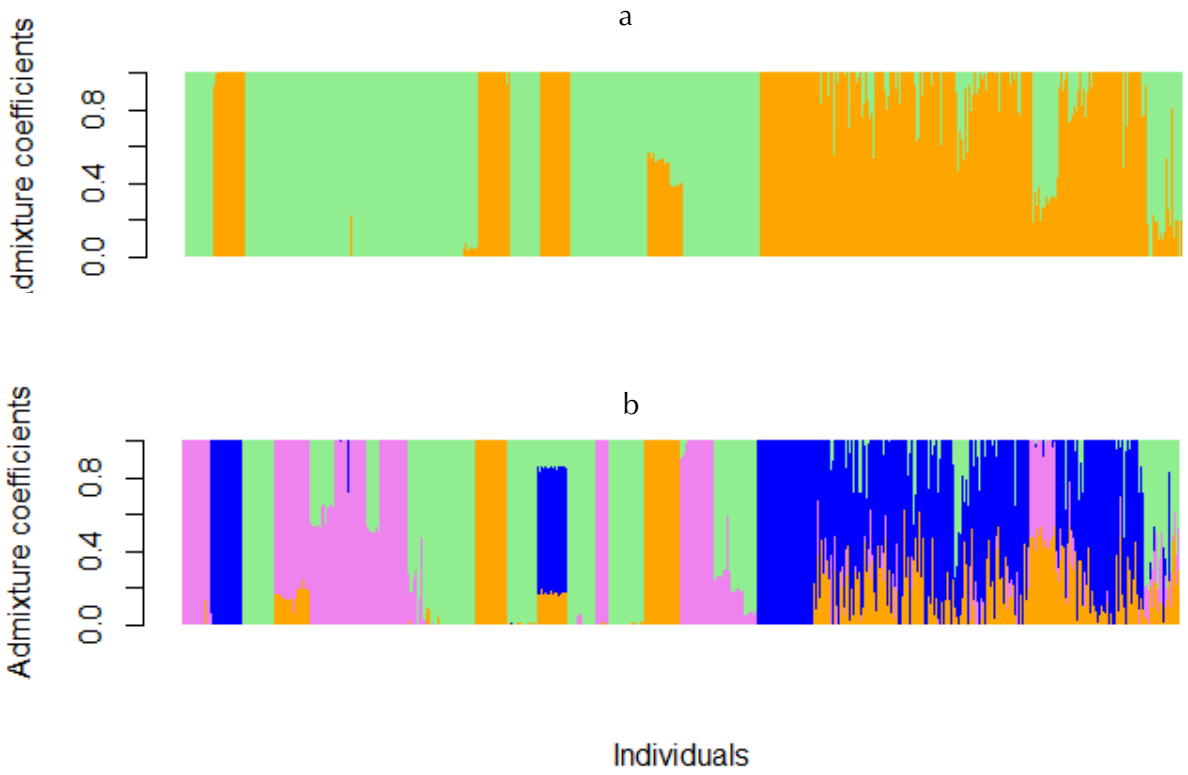

Figure S4.3. AFLP markers bayesian clustering of all the individuals at (a) K = 2, (b) K=4.

Method S1

Flow cytometry ploidy estimations

The relative nuclear DNA content of each sample was measured as follow: approximately 0.2 g of silica dried leaf materials were placed into a clean micro centrifuge tube (2 mL; Sarstedt AG & Co., Nümbrecht, Germany) together with one tungsten carbide bead (QIAGEN®, Hilden, Germany). The tube was submerged in liquid nitrogen for one minute and immediately transferred to a tissue homogenizer (TissueLyser II QIAGEN®, Hilden, Germany) and were beaten for seven seconds at 30 shakes/sec. Then, 400 µL of Otto I buffer (0.1M Citric Acid monohydrate and 0.5% v/v Tween 20) (Otto, 1990) was added to the macerated tissue, mixed and incubated for 10 minutes at room temperature. The homogenate was filtered through a 30 µm nylon mesh (CellTrics®, Sysmex Partec GmbH, Münster, Germany) into a 3.5 mL sample tube (Sarsdtedt AG & Co., Nümbrecht, Germany), and incubated for another 5 minutes before adding 1 mL of Otto II staining buffer (0.4M Na_2HPO_4 dissolved in H_2O plus 4 µg/mL DAPI ($C_{16}H_{15}N_5$), adjusted to pH 8.5) (Otto, 1990) containing DAPI and 2 µL/mL of β-mercaptoethanol. After incubating for 10 minutes at room temperature, each sample was analyzed in the flow cytometer (CyFlow® Cube 6, Sysmex Partec GmbH, Münster, Germany). For each histogram, the relative fluorescence intensity of particles (at least 5000 nuclei) were analyzed with CyView™ data acquisition and data analysis software (Sysmex Partec GmbH, Münster, Germany) and referenced to an external diploid standard ($2n=2x=20$ chromosomes). The standard was measured every 10^{th} sample during the ploidy evaluation process. A maximum CV value of 5% was accepted for each sample peak (G_0/G_1 peak). Peak indexes were calculated as the ratio between the mean peak of the sample / the mean peak of the standard. Samples with a peak index value of 1 were determined as diploids; samples with peak index >1 as polyploid (1.5 for triploids; 2 for tetraploids).

Method S2

Flow cytometric seed screening

Single seeds were placed in a 2 mL centrifuge tube (Sarstedt AG & Co., Nümbrecht, Germany) together with one tungsten carbide bead (QIAGEN®, Hilden, Germany) and 50 µL of Otto I isolation buffer (Otto 1990). Tubes were beaten in a tissue homogenizer (TissueLyser II QIAGEN®, Hilden, Germany) for seven seconds at 30 shakes/sec to obtain a substantial homogenate of the seed tissues. A final 150 µL of Otto I buffer was then added and then each tube was gently mixed and spin-down centrifuged. Sample suspensions were filtered (30 µm mesh CellTrick® filters; Sysmex Partec GmbH, Münster, Germany), stained with 800 µL of Otto II staining buffer (Otto, 1990), and incubated for 5–15 min on dark before analyzed on a CyFlow® Cube 6 flow cytometer (Sysmex Partec GmbH). For each seed histogram the relative fluorescence intensity of at least 3000 nuclei was analyzed with the CyView™ data acquisition software (Sysmex Partec GmbH, Münster, Germany) and referenced to the external standard (a diploid *P. intermedium* plant). The standard was measured at least two times on a daily work basis, at the beginning and the end of the batch of samples. A maximum CV value of 5% was accepted for each sample peak (embryo and endosperm tissue peaks).

List of Publications

Karunarathne P, Schedler M, Martínez EJ, **Honfi AI, Novichkova A, Hojsgaard D,** 2018. Intraspecific ecological niche divergence and reproductive shifts foster cytotype displacement and provide ecological opportunity to polyploids. *Annals of Botany* 121:1183–1196

Karunarathne P, Reutemann V, Schedler M, Glücksberg A, Martínez EJ, Honfi AI, Hojsgaard D, 2018. Sexual modulation and the evolutionary implications of a TUG OF WAR between sexual-apomictic reproductive modes in a polyploid grass species. Manuscript submitted to *New Phytologist*.

Curriculum Vitae

Piyal Karunarathne

- **PhD in Evolutionary Biology** (2015-2018)

  Department of Systematics, Biodiversity and Evolution of Plants
  University of Göttingen, Germany (https://www.uni-goettingen.de)
  Thesis: Cytotype associations, Ecological Divergence and Genetic Variations in the Apomictic Comples Paspalum intermedium Munro ex Morong.

- **Master of Philosophy in Biology (M.Phil.)** (2011-2014)

  Postgraduate Institute of Science, University of Peradeniya, Sri Lanka (www.pgis.lk)
  Thesis: Taxonomy and Phylogenetic Studies on the two genera Alpinia Roxb. and Amomum Roxb. of the family Zingiberaceae in Sri Lanka.

- **BSc (Honors) Special Degree in Biology** (2006-2010)

  Faculty of Science, University of Peradeniya, Sri Lanka (www.fos.pdn.ac.lk)
  Major Subjects: Zoology, Botany, Molecular Biology & Biotechnology
  Completed on 21 July 2010 with Second Class Honours and a cumulative GPA of 3.18/4.00.