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Polyploidy can confer superiority to West African *Acacia senegal* (L.) Willd. trees

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Provisional

1 **Polyploidy can confer superiority to West African *Acacia senegal* (L.)**
2 **Willd. trees**

3
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17 **Running head:** Superiority of polyploid *Acacia senegal*

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21 **Abstract**

22 Polyploidy is a common phenomenon in the evolution of angiosperms. It has been suggested that
23 polyploids manage harsh environments better than their diploid relatives but empirical data
24 supporting this hypothesis are scarce, especially for trees. Using microsatellite markers and flow
25 cytometry, we examine the frequency of polyploids and diploids in a progeny trial testing four
26 different populations of *Acacia senegal*, a species native to sub-Saharan regions of Africa. We
27 compare growth between cytotypes and test whether polyploid seedlings grow better than diploids.
28 Our results show that polyploids coexist with diploids in highly variable proportions among
29 populations in Senegal. *Acacia senegal* genotypes were predominantly diploid and tetraploid, but
30 triploid, pentaploid, hexaploid and octaploid forms were also found. We find that polyploids show
31 faster growth than diploids under our test conditions: in an 18 years old field trial, polyploid
32 superiority was estimated to be 17% in trunk diameter and 9% in height while in a growth chamber
33 experiment, polyploids grew 28 % taller, but only after being exposed to drought stress. The results
34 suggest that polyploid *A. senegal* can have an adaptive advantage in some regions of Africa.

35 **Key words:** adaptation - arid zone trees - drought stress – flow cytometry - microsatellite markers -
36 morphological differentiation - *Senegalia senegal*

Provisional

37 1. Introduction

38 Polyploidy, the achievement of more than two sets of chromosomes through gametic non-reduction
39 and to a lesser degree somatic doubling has important ecological and evolutionary consequences for
40 speciation (Madlung, 2013). In nature, polyploidy arises via intraspecific genome doubling
41 (autopolyploidy) or merging of genomes of distinct species through hybridization and chromosome
42 doubling (allopolyploidy) (Stebbins, 1950). Most angiosperms are believed to have undergone one or
43 more polyploidization events (Soltis *et al.*, 1999). It has been estimated that polyploids form at the
44 frequency of approximately 1 per 100,000 individuals (Ramsey & Schemske, 1998; Levin, 2002) and
45 that 2 – 4 % of all speciation events involve polyploidization (Otto & Whitton, 2000). The high level
46 of polyploidization in the evolutionary history of flowering plants suggests that polyploidy plays an
47 important role in adaptive evolution of plants in natural populations (Van de Peer *et al.*, 2009).
48 Successful polyploidization is generally accompanied by morphological, phenological, physiological,
49 and ecological changes in plants (Levin, 2002), and may produce individuals that can tolerate
50 fluctuating environments (Soltis *et al.*, 2004; Prentis *et al.*, 2008), make use of new niches or by other
51 means become more successful than their progenitor species (Leitch & Leitch, 2008).
52

53 In theory, local co-occurrence of intraspecific cytotypes is evolutionary unstable, driving the minority
54 cytotype towards extinction unless cytotypes have different ecological preferences or strong
55 prezygotic barriers are present between ploidy levels (Husband 2000, Kennedy *et al.*, 2006).
56 Nevertheless, co-distribution of individuals belonging to different ploidy levels in heteroploid species
57 (species with different levels of ploidy) is not uncommon and has been reported in *e.g. Solidago*
58 *altissima*, *Ranunculus parnassifolius* and *Centaurea phrygia*, *C. stoebe* (Halverson *et al.*, 2008; Cires
59 *et al.*, 2010; Koutecky *et al.*, 2012). With the recent introduction of flow cytometry is it now possible
60 to explore the cytotype dynamics in species with mixed ploidy levels in terms of cytotype
61 distribution, hybridization and segregation.
62

63 Morphological changes in polyploids include a general increase of cell size with increased levels of
64 ploidy (e.g. Kudo & Kimura 2002), sometimes leading to changes in the dimensions of plants, such
65 as larger leaf, flower and fruit sizes compared to diploids (Maherali *et al.* 2009, Pettigrew *et al.*
66 2012). Also micro-morphological changes occur, including larger but more dispersed stomata in
67 polyploids than in diploids (Mishra 1997, Pettigrew *et al.* 2012). Such changes are likely to affect
68 plant environment interactions, for example through modification of gas exchange. It was earlier
69 suggested that polyploids withstand harsh environments like subarctic regions, high elevations and
70 xeric environments better than diploids (Love & Love, 1949) perhaps due to their higher levels of
71 heterozygosity and genetic diversity (Lowry & Lester, 2006). This was supported for example by
72 observations of higher colonization potentials in polyploids, increased frequencies of polyploids from
73 warmer to colder latitudes (Manton, 1934, Hagerup, 1939, Brochmann *et al.*, 2004, Lowry & Lester,
74 2006) and larger ecological amplitude of polyploids compared to diploids (e.g. Liu *et al.*, 2011,
75 Schlaepfer *et al.*, 2010).
76

77 Recent studies have found that polyploid cytotypes in heteroploid species complexes of herbaceous
78 plants have better drought tolerance than their diploid progenitors (*Chamerion angustifolium*,
79 Maherali *et al.*, 2009; *Brachypodium distachyon*, Manzaneda *et al.*, 2012), although Buggs & Panell
80 (2007) found that diploid *Mercurialis annua* performed better than hexaploids across a range of
81 natural sites and that the cytotypes did not differ in performance under drought stress. Increased
82 drought tolerance may be related to higher resistance towards cavitation in the xylem as discovered in
83 *Atriplex canescens*, a shrub species from the deserts of Southwestern U.S. (Hao *et al.*, 2013).
84 Nevertheless, it is still discussed under which circumstances polyploidy confer higher fitness
85 (Madlung, 2013), and a comparison of many North American diploid and polyploid species showed
86 no significant differences in extent of range or geographical distribution between ploidy levels

87 (Martin & Husband, 2009). Unfortunately very few studies showed the relative performance of
88 diploids and polyploids under controlled conditions (Soltis *et al.* 2010).

89
90 Variation in ploidy level within species is also known from trees (e.g. the *Adansonia digitata*/*A.*
91 *kilima* complex, Pettigrew *et al.*, 2012, *Betula papyrifera*, Li *et al.*, 1996, *Populus tremula*, Johnsson
92 1940, *Acacia mearnsii*, Beck *et al.* 2003). Because increased cell size is likely to influence hydraulic
93 properties via the influence on conduits (see e.g. Hao *et al.*, 2013, Maherali *et al.*, 2009), studies on
94 trees with their massive xylem, large size and long potential exposure to climatic extremes are
95 particularly interesting. Yet there are few studies comparing performance of trees with different
96 ploidy levels, and the studies that exist focus on short-term responses of seedlings to stress (Li *et al.*,
97 1996, Li *et al.*, 2009). Studies of performance of mature polyploid versus diploid trees are to our
98 knowledge absent.

99
100 Recently it was discovered that *Acacia senegal* (L.) Willd. exists in different levels of ploidy. The
101 species grows naturally in the semi-arid sub-Saharan regions of Africa as well as in India and
102 Pakistan and plays an important role in agroforestry systems by providing fuel, fodder for livestock
103 and restoring soil fertility besides producing Gum Arabic. Gum Arabic is a natural exudate collected
104 from branches and stems after tapping during the dry season, and is only produced when the species
105 is grown under dry conditions (Wekesa *et al.*, 2009). The gum provides an important income for rural
106 people. Most of the populations across the distribution area seem to be composed of diploid trees, but
107 tetraploid individuals ($2n=4x=52$) were discovered in populations from Mali, Sudan and Ethiopia
108 (Assoumane *et al.*, 2013). Due to a limited sample size within each population the ratio between
109 diploid and polyploid individuals was not resolved. Based on chloroplast data, the authors suggest
110 that polyploid *A. senegal* is allopolyploid (Assoumane *et al.*, 2013). The parent species that
111 hybridized with diploid *A. senegal* may have been *Acacia laeta* reported to be a triploid hybrid ($3x =$
112 39) between *A. senegal* and *A. mellifera* (Ross, 1979), but the origin and type of polyploidy in *A.*
113 *senegal* is still not verified.

114
115 Investigating a progeny trial with mature *A. senegal*, we discovered that the trees had mixed ploidy
116 levels (Diallo *et al.*, 2015). As the trial was established with the purpose of breeding for increased
117 gum production, the trees were planted in an experimental design and thus represent a unique
118 possibility for studying the long-term performance of di- and polyploid trees. In this paper, we
119 specifically 1) explore the distribution of cytotypes in trees originating from four different locations,
120 and in their corresponding offspring, 2) compare the growth performance of trees with different
121 ploidy levels, 3) compare the growth of seedlings with different ploidy level under drought stress and
122 4) compare the morphology of plants with different ploidy levels.

123 124 **2. Materials and methods**

125 126 **2.1. Study species**

127
128 *Acacia senegal* (L.) Willd. is a multipurpose tree that belongs to the family Fabaceae. Recently, it has
129 been suggested to transfer the species to the new genus *Senegalia* (Maslin, 2006). Here we maintain
130 the rule of first priority and thus the name *Acacia senegal*. The species is described as consisting of
131 the four varieties *senegal*, *kerensis*, *rostrata* and *leiorhachis* based on differences in inflorescence
132 axis, pod and tree shape and phenology (Odee *et al.*, 2012). Only *A. senegal* var. *senegal* has been
133 reported in Senegal.

134 135 **2.2. The field trial**

136

137 The plant material of *A. senegal* used in this study originated from a progeny trial in Senegal
138 established in 1994 in Dahra, Senegal (15° 20' N and 15° 28' W). The annual precipitation at the site
139 is approximately 410 mm, and the annual mean temperature is 27°C (climate data estimated from
140 Worldclim based on the 1950-2000 period, see Hijmans *et al.* 2005). The soil at the site is sandy, and
141 the natural vegetation in the area consists of mainly *Acacia tortilis* and *Balanites aegyptiaca*
142 (Pontanier *et al.* 2003).

143
144 In November and December 1993, seeds were collected from four populations (provenances)
145 representing the natural distribution area of the species in Senegal (Fig. 1): Ngane, located in the
146 centre of Senegal and characterized by saline soils and 620 mm of rainfall; Diamenar, located in a
147 dryer region in the north with 300 mm of rainfall; Daiba and Kidira, located in the north-eastern and
148 south-eastern parts with 430 mm and 600 mm of rainfall, respectively. At each site seeds were
149 collected from 15 trees considered to have desirable phenotypes based on superior health, trunk
150 diameter, crown diameter and height. In the natural stands diploids and polyploids are
151 indistinguishable with the naked eye, implying no bias for or against any type during seed
152 collections. Seeds were kept in separate lots for each mother tree, thus giving 60 seedlots, and in
153 1994 seeds were pretreated in sulfuric acid (98 %) for 6 min and sown in polyethylene bags with
154 nursery soil. Two seeds were sown per bag, and in cases where both seeds germinated, one of the
155 seedlings was randomly selected and removed. Seedlings were raised in a nursery and in August
156 1994 (during the rainy season), 30 healthy seedlings per seedlot were selected and planted at the
157 Dahra site. Prior to plantation, weeding and clearing were undertaken in the site. The trial was
158 established in a randomized complete single tree block design with all seedlots represented by one
159 tree in each block, replicated thirty times (30 blocks). The initial number of plants was thus $4 \times 15 \times$
160 $30 = 1800$ trees. Trees were spaced 5 x 5 meters from each other.

161
162 One year after planting, in August 1995, survival and height of all trees were assessed. Height was
163 measured as vertical height from the ground to the top of the tree. In February 2012, a second
164 assessment was conducted on the 634 surviving trees. The maximum vertical height was determined
165 using a height rod, trunk diameter at 30 cm from the soil surface was assessed using a diameter tape,
166 and crown diameter was estimated as the average of two perpendicular measures of the edge of the
167 crown projected to the ground. In 2012, cambium samples were taken from every tree for assessment
168 of the ploidy level (see later).

169 170 **2.3 Growth chamber experiment**

171 In November 2012, 108 pods were collected from 76 parent trees in the field trial. The parent trees
172 were selected to cover all four sites of origin and – to the extent possible – different levels of ploidy
173 in all sites of origin. One pod was randomly chosen from each tree except for trees from Ngane
174 where 1-6 pods were collected per tree (due to higher frequency of polyploidy in this provenance as
175 described below).

176
177 The number of seeds per pod was counted and seed dimensions (length and width) were measured
178 using a Vernier caliper. Seeds were pretreated with sulfuric acid (95-97 %) for 10 min to release seed
179 dormancy, kept under sterile conditions in a laminar hood (Thermo Scientific, SAFE 2020, Germany)
180 and germinated in boxes containing sterilized vermiculite and incubated at 29 °C and 16 h
181 photoperiod in a growth chamber. The germination rate was registered, and the ploidy level of all
182 seedlings was determined (see below).

183
184 To compare the growth between different levels of ploidy, 132 seedlings were transplanted in peat
185 soil (Plugg och Såjord) from Weibulls Horto AB, Sweden. 500 g of soil was filled in 17 plastic boxes
186 each. The seedlings consisted of 83 diploids, 46 tetraploids and three hexaploids. Each box was

187 considered as one block and contained eight seedlings representing different sites of origin
188 (provenance), descendance and ploidy levels. By descendance we here understand all seedlings
189 descending from a single mother tree in the Dahra field trial. Because of the restricted number of
190 polyploid seedlings in some sites of origin (provenances), the design was imbalanced, but each box
191 contained at least three sites of origin and one set of diploids and polyploids from the same site of
192 origin. For example, block no. 1 included four tetraploids and one diploid from the Ngane origin, and
193 one diploid of each of the Daiba, Diamenar and Kidira origins, while block no. 17 contained one
194 diploid and one tetraploid of the Kidira origin, and three diploids of each of the Daiba and Diamenar
195 origins. Despite the imbalance in the trial, this design allowed us to assess the growth of the different
196 levels of ploidy because there was always at least one pair of diploid and polyploid seedlings from the
197 same origin in each block. We planted seedlings in boxes to make sure that all plants in a box were
198 exposed to the same water level irrespective of plant size, leaf area and stomatal conductance
199 (Verslues et al. 2006).

200
201 The initial weight of seedlings was recorded before transplanting into soil. We first compared the
202 growth under well-watered conditions (85% of field capacity) for five weeks in the growth chamber
203 at 16 h photoperiod. The temperature varied between 28 and 33 °C, while relative air humidity ranged
204 between 47 and 71%. Boxes were weighed every day and the amount of water lost by
205 evapotranspiration was added to maintain 85% of field capacity. Plant height was measured weekly
206 from the soil surface to the apical bud, and the numbers of leaves and branches were counted after
207 three weeks. Leaf length, leaflet length and width were measured after five weeks of growth under
208 well-watered conditions. Likewise, stomatal size and density were determined on 50 plants (diploid
209 and polyploid) and two randomly chosen leaflets per seedling. Leaflets were stained with Toluidine
210 Blue O dissolved in 0.05 % of benzoate buffer and water at pH 4.4 (O'Brien & McCully, 1981) and
211 viewed with a microscope (Olympus Cx40 RF 200, Japan) under x 40 magnification. Two
212 microscopic grids were examined per leaflet, totaling 200 counts. To determine the size of stomata,
213 20 random individual stomata per seedling were measured (scale bar 100 µm) at magnification x 40
214 and the mean stomatal length and density was calculated.

215
216 After five weeks, drought stress was applied by reducing the amount of added water to 47% of field
217 capacity. In a pilot study, this was shown to be close to the wilting point. Again, in order to maintain
218 the field capacity at 47%, boxes were weighed every day and the amount of water lost was added.
219 Plant height was recorded weekly for six weeks and the fresh and dry biomasses of seedlings (after
220 drying at 80°C for 48 h) were assessed at the end of the experiment.

221

222

223 **2.4. Ploidy level assessment**

224

225 For 59 trees from the Dahra field trial, twigs of 20 cm length with vegetative buds were collected in
226 April 2014 and placed in a lab with their proximal end in water. After two weeks of forcing, one
227 complete leaf was collected from each twig and analyzed by flow cytometry. Seeds descending from
228 the mother trees in the Dahra field trial were germinated as described above, and flow cytometry was
229 performed on 3 weeks old leaves in the lab.

230

231 Flow cytometry was performed using a Partec PA II flow cytometer equipped with an HBO-100
232 mercury arc lamp (Partec GmbH, Germany) and filter combination for DAPI staining (Partec 06-03-
233 310). Fresh leaf samples of two-weeks-old plants or from forced twigs were chopped for 30 s in a
234 petri dish containing 0.6 ml of Citric acid buffer and left for 5 min to allow nuclei release. The nuclei
235 were stained by adding 2.5 ml of fluorescent solution containing 5 µM DAPI (4,6-Diamino-2-
236 phenylindol dihydrochlorid) and left for another 5 min (Otto, 1990). The suspension of nuclei was

237 passed through a nylon filter with pore size 50 μm to remove large debris. The DAPI binds to the A-
238 T bases of DNA and the intensity of the fluorescence emitted will reflect the number of bounds and
239 therefore also the DNA content in such DAPI-labelled nuclei. The relative fluorescence of total DNA
240 of single nuclei was analyzed and in each sample the DNA content of 5000 nuclei was checked.
241 Samples of *Miscanthus sinensis* with known ploidy (diploid) was used as an internal standard. The
242 standard produced two peaks: a major peak corresponding to 2x DNA quantities of the majority of
243 the cells and a minor peak corresponding to 4x DNA quantities from cells in mitotic interphase.
244

245 The gain was adjusted so that the peak of diploid *A. senegal* was localized on channel 50
246 corresponding to one large (2C) and one small (4C) peak. Plants were regarded as tetraploids if
247 histograms showed one major 4C peak, a small 8C peak and no 2C peak.
248

249 To assess the exact DNA content of the genome, a subset of 26 leaf samples from 13 DAPI-examined
250 plants (3 diploids, 3 triploids, 3 tetraploids, 1 pentaploid and 3 hexaploids) was analyzed using flow
251 cytometry with propidium iodide dye according to the protocol by Doležel *et al.* (2007).
252

253 All 634 living trees in the field trial were genotyped with 8 polymorphic microsatellite markers
254 (SSR) as described in Diallo *et al.* (2015). By comparing the flow cytometry results with the SSR
255 markers for the 59 mature trees, we concluded that polyploids could always be separated from
256 diploids by the presence of more than two alleles per locus in at least 1 of the 8 loci (Appendix 1).
257 Based on their SSR genotypes, we therefore assigned all 634 trees in the field trial to either diploid or
258 polyploid status, but not distinguishing between tri-, tetra-, penta- or hexaploids.
259

260 2.5. Statistics

261

262 For the data from the field trial, a generalized linear analysis of variance was applied to test
263 differences between the growth of diploid and polyploid trees in the trial. Trees that were assessed in
264 1995 but were not alive in 2012 were excluded, as their ploidy level was unknown. The analysis
265 included the effects of ploidy level (diploid or polyploid), site of origin (Daiba, Diamenar, Kidira or
266 Ngane) and block (30 levels) according to the following model:
267

$$268 Y = \text{Ploidy} + \text{Site of origin} + \text{Block} + \text{Error} \quad (1)$$

269

270 Where the effects of ploidy and site of origin were considered as fixed, block was considered as
271 random, and the error followed a normal distribution with expectation zero. The average performance
272 of diploids and polyploids was estimated as least square means from the analysis, *i.e.* averages
273 corrected for systematic differences among provenances.
274

275 Data from the growth chamber experiment were analyzed in two steps. The first step was a model
276 with the effects of descendance and blocks:
277

$$278 Y = \text{Descendance} + \text{Block} + \text{Error} \quad (2)$$

279

280 Where descendance was considered a fixed effect and the block was considered as random. From this
281 model, we calculated the least square mean values of all variables for each descendance. Due to the
282 limited number of samples for stomatal density and length, calculation of least square means was not
283 possible and instead the mean values were calculated. Mean values were also calculated for seed
284 traits. Next, as each descendance was either diploid or polyploid and originated from one of the four
285 sites of origin, we applied the following model:
286

287 $Y = \text{Ploidy} + \text{Site of origin} + \text{Error}$ (3)

288

289 Where Y denotes the least square means (or means) of the descendant families from model (2), and
290 ploidy and site of origin were considered as fixed effects.

291

292 All analyses were performed using the GLM procedure in the SAS 9.3 Software (SAS Institute Inc.
293 2014). Assumptions of variance homogeneity and normality of residuals were accepted for all the
294 studied characters based on visual inspection of residual plots. However, for the ratio between fresh
295 and dry weight in the growth chamber experiment a single outlier (Kidira family 7) was identified
296 and deleted from the data.

297

298

299 **3. Results**

300 **3.1. Ploidy level**

301 Both diploid and polyploid individuals were found among trees from all four origins, but at very
302 different frequencies: 136 of 164 trees (83%) originating from Ngane were polyploid, compared to 3
303 of 178 trees in Diamenar (2%), 14 of 146 trees in Daiba (10 %) and 16 of 117 trees in Kidira (14%).

304

305 Flow cytometric analyses of seedlings allowed separation between diploid, triploid, tetraploid,
306 pentaploid and hexaploid individuals corresponding to a mean 2C DNA content of 1.25 ± 0.02 pg,
307 1.96 ± 0.05 pg, 2.60 ± 0.03 pg, 3.19 pg and 3.83 ± 0.08 pg respectively. All offspring from diploid
308 mothers were diploid, while tetraploid mothers produced either offspring with the same ploidy level
309 (tetraploid) or higher levels (pentaploid, hexaploid and octaploid). Seeds from the same pod collected
310 on the tetraploid mother NG16_B19 gave rise to one tetraploid and one hexaploid seedling, while all
311 seedlings coming from the tetraploid mother DA15_B3 were hexaploid. Of 13 offspring tested from
312 the tetraploid parent NG10_B8, 11 were tetraploid, one was pentaploid and one was hexaploid; of the
313 16 plants examined from the tetraploid mother NG20_B3, one was octaploid and 15 were tetraploid.
314 Out of three seedlings tested from the triploid individual DA1_17, two were triploid and one was
315 tetraploid (Appendix 1).

316 **3.2. Growth differences between diploid and polyploid trees in the progeny trial**

317 The polyploid trees in the Dahra trial were significantly taller than the diploid trees 1 year after
318 planting in 1995. In 2012, after 18 years, differences between diploid and polyploid trees for both
319 height and trunk diameter were still significant, whereas crown diameter did not differ between
320 ploidy levels. The superiority of polyploids compared to diploids based on least square mean
321 estimates after 1 year was 18% for height, while 9% for height and 17% for trunk diameter after 18
322 years. The true differences between average of diploid and polyploid trees (i.e not corrected for
323 systematic effects of provenances) were substantially larger: 49% for height at age 1; and 15% and
324 24% for height and diameter respectively at age 18 (Table 1, Fig. 2).

325

326 **3.3. Phenotypic differences between diploid and polyploid seedlings in the drought stress test**

327

328 Polyploids differed significantly from diploids in seed length and width, initial fresh weight (at week
329 0 when transplanted to the boxes), leaflet length, stomatal density and length. Heights were similar
330 until weeks 10 and 11. Total fresh weight was borderline significant at the end of the trial, and the
331 fresh weight / dry weight ratio differed significantly between ploidy levels (Table 2, Fig. 2).

332

333 The morphological parameters showed that the polyploids tended to be larger than the diploids
334 (Table 2). Seed length and width were 12% and 10% larger in polyploids compared to diploids,
335 respectively. Polyploids had leaflets that on average were 19% longer than in diploid individuals, and
336 the differences in stomatal density and length were pronounced: polyploid individuals were
337 characterized by 54% wider stomata but with lower stomatal density (31% less) compared to
338 diploids.

339

340 Prior to drought stress, the plant height was similar for both ploidy levels, but after 10 and 11 weeks
341 (corresponding to 5 and 6 weeks of water deficit), the tetraploids has grown taller than diploids (22
342 and 28 %, respectively) (Table 2, Fig. 3). At the end of the trial, polyploid *A. senegal* seedlings had
343 60% larger fresh weight than diploids. Differences in dry weight were smaller because the fresh
344 weight / dry weight ratio was 9% larger in polyploids than in diploids.

345

346 4. Discussion

347

348 4.1. Evolution of polyploidy in *A. senegal*

349

350 Our results showed that *A. senegal* can occur in more than two levels of ploidy, which supplement
351 the results of Assoumane *et al.* (2013) and Odee *et al.* (2015) who reported diploid and tetraploid
352 individuals. In our study, we found that diploids were most frequent followed by tetraploids.
353 Pentaploids, triploids, and hexaploids were also present among seedlings although in small quantities.
354 Based on small sample sizes, Odee *et al.* (2015) showed co-existence of ploidy types. This result is
355 qualified by the present study, where we show that the frequency of polyploid individuals can vary
356 significantly among natural populations in Senegal. Polyploids were dominant in the population from
357 the central Senegal (Ngane) characterized by saline soils, whereas higher proportions of diploids
358 were found in Diamenar (North), Daiba (North-east) and Kidira (South-east). Nevertheless, a few
359 diploid individuals from the saline site (Ngane) were also identified and polyploids occurred in low
360 frequency in non-saline areas (Kidira, Diamenar and Daiba sites).

361

362 Our data comparing the ploidy level of trees and their offspring indicated only very limited
363 hybridization between cytotypes, even in a setup where diploid and polyploid trees were grown side
364 by side in a field trial. Further, offspring from diploid mothers were always diploid suggesting that
365 hybridization between cytotypes with diploid maternal trees must be very rare if at all possible. The
366 higher ploidy levels occasionally found in offspring from tetraploid mothers on the other hand
367 suggest that tetraploid mothers rather frequently produce some gametes that are unreduced.
368 Hexaploid seedlings from tetraploid mothers may have been formed by unreduced egg cells (4n)
369 sired by reduced pollen gametes from a tetraploid pollen donor. The identified pentaploid seedling
370 could have been formed by a pollination event involving a hexaploid pollen donor from the field trial
371 (however not among the trees that were tested with FCM). Alternatively the pentaploid seedling
372 could originate from a fusion between an unreduced egg cell from the tetraploid mother and a
373 reduced pollen gamete from a diploid pollen donor i.e. reflecting cytotype hybridization. In relation
374 to this aspect, we found a single triploid mother DA1_B17 which might have been formed by
375 cytotype hybridization in the previous generation. This triploid tree produced both triploid and
376 tetraploid seedlings. Triploids have previously been reported when conspecific diploids and
377 tetraploids co-occur in the same area as *e.g. Chameron angustifolium* (Husband, 2004) and may play
378 an important role as a triploid-bridge allowing gene flow through mating between diploid and
379 polyploid individuals (Henry *et al.*, 2005) with recurrent polyploid formation in the population.

380

381 The frequency of triploid seedlings (2 out of 162 – both from a triploid mother) observed in our study
382 is, however, low compared to the reported frequencies of triploids in species with mixed populations

383 (range of 2 - 22 %) as reviewed by Soltis *et al.* (2010). Also, no triploid seedlings were observed
384 from either diploid or tetraploid mother trees, which support the presence of a significant
385 reproductive barrier. Stebbins (1971) predicted that mating between ploidy levels is likely only from
386 diploid fathers to polyploid mothers. Unidirectional mating from diploids to tetraploids is known
387 from other species complexes such as *Sorghum* (diploid *Sorghum bicolor* and tetraploid *Sorghum*
388 *halepense*) (Arriola & Ellstrand 1996), *Capsella rubella* (to the allotetraploid descendant *C. bursa-*
389 *pastoris*) (Slotte *et al.*, 2008), and *Arabidopsis arenosa* (Jørgensen *et al.*, 2011). As pollen is
390 aggregated in polyades in *A. senegal* and the stigmatic cavity is cup-shaped (Tandon *et al.*, 2001)
391 morphological size differences between cytotypes could also restrict hybridization. Additional
392 detailed studies are needed to clarify the strength of the reproductive barriers between cytotypes of *A.*
393 *senegal*, and if pollination is always unidirectional under natural conditions.

394

395 **4.2. Adaptive potential and evolutionary success of polyploids**

396

397 Polyploid trees often occupy drier habitats than their diploid relatives (e.g. Li *et al.*, 1996, Pettigrew
398 *et al.*, 2012). Experiments have shown different performance of diploids and polyploids under
399 drought stress, but are limited by their short duration and experimental setup (Li *et al.*, 1996, Li *et al.*,
400 2009). Unfortunately, there is very limited evidence from long-term field trials on the relative
401 performance of trees with different ploidy levels. The faster growth of polyploid *A. senegal* in our
402 study is to our knowledge the first observation of superiority of mature natural polyploid trees in a
403 field trial and indicates that at least under some conditions, trees with high ploidy levels will have an
404 adaptive advantage. Although the effects of ploidy level and origin were to some extent confounded
405 due to the observed unequal distribution of polyploids, our statistical analysis showed that the
406 positive effect of being polyploid remained even when accounting for the effect of origin.

407

408 The field trial represents relatively dry conditions close to the Northern limit of distribution of the
409 species towards the Sahara desert. Since the relative performance of diploid and polyploid *A. senegal*
410 has not been investigated under wetter conditions, it is not possible to conclude whether the better
411 growth is found only under dry conditions or indicates a general superior performance.

412

413 Still, the growth chamber experiment showed that polyploid plants only grew faster than diploids
414 after the plants were subjected to water stress. The limited number of polyploid plants did not allow
415 us to include a control treatment where seedlings were continuously raised without water stress, and
416 we hence do not separate potential ontogenetic effects from effects of drought. The question of
417 whether the adaptive advantage of polyploids is limited to dry conditions or applies over a broader
418 range of environments therefore remains unresolved. Reciprocal experiments ('optimal' versus a
419 single abiotic stress factor) to test the relative performance of di- and polyploids under different stress
420 situations are needed to conclude whether polyploids are generally superior or if it is only the case
421 under dry conditions (*cf.* Soltis *et al.*, 2010). Investigations in other heteroploid tree species or
422 species complexes are needed to reveal if the observed effects of polyploid in *A. senegal* reflect a so-
423 far undiscovered pattern in trees species growing under stressful conditions in Sahel.

424

425 Polyploidy is often associated with a difference in plant phenotype (e.g. increase in cell size, enlarged
426 floral structure, pollen, stomata and robust stems) when compared to the diploid relatives (Ramsey &
427 Schemske 1998; Madlung, 2013). In our study, we found the first evidence of phenotype
428 differentiation between cytotypes in *A. senegal*, as seeds, leaflets and stomata were larger and
429 stomatal densities were smaller in tetraploids of *A. senegal*. This confirms results reported for other
430 tree species, such as *Adansonia digitata* (Pettigrew *et al.*, 2012), *Betula papyrifera* (Li *et al.*, 1996),
431 *Acacia mangium* (Harbard *et al.*, 2012) and *Acacia maerensii* (Beck *et al.*, 2003). Studies based on
432 neopolyploids have shown that many of these polyploid characteristics are directly linked to

433 increased genome size (e.g., Harbard *et al.*, 2012). It is unknown whether the size differences in *A.*
434 *senegal* are caused by increased genome size, increased genetic diversity or a combination of both.

435
436 The observed phenotypic differences are likely to lead to differences in physiology, as gas movement
437 in and out of leaves is affected by leaf size, size and distribution of stomata. It has been hypothesized
438 that the fewer, but larger stomata observed in polyploids can change stomatal conductance and
439 confer increased water use efficiency to polyploids under drought stress (e.g. Li *et al.*, 1996; Li *et al.*,
440 2009; Pettigrew *et al.*, 2012). Assuming that width and depth of the stomatal pore is proportional to
441 the length, it can easily be estimated following Franks & Farquahar (2001) and Franks & Beerling
442 (2009)) that stomatal conductance is expected to be 5% larger in polyploids than diploids. On the
443 other hand, estimates based on leaf dimensions (Nobel, 2009) suggest that leaf boundary layer
444 conductance will be reduced by 9% in polyploids compared to diploids. Hence the effects of leaf size
445 tend to negate effects of changed stomatal size and density, and the expected overall effects of ploidy
446 level on water use efficiency are therefore unclear. Detailed anatomical studies, coupled with
447 assessments of gas exchange on trees with different levels of ploidy will be important in order to
448 infer on potential mechanisms behind the putative selective advantage of polyploidy in *Acacia*
449 *senegal*. For example, in the herbaceous perennial *Chamerion angustifolium*, Maherali *et al.* (2009)
450 found that tetraploids characterized by large stomata and wide xylem vessels did not differ from
451 diploids in stomatal conductance and gas exchange when grown under drought conditions. However,
452 increased hydraulic conductivity was believed to cause increased drought resistance of tetraploids in
453 this species.

454
455 Another observed phenotypic difference with potential physiological consequences is the larger fresh
456 to dry weight ratios of polyploids compared to diploids (Table 2) causing the ploidy levels to differ
457 almost significantly in fresh weight, but not in dry weight. High water contents indicate either a
458 larger capacity for osmotic adjustment or an increased elasticity of cell walls (Verslues *et al.* 2005),
459 and Li *et al.* (1996) suggested that polyploids might have a larger ability to adjust their osmotic
460 potential under drought stress. If this is indeed the case, it may explain part of the better performance
461 of polyploids in *A. senegal*.

462
463 In conclusion, the co-existence of different ploidy levels in natural populations was confirmed while
464 the pattern of segregation supports that gene flow between cytotypes is limited. Our results document
465 increased growth of polyploid *A. senegal* both in the field trial and under growth chamber conditions,
466 but it remains to be verified if superiority of polyploids is expressed only under relatively dry
467 conditions or applies more generally.

468 469 **Author contributions**

470
471 AD, LN, EK and AR conceived the ideas; AD, LN and KP collected the data; AD, EK and AR
472 carried out the statistical analyses; all authors analyzed and interpreted the data, and all authors
473 contributed to writing of the paper.

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480 481 **Conflict of interest**

482

483 The authors declare no conflict of interest.

484

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486

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689

690 **Figure legends**

691

692 **Figure 1.** Location of the progeny trial (Dahra) and the four tested provenances of *Acacia senegal* in
693 Senegal

694

695 **Figure 2.** Performance of polyploids expressed in percent of diploid performance. The vertical dotted
696 line (100%) denote diploids. Error bars denote the 95% confidence limits of differences between
697 polyploids and diploids.

698 **Figure 3.** Variation in height between diploid (filled circles) and polyploid seedlings (open circles) of
699 *Acacia senegal* under drought stress conditions. The arrow indicates onset of the drought stress,
700 while error bars denote SD.

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704 **Tables**

705

706 **Table 1.** F-tests for significance in growth traits between ploidy levels and provenances in the field trial and
 707 LS estimated averages of diploid and polyploid trees

Traits	Ploidy level			Provenance			Ploidy (Average performance)			
	<i>Df</i> ;	<i>F</i>	<i>P>F</i>	<i>Df</i> ;	<i>F</i>	<i>P>F</i>	Diploid (LSmean)	Polyploid (LSmean)	Diploid (Mean)	Polyploid (Mean)
Height 1995 (cm)	1; 29	5.96	0.015	3; 29	14.61	0.001	38.2(1.18)	45.0(2.29)	38.2 (16.3)	57.0(22.3)
Height 2012 (m)	1; 29	9.52	0.002	3; 29	4.24	0.006	4.52(0.05)	4.92(0.11)	4.57(0.05)	5.25(0.07)
Diameter 2012 (cm)	1; 29	17.29	0.001	3; 29	3.64	0.013	11.86(0.19)	13.92(0.41)	11.57(0.16)	14.34(0.28)
Crown diameter 2012 (m)	1; 29	0.06	0.804	3; 29	1.76	0.152	5.50(0.07)	5.55 (0.14)	5.40(0.06)	5.61(0.09)

708 Least square means (LS means), Simple average (Mean) and standard errors (SE) are estimated from the
 709 analysis of variance. *Df*: Degrees of freedom; *F*: *F*-value.

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714 **Table 2.** F-tests for significance of morphological differences between ploidy levels and provenances of
 715 *Acacia senegal* in the growth chamber trial.

Traits	Ploidy level			Ploidy LS means	
	Df; Error	F	P>F	Diploid	Polyploid
Seed traits					
Number of seeds per pod	1; 55	0.34	0.56	4.4 (0.2)	4.1 (0.5)
Seed length (mm)	1; 49	13.6	0.0006	7.7 (0.1)	8.7 (0.2)
Seed width (mm)	1; 49	6.5	0.01	7.8 (0.1)	8.6 (0.2)
Germination rate (%)	1; 55	0.94	0.34	89 (3)	95 (6)
Growth and morphology at 87 % field capacity					
Initial total fresh weight (g)	1; 49	12.2	0.001	0.17 (0.01)	0.27 (0.02)
No. leaves	1; 48	0.02	0.89	16.0 (1.0)	16.3 (2.2)
No. branches	1; 48	0.00	0.99	6.2 (0.4)	6.2 (0.8)
Leaf length (cm)	1; 31	0.03	0.87	1.9 (0.1)	1.9 (0.2)
Leaflet length (mm)	1; 31	5.1	0.03	6.1 (0.2)	7.3 (0.5)
Leaflet width (mm)	1; 31	2.0	0.17	1.8 (0.1)	2.1 (0.1)
Stomatal density (mm ⁻²)	1; 31	13.7	0.0008	204 (7)	139 (15)
Stomatal length (µm)	1; 31	57	<0.0001	46 (1)	71 (3)
Growth at 47 % field capacity					
Height at week 8 (cm)	1; 48	0.97	0.33	15.9 (0.6)	17.6 (1.4)
Height at week 9 (cm)	1; 48	2.4	0.13	17.2 (0.7)	20.0 (1.5)
Height at week 10 (cm)	1; 48	4.1	0.05	18.1 (0.7)	22.2 (1.6)
Height at week 11 (cm)	1; 48	6.3	0.02	19.1 (0.8)	24.5 (1.7)
Total fresh weight (g)	1; 48	3.6	0.06	1.0 (0.1)	1.6 (0.2)
Total dry weight (g)	1; 48	1.6	0.21	0.33 (0.03)	0.44 (0.07)
Fresh weight/dry weight ratio	1; 47	5.4	0.03	3.30 (0.05)	3.60 (0.10)

716 Least square means (LS means) and standard errors (SE) are given for the two ploidy levels. Df: Degrees of
 717 freedom and F: F-value.

718

719 **Appendix 1.** Ploidy levels in a sub-set (76 parents) of *Acacia senegal* in the progeny trial and in their offspring revealed by eight polymorphic
 720 microsatellites and flow cytometry (FCM) respectively
 721

Provenance	Family	Ind.	Parent ploidy levels			Offspring ploidy levels							
			No. loci with 1-2 alleles	No. loci with more than 2 alleles	FCM on twig	No. Pods tested	No. offspring tested	Diploid (2n)	Triploid (3n)	Tetra (4n)	Penta (5n)	Hexa (6n)	Octo (8n)
Ngane	NG4	*B14	5	3	Tetraploid	1	2	-	-	2	-	-	-
Ngane	NG4	B16	4	4	Na	5	11	-	-	10	-	1	-
Ngane	NG7	*B22	8	0	Diploid	1	2	2	-	-	-	-	-
Ngane	NG10	*B8	7	1	Tetraploid	6	13	-	-	11	1	1	-
Ngane	NG11	*B17	8	0	Diploid	1	2	2	-	-	-	-	-
Ngane	NG14	B1	5	3	Na	5	12	-	-	11	-	1	-
Ngane	NG14	*B2	5	3	Tetraploid	1	2	-	-	2	-	-	-
Ngane	NG15	*B16	5	3	Tetraploid	1	2	-	-	2	-	-	-
Ngane	NG16	B3	5	3	Na	4	9	-	-	9	-	-	-
Ngane	NG16	*B19	4	4	Tetraploid	1	2	-	-	1	-	1	-
Ngane	NG17	*B8	5	3	Tetraploid	1	2	-	-	2	-	-	-
Ngane	NG18	*B25	4	4	Tetraploid	1	2	-	-	2	-	-	-
Ngane	NG19	B1	4	4	Na	4	8	-	-	6	-	2	-
Ngane	NG19	* B24	4	4	Tetraploid	1	2	-	-	2	-	-	-
Ngane	NG20	*B3	4	4	Tetraploid	6	16	-	-	15	-	-	1
Ngane	NG21	* B4	4	4	Tetraploid	6	13	-	-	12	-	1	-
Ngane	NG22	*B6	5	3	Tetraploid	1	2	-	-	2	-	-	-
Ngane	NG25	*B30	4	0	Diploid	4	10	10	-	-	-	-	-
Ngane	NG26	*B17	4	4	Tetraploid	1	2	-	-	2	-	-	-
Diamenar	DIA2	B1	8	0	Na	1	6	6	-	-	-	-	-
Diamenar	DIA2	*B2	8	0	Diploid	1	2	2	-	-	-	-	-
Diamenar	DIA6	*B2	8	0	Diploid	1	2	2	-	-	-	-	-
Diamenar	DIA6	B3	8	0	Na	1	4	4	-	-	-	-	-
Diamenar	DIA7	*B27	8	0	Na	1	2	2	-	-	-	-	-
Diamenar	DIA8	*B21	8	0	Na	1	2	2	-	-	-	-	-
Diamenar	DIA11	* B5	8	0	Na	1	2	2	-	-	-	-	-
Diamenar	DIA13	*B26	8	0	Na	1	2	2	-	-	-	-	-
Diamenar	DIA14	*B6	8	0	Na	1	2	2	-	-	-	-	-
Diamenar	DIA15	B8	8	0	Na	1	2	2	-	-	-	-	-
Diamenar	DIA15	*B21	8	0	Diploid	1	2	2	-	-	-	-	-
Diamenar	DIA17	*B17	8	0	Na	1	2	2	-	-	-	-	-
Diamenar	DIA18	B4	8	0	Diploid	1	2	2	-	-	-	-	-
Diamenar	DIA20	*B23	8	0	Na	1	2	2	-	-	-	-	-

Diamenar	DIA22	*B6	8	0	Na	1	3	3	-	-	-	-	-
Diamenar	DIA22	*B14	8	0	Na	1	2	2	-	-	-	-	-
Diamenar	DIA26	*B2	8	0	Na	1	2	2	-	-	-	-	-
Diamenar	DIA27	*B5	8	0	Na	1	2	2	-	-	-	-	-
Diamenar	DIA29	*B5	8	0	Na	1	2	2	-	-	-	-	-
Daiba	DA1	B17	7	1	Triploid	1	3	-	2	1	-	-	-
Daiba	DA1	*B19	8	0	Diploid	1	5	5	-	-	-	-	-
Daiba	DA2	*B5	8	0	Diploid	1	3	3	-	-	-	-	-
Daiba	DA4	*B12	8	0	Diploid	1	3	3	-	-	-	-	-
Daiba	DA4	*B22	7	1	Na	1	3	-	-	3	-	-	-
Daiba	DA6	*B6	8	0	Diploid	1	2	2	-	-	-	-	-
Daiba	DA7	*B1	8	0	Diploid	1	2	2	-	-	-	-	-
Daiba	DA8	*B17	8	0	Diploid	1	2	2	-	-	-	-	-
Daiba	DA13	*B9	8	0	Diploid	1	2	2	-	-	-	-	-
Daiba	DA15	*B3	7	1	Tetraploid	1	2	-	-	-	-	2	-
Daiba	DA15	B11	8	0	Diploid	1	5	5	-	-	-	-	-
Daiba	DA16	*B15	8	0	Diploid	1	2	2	-	-	-	-	-
Daiba	DA17	*B2	8	0	Diploid	1	2	2	-	-	-	-	-
Daiba	DA18	B2	8	0	Diploid	1	4	4	-	-	-	-	-
Daiba	DA18	B5	8	0	Diploid	1	1	1	-	-	-	-	-
Daiba	DA19	*B6	8	0	Diploid	1	2	2	-	-	-	-	-
Daiba	DA20	*B13	8	0	Diploid	1	4	4	-	-	-	-	-
Daiba	DA25	*B8	8	0	Diploid	1	3	3	-	-	-	-	-
Daiba	DA25	B12	8	0	Diploid	1	3	3	-	-	-	-	-
Daiba	DA26	B2	8	0	Diploid	1	5	5	-	-	-	-	-
Daiba	DA26	B9	8	0	Diploid	1	3	3	-	-	-	-	-
Kidira	K1	*B3	8	0	Diploid	1	2	2	-	-	-	-	-
Kidira	K3	*B2	8	0	Diploid	1	2	2	-	-	-	-	-
Kidira	K4	*B23	8	0	Diploid	1	2	2	-	-	-	-	-
Kidira	K5	*B7	8	0	Diploid	1	2	2	-	-	-	-	-
Kidira	K7	*B8	8	0	Diploid	1	2	2	-	-	-	-	-
Kidira	K8	*B7	8	0	Diploid	1	2	2	-	-	-	-	-
Kidira	K9	*B5	8	0	Diploid	1	2	2	-	-	-	-	-
Kidira	K14	*B6	8	0	Diploid	1	2	2	-	-	-	-	-
Kidira	K16	*B15	8	0	Diploid	1	4	4	-	-	-	-	-
Kidira	K17	B14	8	0	Diploid	1	2	2	-	-	-	-	-
Kidira	K20	*B9	8	0	Diploid	1	4	4	-	-	-	-	-
Kidira	K21	B13	8	0	Diploid	1	5	5	-	-	-	-	-
Kidira	K21	*B22	4	4	Tetraploid	1	2	-	-	2	-	-	-
Kidira	K22	*B9	8	0	Diploid	1	2	2	-	-	-	-	-
Kidira	K23	B3	6	2	Na	1	Na	Na	-	Na	-	-	-

Kidira	K23	B6	8	0	Diploid	1	4	4	-	-	-	-	-
Kidira	K25	*B16	4	4	Tetraploid	1	2	-	-	2	-	-	-

722 In bold: The pure diploid Ngane families (NG7 & NG11). Dark grey: triploid mother producing both triploid and tetraploid offspring. Light grey: Families with mixed
723 ploidy levels. Individuals marked with * were used in the drought stress trial. Na: Not assessed.

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Provisional

Figure 01.JPEG

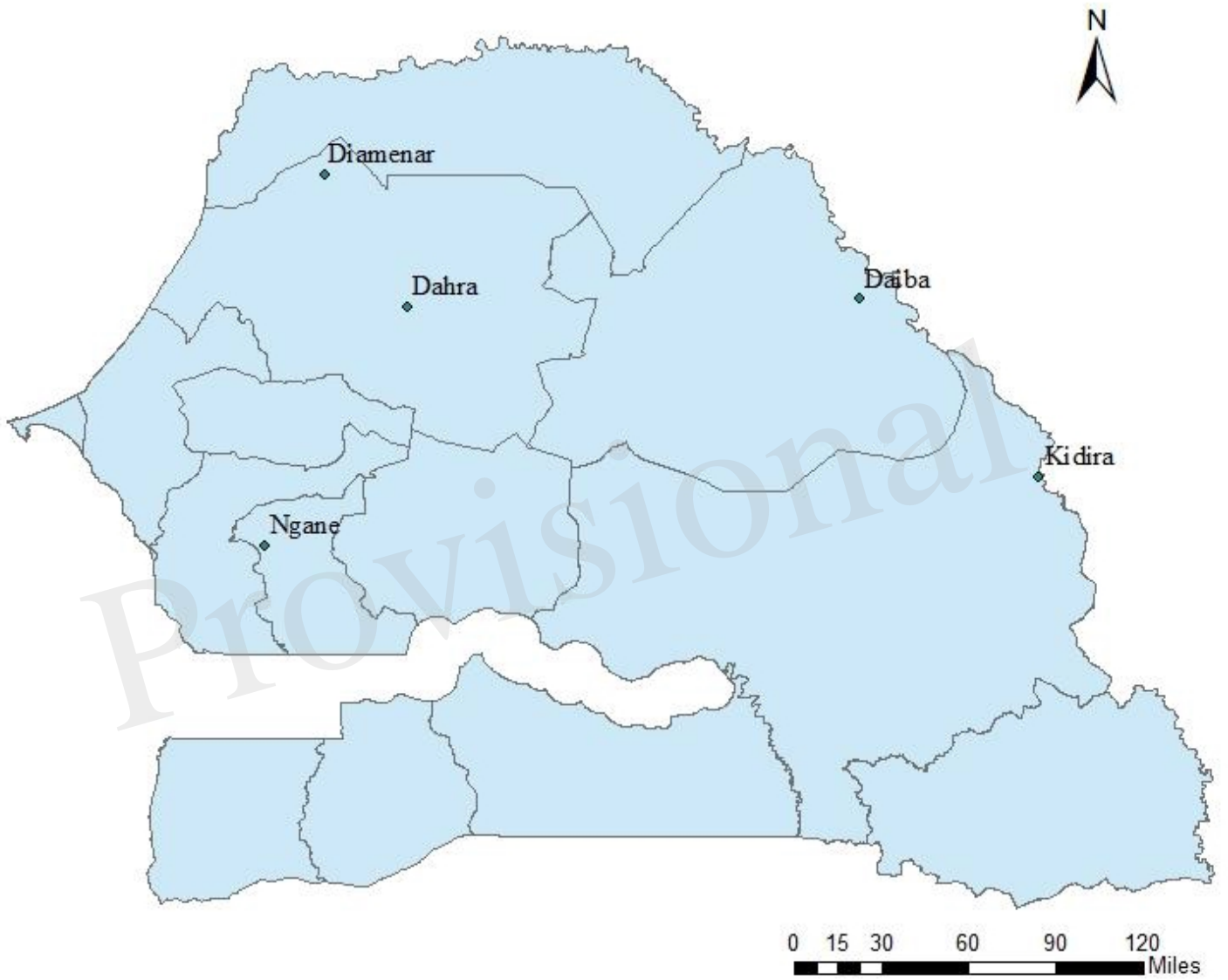


Figure 02.TIF

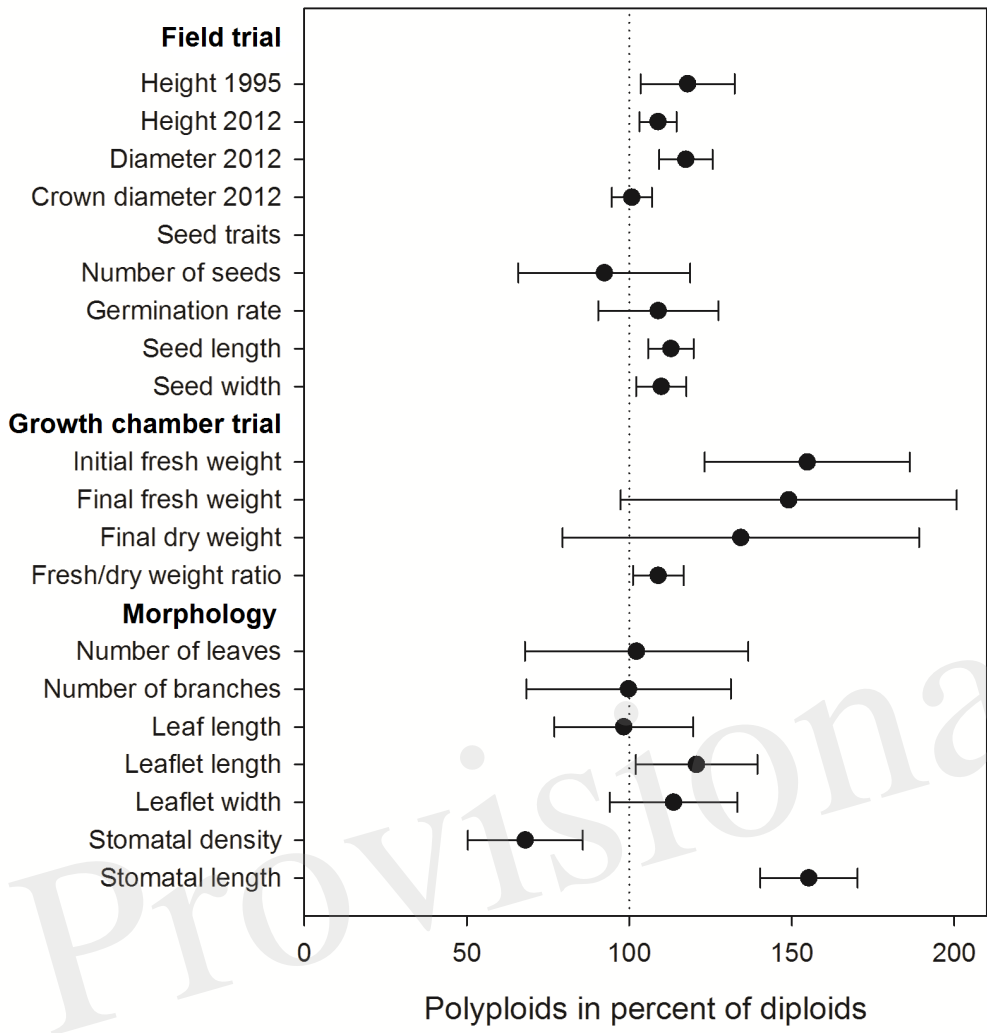


Figure 03.JPEG

