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1 Frequent fires prime plant developmental responses to burning

2

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10 **Summary**

- 11 • Coping with temporal variation in fire requires plants to have plasticity in traits that
12 promote persistence, but how plastic responses to current conditions are affected by
13 past fire exposure remains unknown. We investigate phenotypic divergence between
14 populations of four resprouting grasses exposed to differing experimental fire
15 regimes (annually-burnt or unburnt for >35 years), and test whether divergence
16 persists after plants are grown in a common environment for one year.
- 17 • Traits relating to flowering and biomass allocation were measured before plants were
18 experimentally burnt, and their regrowth was tracked. Genetic differentiation
19 between populations was investigated for a subset of individuals.
- 20 • Historic fire frequency influenced traits relating to flowering and below-ground
21 investment. Previously burnt plants produced more inflorescences and invested
22 proportionally more biomass below ground, suggesting greater capacity for
23 recruitment and resprouting than unburnt individuals. Tiller-scale regrowth rate did
24 not differ between treatments, but prior fire exposure enhanced total regrown
25 biomass in two species. We found no consistent genetic differences between
26 populations suggesting trait differences arose from developmental plasticity.
- 27 • Grass development is influenced by prior fire exposure, independent of current
28 environmental conditions. This priming response to fire, resulting in adaptive trait
29 changes, may produce communities more resistant to future fire regime changes.

30

31 **Keywords:** Flowering, Functional traits, Phenotypic plasticity, Poaceae, Resprouting

32

33 **1. Introduction**

34 Fire is a major and ancient environmental perturbation that plants have adapted to through
35 changes to their functional traits (1,2). There is growing evidence of woody plant adaptation
36 to fire, where different fire regimes create intraspecific trait variation that is genetically
37 determined and thus subject to natural selection (3,4,5). However, whether exposure to fire
38 has a priming effect on plastic responses remains unexplored. An expanding body of
39 evidence shows that plants may respond to extreme climatic events differently depending
40 upon their previous experiences (6,7,8,9), and these primed responses can have positive
41 effects on plant performance during environmental perturbations and avoid the costs
42 associated with maintaining a constant high level of phenotypic plasticity (10).

43 Fire-prone savanna grasses are an ideal study system for exploring the role of fire on
44 priming, as these species persist through the most frequent fire regimes on Earth (11) even
45 though fires usually remove all above-ground biomass and can kill mature grass plants (12).
46 Grasses use two main mechanisms for persisting through fire: resprouting from protected
47 organs or recruiting from a fire-resistant seed bank. The majority of grasses that inhabit fire-
48 prone grasslands and savannas are perennial, resprouting species (13,14). For these species,
49 traits that allow an individual to resprout quickly after fire, such as high specific leaf area
50 (SLA) and large below-ground reserves, are likely to be favoured (15,16) as they allow
51 access to the sunlight- and nutrient-rich environment immediately after fires (17,18). Whilst
52 successful recruitment in many perennial grasslands is infrequent because seedlings must
53 compete with established plants (19,20), traits that enhance recruitment after fire, such as the
54 stimulation of flowering and germination (21,22) can help seedlings to access the high post-
55 fire resources.

56 Here we test the hypothesis that fire causes divergences in recruitment and
57 regeneration traits that favour persistence in fire-prone savannas. Our work sampled four
58 savanna grasses from experimental field plots that had been either unburned or annually
59 burned for >35 years. Cuttings from these plants were grown in a common environment for
60 one year, after which traits were remeasured to determine whether differences between
61 populations persisted independently of the current environment. In comparison to unburnt
62 plants, we predicted that annually burnt plants would have traits advantageous under
63 recurrent fires, including rapid post-fire resprouting (high investment in below-ground
64 biomass, high regrowth rate, low SLA) and recruitment (fire-stimulated flowering). To
65 evaluate whether phenotypic differences had a genetic component or arose from plasticity,
66 we tested for allelic divergence among populations.

67 2. Material and methods

68

69 (a) Plant collection and establishment

70 Plants of four Poaceae species (*Cymbopogon pospischilii*, *Digitaria eriantha*, *Melica*
71 *racemosa* and *Themeda triandra*) were collected on 07/07/15, from experimental burn plots
72 (based at the University of Fort Hare Research Farm, Eastern Cape, South Africa; 32° 47' S,
73 26° 52' E) experiencing two contrasting fire frequency treatments: annual burn and no burn.
74 The latter had not been burned in the 35 years since the plots were created in 1980 (23; see
75 Table S1 for details about the plots including climate data). Both of these treatments
76 represent a departure from the natural fire return interval of the semi-arid savanna site
77 (approximately 15-20 years; W.S.W. Trollope, 2017, pers. comm). Each treatment is
78 replicated twice with the 100 x 50m plots arranged in a randomised block design alongside
79 four other fire frequency treatment plots that were not sampled here. The site varied little in
80 slope and soil chemical and physical properties (24,25). The four species occurred
81 abundantly in all treatment plots, and are perennial, resprouters from three grass subfamilies
82 (Table S2). Based on the reported longevity of these species, it is likely that the populations
83 have undergone several rounds of reproduction and recruitment in the 35 years of treatment
84 (26). Thirty-five mature individuals of each species were dug up from open areas of
85 grassland, minimizing root damage, from across the two replicate plots (n=17 or 18 from
86 each plot). Within 48 hours of collection, a clump of 5 tillers was removed from each
87 individual. The roots were washed carefully to remove soil, and limit the effects of any soil
88 nutrient differences on plant growth. The clumps were subsequently planted into 10L pots
89 containing locally sourced topsoil. A voucher specimen of each species was created (see
90 Table S2 for specimen details).

91 To determine whether there were differences in plant traits between annual-burn and
92 no-burn populations at the time of sampling from the experimental burn plots, traits were
93 measured on 14 plants per treatment per species (n=7 from each plot). For this, the remainder
94 of biomass (after the five-tiller clump had been removed) from each plant was used to
95 measure plant height and above-ground dry biomass (after drying for 48 hours at 70°C).

96 The plants were grown for 12 months (July 2015 – July 2016) in a common
97 environment (a naturally lit polytunnel at Rhodes University, South Africa) in a fully
98 randomized block design, and were weeded and watered regularly. In the polytunnel, average
99 monthly temperatures ranged from 14°C (July) to 26°C (January) and average relative

100 humidity was 68% (as recorded by thermochron data loggers: i-buttons, model DS1923,
101 Maxim Integrated Products, California, USA). A 12-month growth period was chosen so that
102 plants could become well-established in the pots and to minimise any environmental effects
103 carrying over from the different field treatments. Thirty-seven of the 280 plants died during
104 this period, but mortality was not associated with a particular species, treatment or plot
105 (ANOVA: $P > 0.05$). Watering was reduced and eventually stopped two weeks prior to
106 burning to imitate the winter dry season and to force the plants into a phenological stage most
107 relevant to burning.

108

109 (b) Pre-fire traits

110 On the day prior to the experimental burns, the number of flowering tillers was recorded and
111 a sample of above-ground biomass (~1/4 of the total biomass) was removed for all plants.
112 The harvested biomass was dried (for 48 hours at 70°C) and measured. For each species,
113 eight annual-burn and no-burn plants were also randomly chosen, destructively harvested and
114 used to measure above- and below-ground dry biomass. Roots were carefully washed over a
115 fine sieve and then dried at 70°C for 7 days. Root dry mass was measured and expressed as a
116 proportion of the total dry plant biomass.

117

118 (c) Experimental burn and post-fire regrowth

119 Plants were burned in a random order on a warm day with little wind (04/07/16). An area of
120 land was cleared of vegetation and series of holes were dug. Each plant was carefully
121 removed from its pot and lowered into a hole, the depth of which was adjusted to ensure
122 plants sat flush with the soil surface and thus burning was even. Each plant was burned
123 sequentially in a controlled way (see Fig. S1 for diagram of the set-up). After burning, plants
124 were returned to their pots (with any ash on the soil surface removed to standardize any
125 fertilizing effect) and were returned to the polytunnel in a randomized block design and
126 watered.

127 Most plants had initiated regrowth six days after the burns. For each individual, the
128 length of five regrowing leaves was measured using digital callipers six days after the burn
129 and on a further four occasions (each being 5-7 days apart), with the final measurement taken
130 30 days after the burn. Daily average temperatures were slightly higher (2.7°C on average) in
131 the polytunnel than outside (see Fig. S3), thus the plants experienced conditions similar to
132 early spring without late season frosts. Ten of 47 *M. racemosa* and six of 44 *T. triandra*

133 failed to resprout within 30 days, but mortality was not associated with treatment (ANOVA:
134 $P > 0.05$).

135 After the last measurement was taken, all regrown biomass was removed from each
136 plant and stored in sealed plastic bags containing moist tissue paper. Total regrown leaf area
137 was then measured within 72 hours using digital images and the program WinDIAS (Delta-T
138 Devices, Cambridge, U.K.). The regrown material was subsequently dried at 70°C for 48
139 hours and the dry mass was determined. Specific leaf area (SLA) was calculated by dividing
140 the regrown leaf area by the regrown dry mass.

141 A regrowth rate was calculated using the leaf length and air temperature data. Daily
142 minimum and maximum temperature values were used to calculate growing degree-days
143 (GDD, °C-day) for each time period between measurements using the equation:

144

$$145 \quad GDD = \left[\frac{T_{MAX} + T_{MIN}}{2} \right] - T_{BASE}$$

146

147 Where T_{MAX} and T_{MIN} are daily maximum and minimum air temperature respectively. 10°C
148 was selected for T_{BASE} (the base temperature for growth), which represents an intermediate
149 value of published temperate and tropical grass T_{BASE} values (27,28,29). Individual average
150 rates of leaf length regrowth were calculated by fitting linear models to the cumulative leaf
151 length and GDD data.

152 To convert the rate of leaf length regrowth to a rate of leaf biomass regrowth, the
153 fresh length and dry mass of three leaves of each individual were measured. The relationship
154 between leaf length and dry mass was determined for each species by fitting linear models to
155 the log-transformed data. The fit of the models to the data was good (R^2 values > 0.87 for all
156 species; Fig. S2), and the slopes of these relationships were used to convert leaf length
157 regrowth rate into leaf biomass regrowth (in mg GDD⁻¹).

158

159 (d) Statistical analysis

160 All analyses were performed using R (version 3.4.1; 30). The effect of fire frequency on plant
161 traits (all biomass measurements, plant height, number of flowering tillers, regrowth rate,
162 regrown leaf area and SLA) was determined by fitting a linear mixed-effects model to the
163 data (“lme4” package; 31). The fixed effects were “treatment” (annual burn vs no burn) and
164 “species”, and an interaction term between these effects was added if it improved the quality
165 of the model (as indicated by the Akaike information criterion value). “Plot” (i.e the replicate

166 plot the plant was taken from) was added as random effect. “Plant size” (the sampled subset
167 of above-ground biomass taken from each plant before being burned) was also added as a
168 fixed effect for models in which the trait is likely influenced by plant size (number of
169 flowering tillers, height and some regrowth traits after fire). To determine whether fire
170 frequency was significantly influencing plant traits, this model was then compared to a grand
171 mean model using a parametric bootstrapping method (“pbkrtest” package, 32) with 10,000
172 simulated generations.

173

174 (e) DNA extraction and RAD-seq

175 For each species, total genomic DNA was extracted from leaf material for a subset of
176 individuals (n=3-5) per treatment (using the DNeasy Plant Mini Kit, Qiagen) and double-
177 digested restriction-associated DNA libraries were built (following 33). DNA extract (150-
178 350 ng DNA) was double-digested using *EcoRI* and *MseI* after which barcoded adaptors
179 were ligated to the *EcoRI* side and a common adaptor to the *MseI* side (following 34). The 34
180 libraries were pooled with 62 libraries from different projects and the library pool was gel
181 size selected (300-600 bp), purified (using QIAquick Gel Extraction kit) and paired-end
182 sequenced on an Illumina HiSeq2500 lane at Edinburgh Genomics (University of Edinburgh,
183 UK), following standard protocols.

184 Sequencing data was cleaned such that adaptor and primer sequences were removed
185 and low quality (<3) bases were trimmed from 3’ and 5’ ends, as well as bases with a
186 minimum quality of 15 in a four-base sliding window. Reads shorter than 36 bases after
187 trimming were removed. The library pool was de-multiplexed and the barcodes were
188 removed (using the program “Stacks”, 35). Nuclear reads were selected (see Table S3 for
189 details on chloroplast read removal) and used to *de novo* assemble nuclear RAD loci in
190 ipyrad (v.0.7.2; 36). A cluster threshold (sequence similarity for homology) of 0.85 was used
191 and only loci with a cluster depth below 100 and less than 50% missing data were output.

192 One random single nucleotide polymorphism (SNP) with a minor allele count of three
193 was extracted using VCFtools (37) from each of the assembled RAD loci. The SNPs were
194 then used in a principal component analysis (R package “adegenet”; 38) to test whether the
195 two treatments were genetically distinct. An analysis of similarity was used to evaluate the
196 significance of sample clustering (R package “vegan”; 39). Signatures for genetic differences
197 were further evaluated by calculating the genetic distances between the treatments for each
198 species. Pairwise F_{ST} for each SNP were calculated in VCFtools, and an average F_{ST} across

199 all SNPs was estimated. Jackknifing was used to evaluate the significance of average F_{ST}
200 values, by randomly sampling individuals for each species with replacement into 1,000 two-
201 population comparisons and the average F_{ST} between the two populations calculated.
202 Significance was evaluated as the percentage of the jackknifed F_{ST} values that were greater
203 than or equal to the observed F_{ST} . The number of SNPs showing extreme F_{ST} values (>0.8)
204 was also assessed. The p-values for each SNP were calculated as the proportion of jackknifed
205 F_{ST} values above the observed F_{ST} . Comparisons of observed and expected p-values were
206 then used to evaluate the power of the genetic data to detect differentiation between
207 treatments.

208 SNPs were concatenated to an alignment and used to estimate a maximum likelihood
209 phylogenetic tree for each species using RAxML v.8.2.11 (40) under a GTR+G substitution
210 model and 100 fast bootstrap replicates were used to evaluate node support.

211 3. Results

212 (a) Traits in plants sampled from the field

213 Plants in the annual-burn and no-burn populations differed significantly in their initial (field-
214 state) traits. In comparison to annual-burn plants, no-burn plants were taller (+29.6%;
215 likelihood ratio test (LRT)=35.1, $df=1$, $P<0.001$) and had higher above-ground dry biomass
216 (+33%; LRT=62.5, $df=1$, $P<0.001$; all model coefficients given in Table S4).

217

218 (b) Traits in a common environment

219 After plants had been reduced to a small, uniform number of tillers and grown in a common
220 environment for 12 months, significant trait differences persisted between the no-burn and
221 annual-burn populations. After this growth period, all plants were well established and had
222 greatly increased in size (~500-700% increase from the initial number of tillers, data not
223 shown). Past fire frequency had a significant effect on the number of flowering tillers, with
224 annual-burn plants having 50% more flowering tillers on average than no-burn plants (LRT =
225 11.11, $df=1$, $P<0.001$; Fig. 1A). Annual-burn plants also invested significantly more of their
226 total biomass below-ground (+23% on average; LRT=19.98, $df=1$, $P<0.001$; Fig. 1C) than
227 no-burn plants. However, the treatment had no effect on total (above- and below-ground) dry
228 biomass (LRT=0.62, $P=0.43$; Fig. 1B) or on plant height (LRT=0.09; $df=1$, $P=0.77$; model
229 coefficients in Table S5).

230

231 (c) Traits after experimental fire

232 Populations with a different fire history behaved similarly after the experimental fire. All
233 regrowth traits differed significantly between species (ANOVA: $P<0.05$), but were
234 unaffected by the fire frequency previously experienced in the field. Treatment had no
235 significant effect on tiller-scale regrowth rate (LRT=0.69, $df=1$, $P=0.41$, Fig. 1E), regrown
236 leaf area (LRT=0.11; $df=1$, $P=0.73$) or regrown leaf SLA (LRT=1.22, $df=1$, $P=0.27$, Fig. 1F;
237 all model coefficients in Table S6). Overall, there was no treatment effect on regrown dry
238 biomass across species (LRT=0.46; $df=1$, $P=0.49$) but significant intra-specific differences
239 existed within *C. pospischilii* and *T. triandra* populations where annual-burnt plants regrew a
240 larger aboveground biomass after fire in comparison to no-burn plants ($P<0.05$; Fig. 1D).

241

242 (d) Population genetic analyses

243 The species *M. racemosa* was excluded from the genetic analyses, because sample failure
244 resulted in a low sample size. The number of assembled RAD loci and retained SNPs differed
245 between the remaining species (Table 1), as expected due to variation in sequencing quality
246 and depth, and divergence between individuals within each species. We observed deviation
247 from the null hypothesis with an excess of SNPs with low p-values. At the whole genome
248 level, there is therefore power in our genetic data to detect differentiation between treatments
249 (Fig. S4). However, we found no significant clustering of individuals within species based on
250 treatment (Fig. 2, analysis of similarity: *C. pospischilii*, $P=0.22$; *D. eriantha*, $P=0.42$; *T.*
251 *triandra* $P=1$), and none of the species showed a significant genetic difference (as estimated
252 by average F_{ST} and number of F_{ST} outliers) between the treatments (Table 1). Furthermore,
253 with a few exceptions, the bootstrap support in the maximum likelihood trees were generally
254 low (<95) indicating that there is no significant phylogenetic clustering in the investigated
255 species.

256

257 **4. Discussion**

258 This study of grass functional traits under differing fire frequencies supports the hypothesis
259 that fire has strong direct effects upon plant structure and function (2). Previous studies have
260 found evidence of a genetic basis for fire-related traits such as serotiny in pines (3) and
261 flammability in a Mediterranean shrub (5). However, we found no detectable genetic
262 differences between plants that had experienced one or the other of the two fire regimes.
263 Given the statistical power of our test, this is strong evidence that the selective pressure
264 imposed by the past fire regime has not led to consistent genetic differences between the two
265 treatments. Previous evidence of selection for fire-related traits is from obligate seeder
266 species (5). Such species are expected to experience stronger selection pressures for fire
267 adaptations than resprouting species, such as those studied here, due to their short and non-
268 overlapping generations, and the higher cost of being burned. The absence of detectable
269 genetic differentiation between the annual-burn and no-burn grasses may therefore be a result
270 of their resprouting mode of persistence through fire. Alternatively, as grasses are wind
271 pollinated, gene flow among populations in the different fire treatments may have
272 counteracted the effects of selection.

273 The trait differences observed between the contrasting fire treatments could potentially be
274 explained by environmental effects carried over from the long-term treatments into the

275 common environment, rather than by differential developmental responses to these
276 treatments. However, this is unlikely for three reasons. Firstly, we washed the roots of soil
277 before potting the plants. Fire causes a release of nutrients into the soil, and may result in
278 increased soil fertility and faster plant growth in burnt areas (41,42). However, any possible
279 carry-over fertilisation effects were limited by soil removal from the roots. Secondly, we
280 found no significant difference in the total (above- and below-ground) biomass between the
281 treatments after one year in a common environment, implying that any carryover of internal
282 resource stores from annually burnt plots did not enable plants to grow larger. Finally, the
283 long period of growth in a common environment resulted in the initial transplanted biomass
284 (five tillers) constituting only a small fraction of the final plant biomass (30-40 tillers). Whilst
285 efforts were made to limit variation in the age of plants removed from the burn plots (by
286 selecting plants of a similar basal diameter), we are unable to directly determine age and
287 whether this differed by treatment. Individuals in the frequently burned plots could be
288 younger and therefore differ in allocation strategies. However, as plants were standardised
289 by tiller number before being grown in a common environment, and plant size
290 (aboveground dry biomass) was included as a fixed effect in appropriate analyses, there
291 can only be age effects and not size effects. Furthermore, many age-related changes in
292 allocation strategy and growth can be explained by size (43).

293 This study constitutes the first documentation, as far as we are aware, of plants having a
294 primed response to fire, as found for some other abiotic stresses such as drought and
295 inundation (e.g. 10,44,45). Traits relating to flowering and growth allocation differed across
296 all of the species according to prior fire exposure. These differences continued at least until
297 the end of the study providing an example of a persistent phenotypic change, but could be
298 maintained for one or more generations as has been found in other cases of environmentally
299 induced carry-over effects (46,47,48). Whilst fire is a major disturbance to plants, these
300 developmental changes may mean current performance can be maximised, through improved
301 tolerance and/or responses to future fires, whilst avoiding the potential costs of maintaining a
302 life-long high-fire-suited phenotype. The priming mechanism is not addressed here, but such
303 responses could involve epigenetic, metabolic, physiological or morphological changes
304 (7,8,9). The roles of epigenetic and chromatin modifications are particularly recognised in
305 plant stress responses, and therefore represent a likely mechanism for the traits differences
306 seen between plants with and without prior fire exposure.

307 Similar to findings on the effect of crown fires on woody species (49,50,51), this study shows
308 that fires cause trait divergence in the above- and below-ground allocation strategies of
309 herbaceous plants. Annually burned plants invested more of their biomass below ground
310 compared to no-burn plants, which likely equates to them having greater stored energy
311 reserves to initiate and support early resprouting. The greater frequency of disturbance
312 experienced by the annual-burn plants means they are regularly subjected to the near
313 complete removal of above-ground biomass and frequently encounter the competitive, post-
314 fire environment. Thus, greater investment below ground results in a smaller proportion of
315 total plant biomass being consumed by fire. However, a higher proportional investment in
316 root biomass in annual-burn plants did not cause the faster initial resprouting rate compared
317 to no- burn plants that we expected. Similarly, specific leaf area, a trait indicative of resource
318 acquisition (52), did not differ between annual-burn and no-burn plants. Recurrent fire could
319 instead select for more vigorous resprouting (i.e. greater resprouted biomass, as found for two
320 of the four species examined here, 53) rather than a faster rate of resprouting at the tiller
321 scale. Such differences could be due to a greater number of resprouting tillers rather than a
322 faster rate of regrowth per tiller. Interestingly, the two species in which annually-burnt
323 populations regrew significantly more biomass after fire than unburnt populations both
324 belong to the monophyletic group *Andropogoneae*. Similarly, in a previous comparative
325 analysis of grass fire responses of different lineages (54), regrowth was stimulated by fire
326 only in the *Andropogoneae* species studied. In fire-prone areas, the rapid creation of a large,
327 flammable fuel load by these shade-intolerant species may aid in the maintenance of an open
328 canopy by burning off standing dead and woody biomass (55).

329 Grasses showed plasticity in reproduction, dependent upon previous fire experience, that is
330 likely to be adaptive in fire-prone environments. A history of high fire frequency favours
331 grass traits relating to vigorous post-fire recruitment, with the heightened flowering in
332 annual-burn plants suggesting that flowering and seed production is stimulated by fire. Fire-
333 stimulated flowering has been demonstrated in other savanna grass species (56), but this
334 study represents the first documentation of fire having a priming effect on grass flowering. In
335 many perennial grasslands, successful recruitment is a rare event (19,20), but fire may
336 enhance seedling establishment through reduced below-ground competition with resprouters
337 (57).

338 Fire-prone savannas are vulnerable to global change drivers (58), with fire regimes changing

339 in frequency and intensity (59). As fire behaviour influences plant traits, a consequence of
340 such changes may be transformed community functional diversity. However, the finding here
341 that grasses may have a primed response to fire, resulting in adaptive trait changes, may lead
342 to a community composition that is more resistant to future fire regime changes (8).

343

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572 in global burned area. *Sci.* **356** (6345): 1356-1362.

573 **Table 1. Assembly statistics for the analysis of genetic differences between populations**
574 **of three savanna grass species under contrasting fire regimes [annual burn (AB) and no**
575 **burn (NB)].** RAD; restriction-site associated DNA sequencing, SNPs; single nucleotide
576 polymorphisms. *Melica racemosa* was removed from this analysis because failure of some
577 samples resulted in a low sample size. ^a $F_{ST} > 0.80$
578

Species	Number individuals (NB:AB)	Number assembled nuclear RAD loci	Number SNPs	Average F_{ST} (p-value)	Nr. Outlier F_{ST} (p-value) ^a
<i>Cymbopogon pospischilii</i>	10 (5:5)	21,649	5,528	0.083 (0.074)	33 (0.136)
<i>Digitaria eriantha</i>	9 (5:4)	11,716	4,611	0.095 (0.107)	22 (0.361)
<i>Themeda triandra</i>	8 (5:3)	40,031	9,977	0.107 (0.076)	104 (0.122)

579

580

581 **Figure legends**

582 **Figure 1. Grass traits differ in populations exposed to contrasting fire frequencies.**

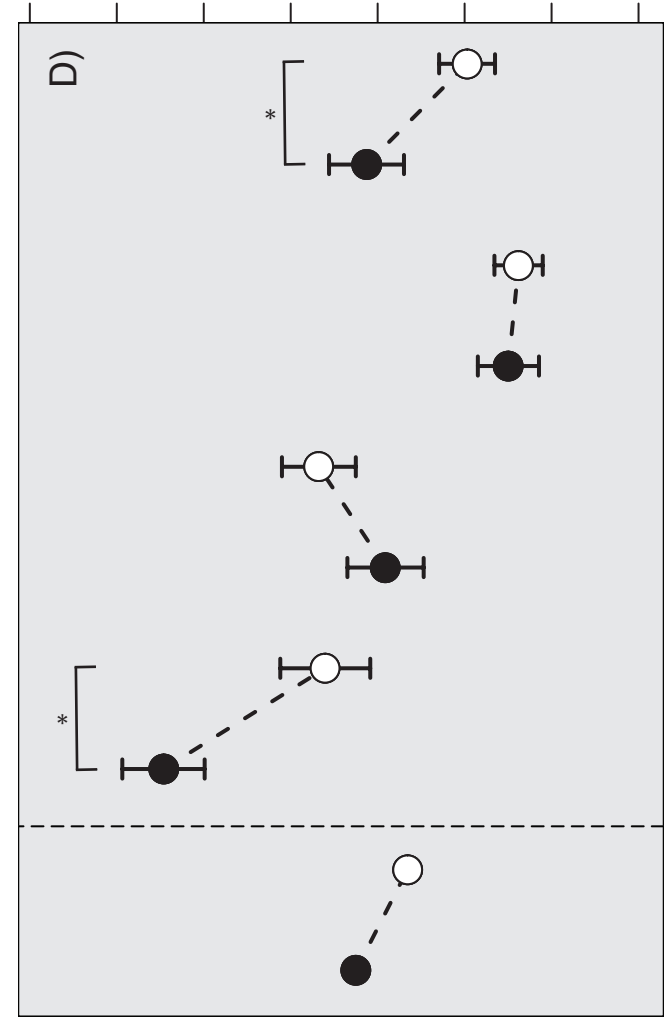
583 Annual-burn plants had significantly more flowering tillers ($P < 0.001$; panel **A**) than no-burn
584 plants. Total dry biomass did not differ between treatments (panel **B**) but the allocation of
585 biomass differed significantly ($P < 0.001$; panel **C**) with annual-burn plants investing a higher
586 proportion of their dry biomass below ground in comparison to no-burn plants. After burning
587 all individuals, there was no overall effect of treatment on total regrown dry biomass (panel
588 **D**; although, for two species, annual-burn plants did regrow significantly more biomass than
589 no-burn plants; $P < 0.05$), regrowth rate (panel **E**) or the specific leaf area of regrown leaves
590 (panel **F**) *C.*=*Cymbopogon*; *D.*=*Digitaria*; *M.* =*Melica*; *T.*=*Themeda*. *, $P < 0.05$; **, $P < 0.01$;
591 ***, $P < 0.001$.

592

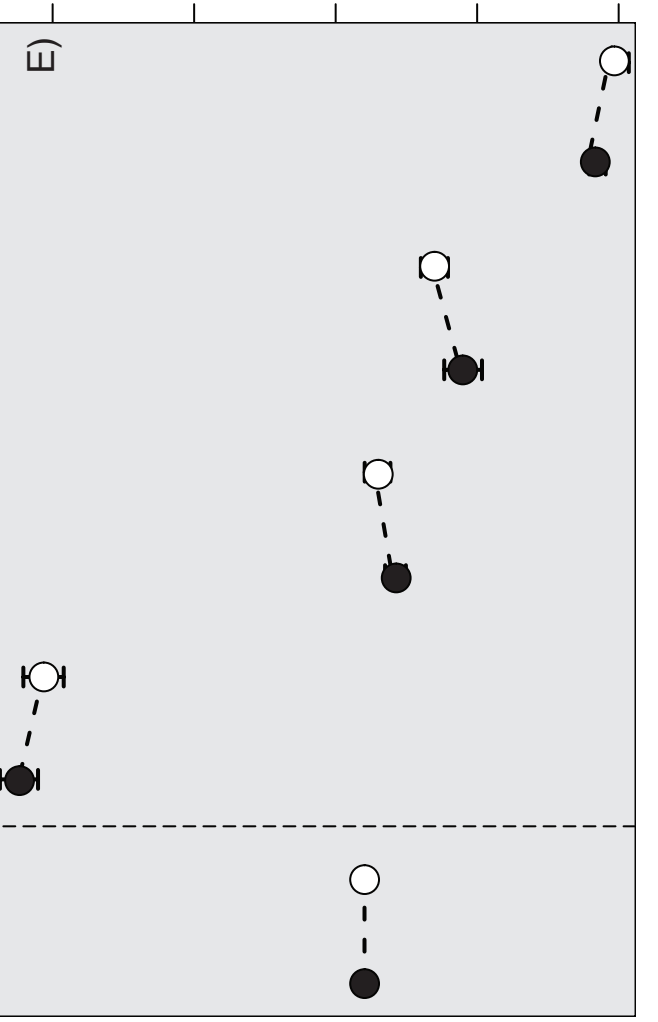
593 **Figure 2. No genetic differentiation between grass populations under contrasting fire**

594 **frequencies.** Phylogenies and PCA plots reveal no clustering based on treatment for
595 individuals of *Cymbopogon pospischilii* (**a**), *Digitaria eriantha* (**b**), and *Themeda triandra*
596 (**c**). Analysis of similarity (anosim) results are indicated in the top left of the PCA plots.
597 Values on nodes represent support evaluated with 100 bootstrap replicates (only support
598 values > 50 are shown). PCAs are based on all single nucleotide polymorphisms.

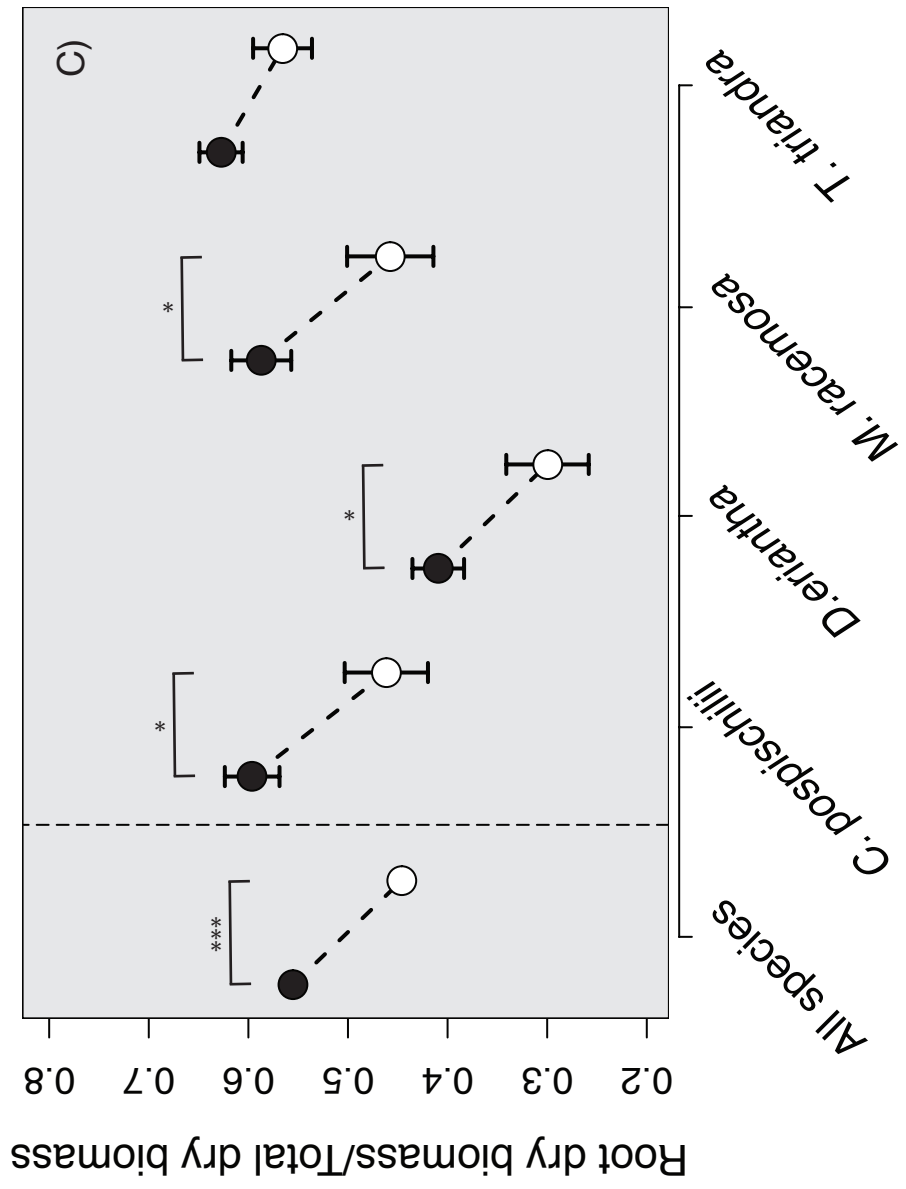
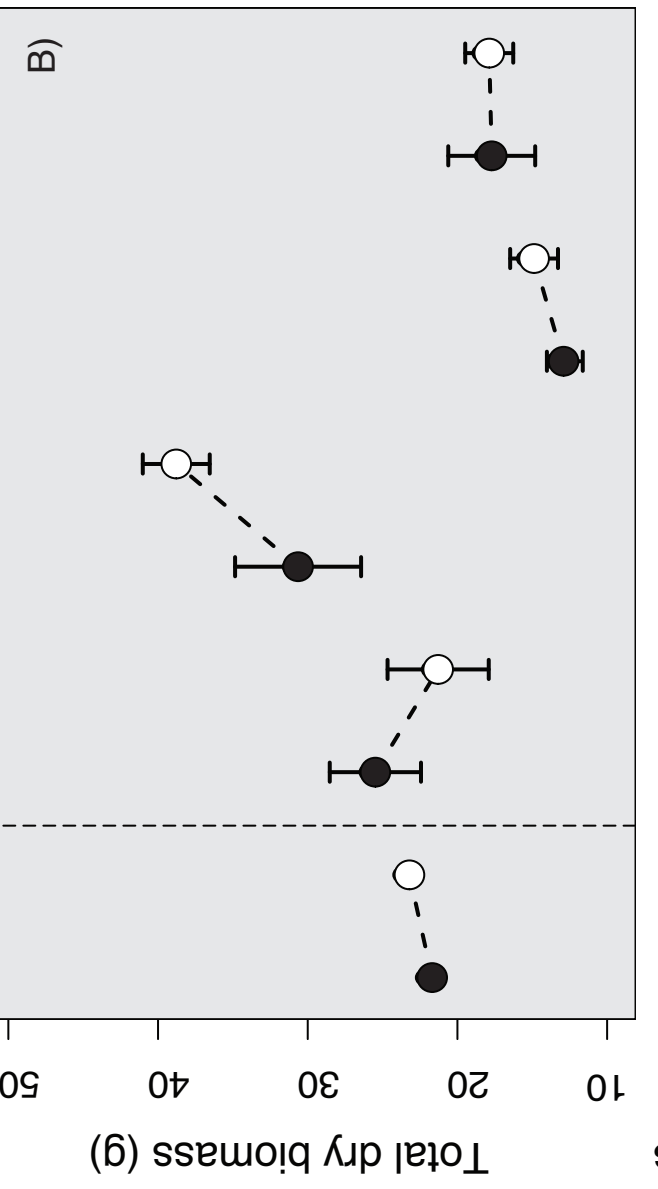
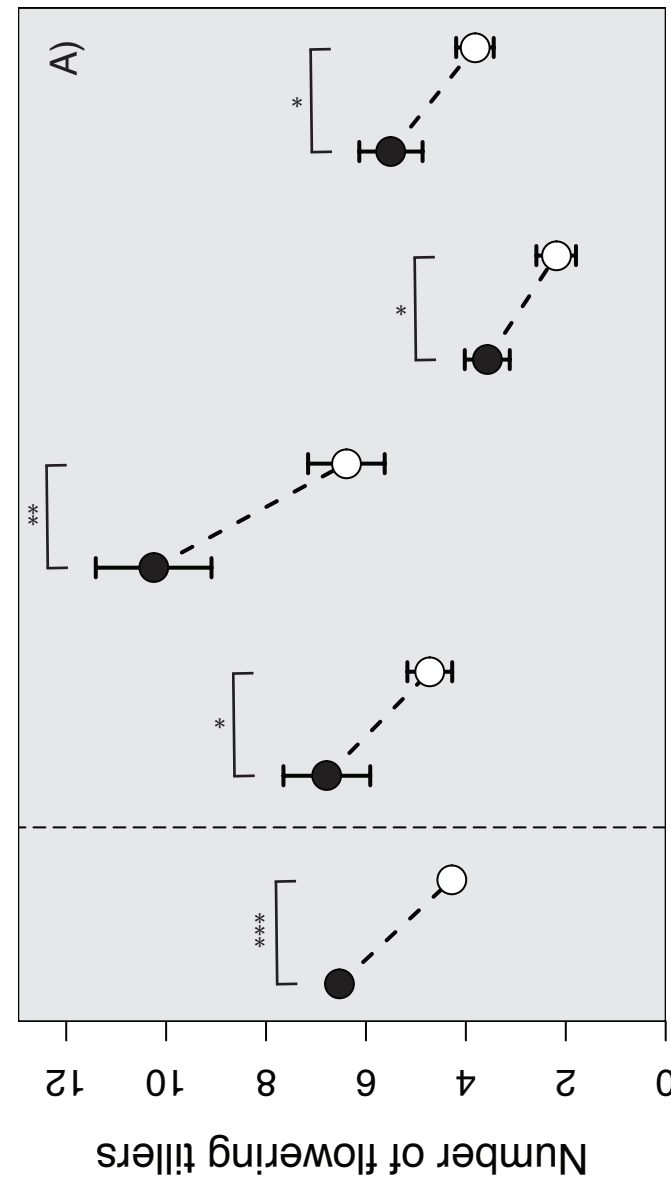
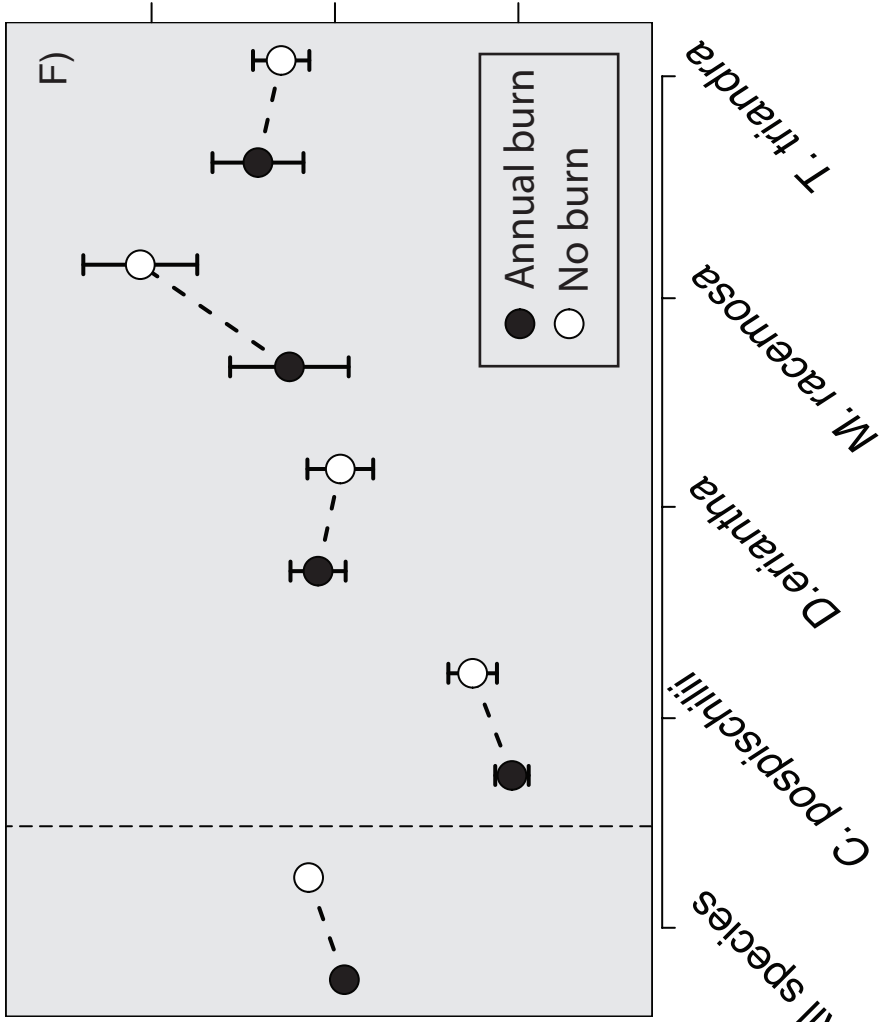
Total regrown dry biomass (g)

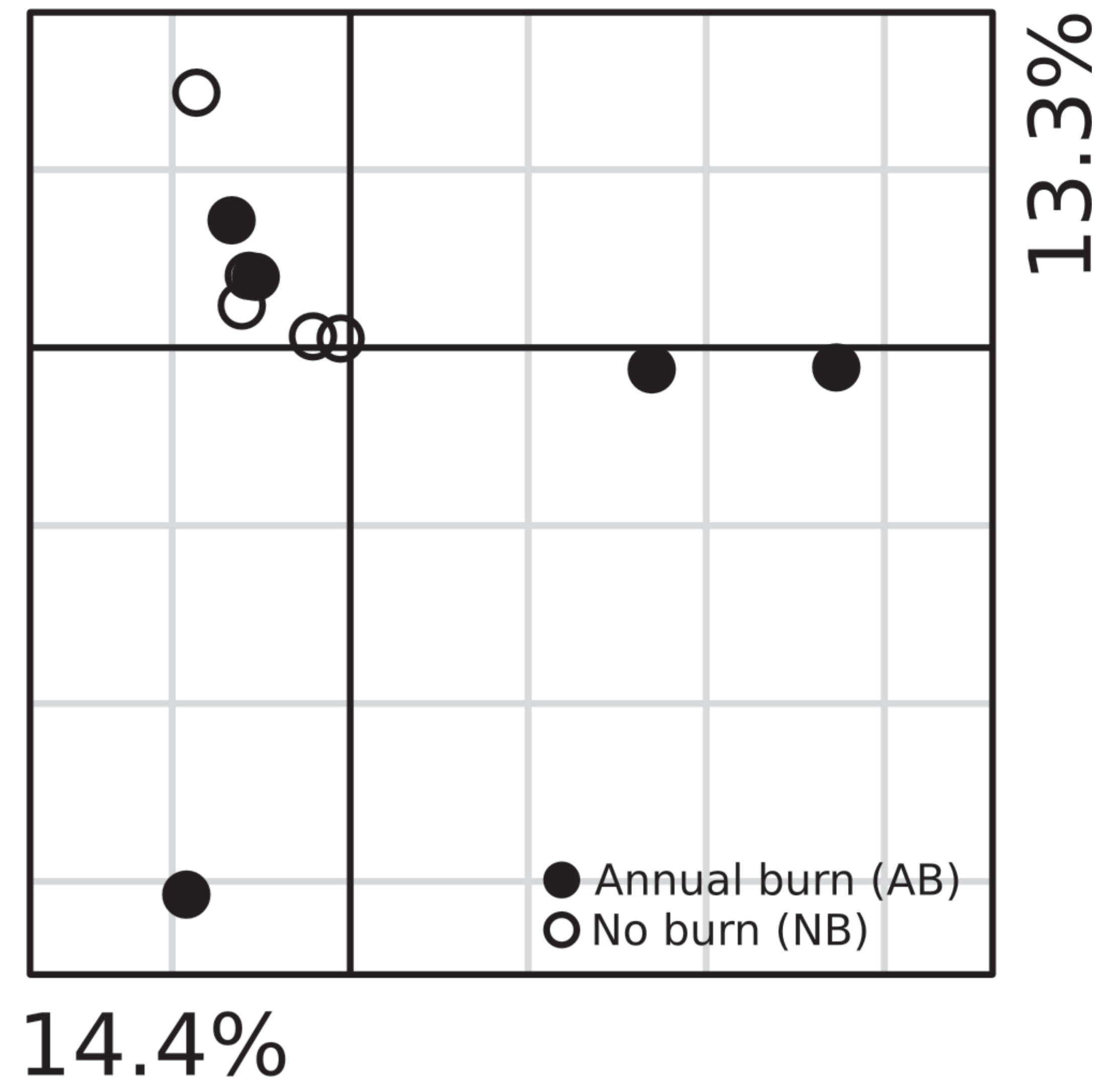
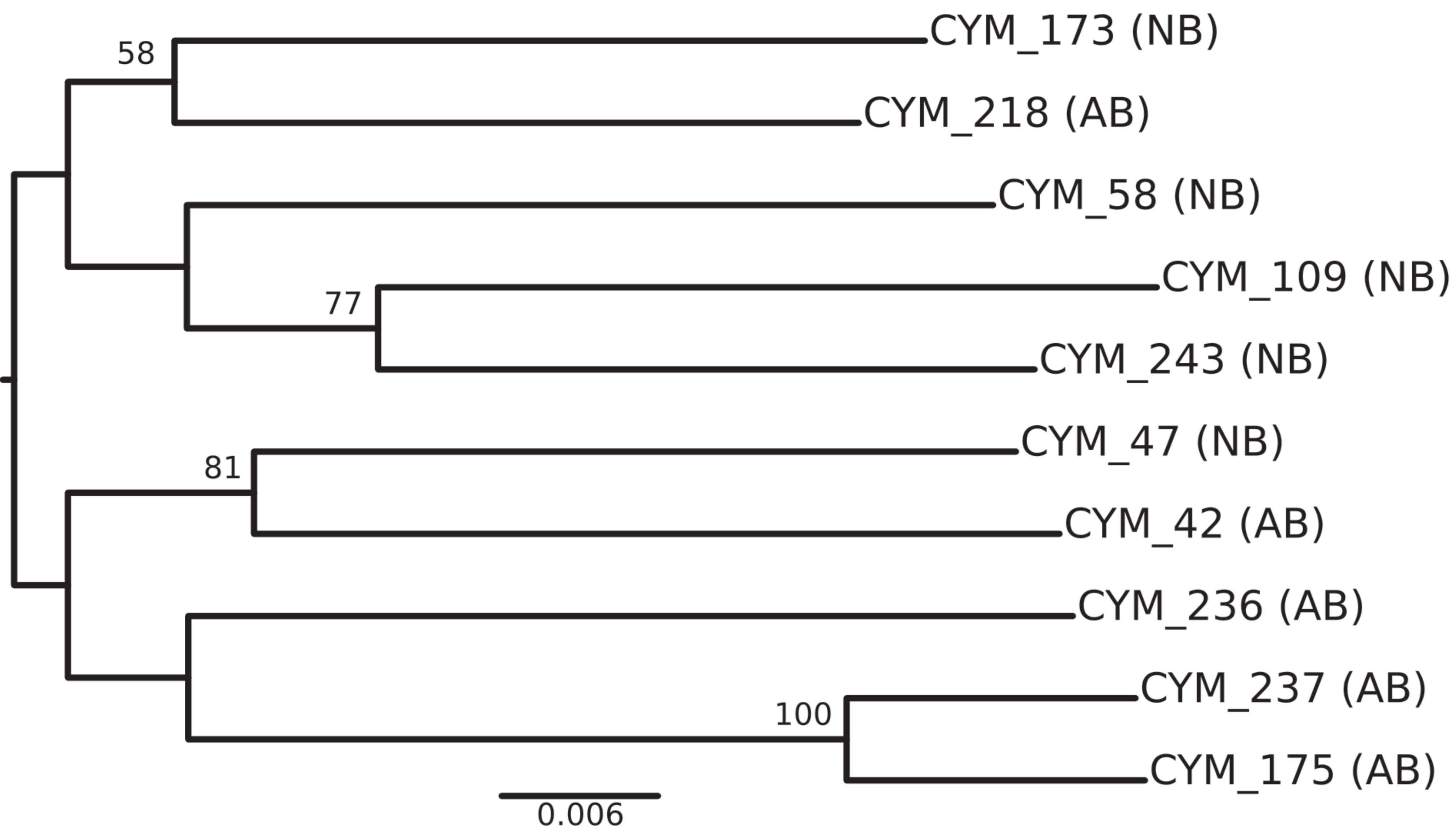
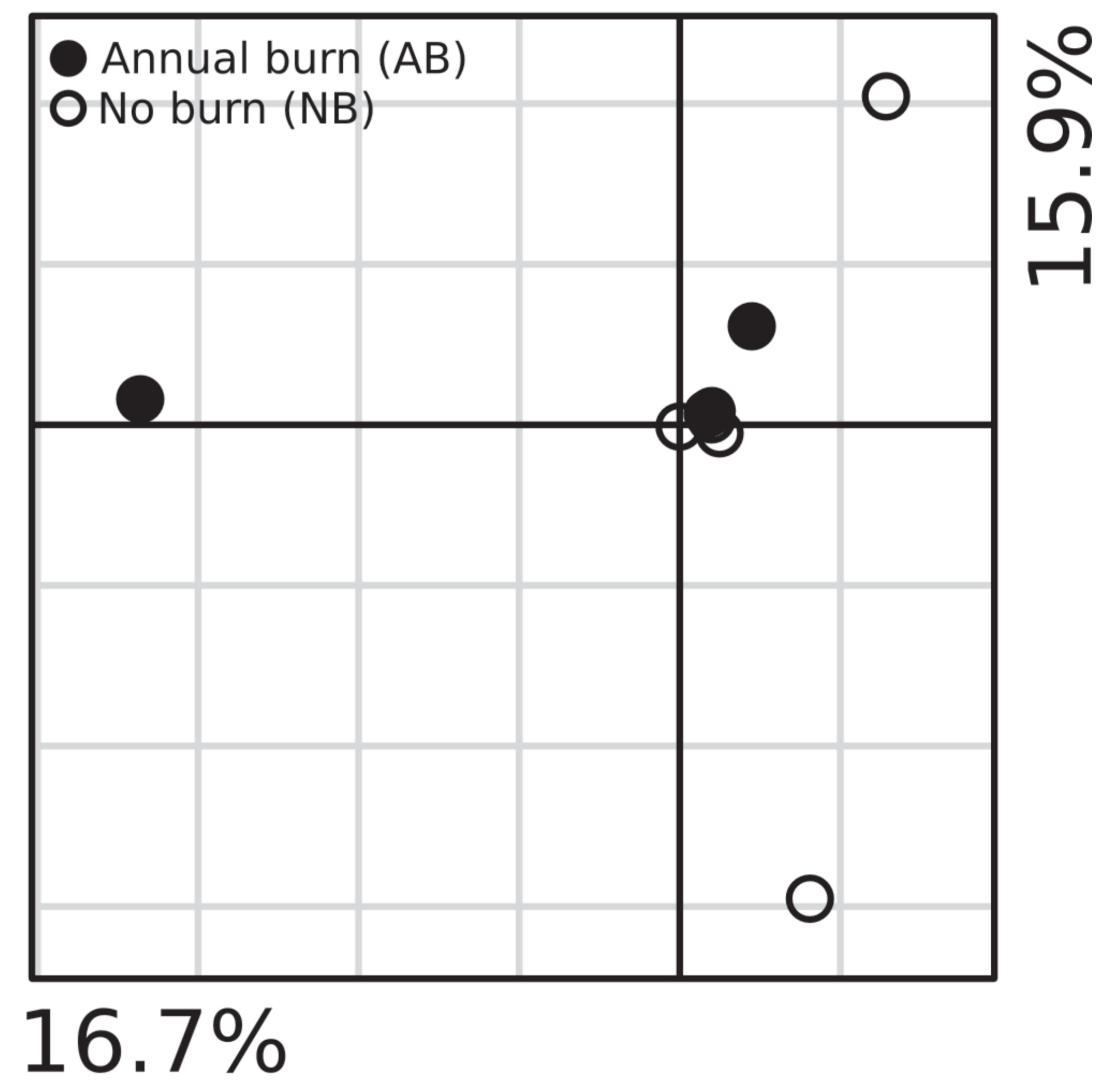
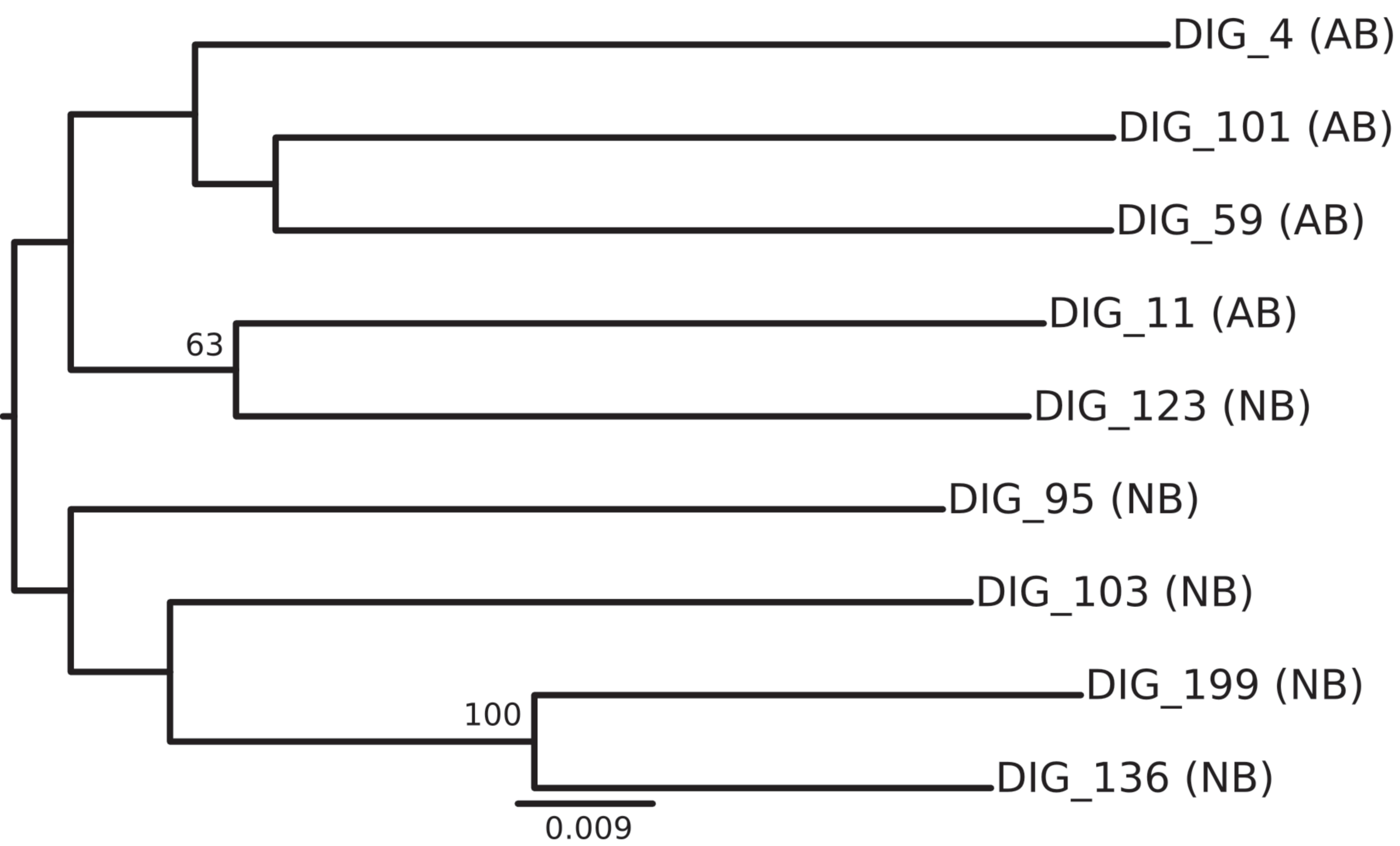
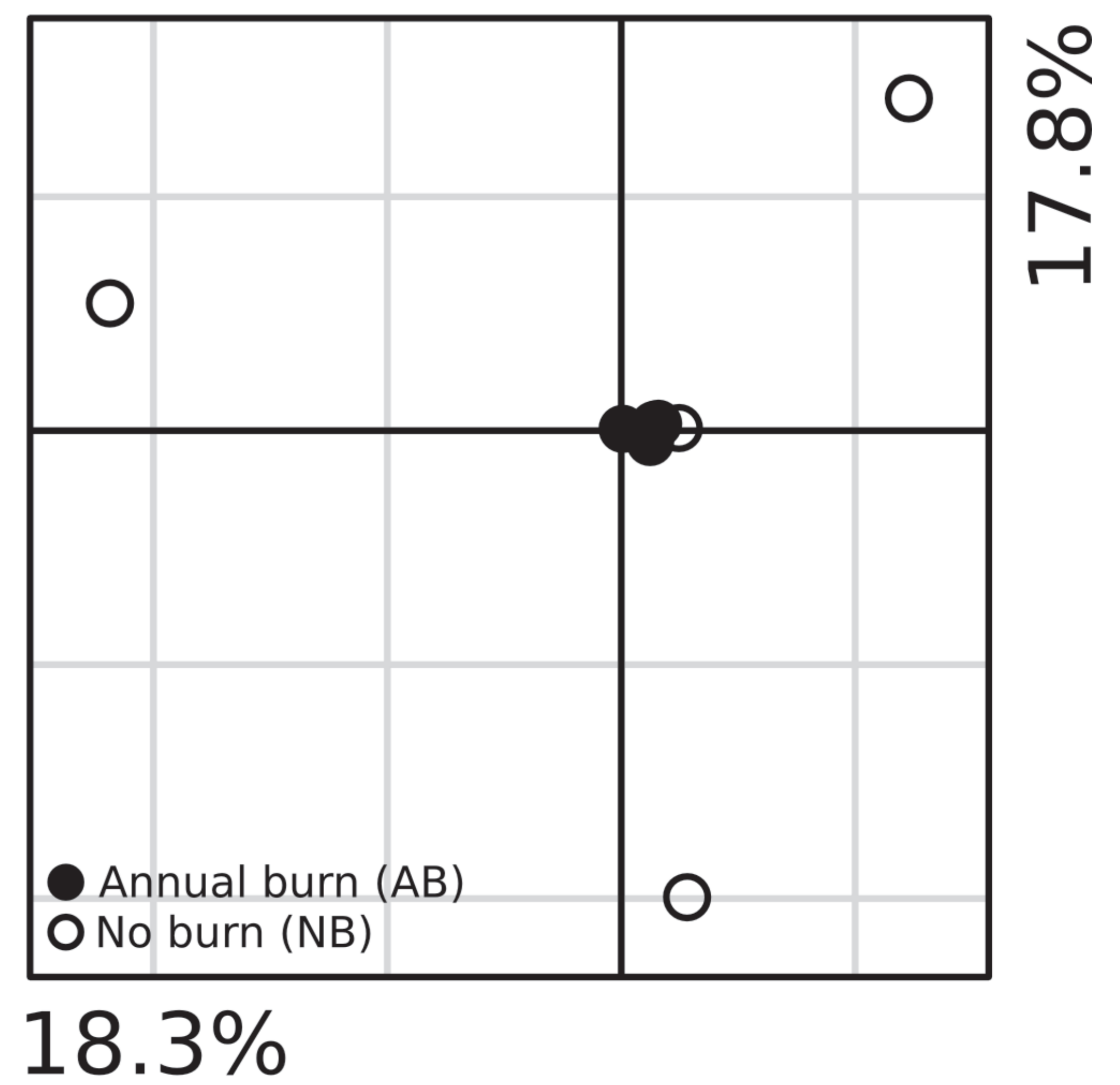
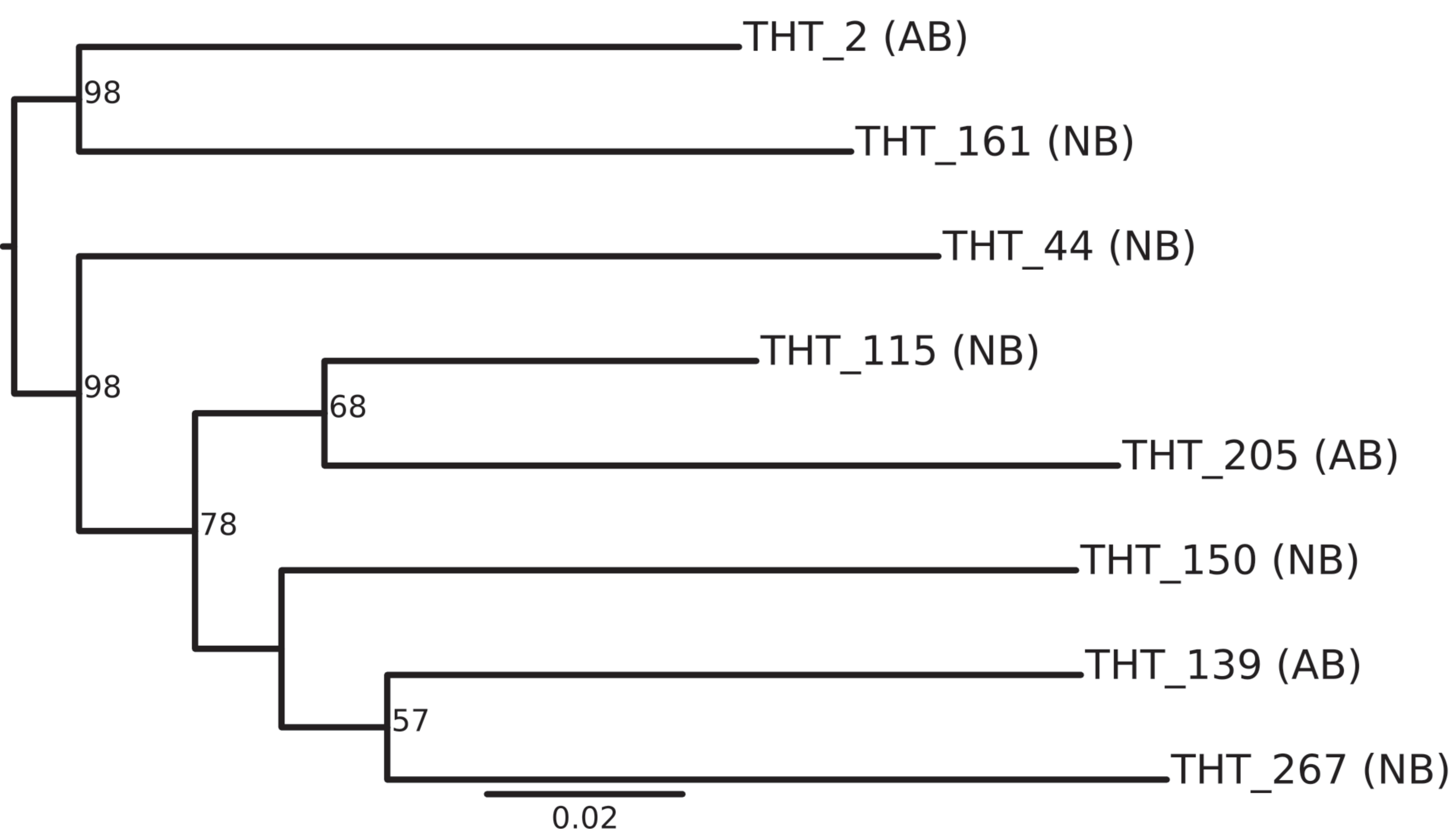


Regrowth rate (mg GDD⁻¹)



Specific leaf area (cm² g⁻¹)



a)**b)****c)**

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Supporting Information

Frequent fires prime plant developmental responses to burning

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Table S1. Climate and treatment data for the experimental burn plots. These plots (based at the University of Fort Hare Research Farm, Eastern Cape, South Africa; 32° 47' S, 26° 52' E), set up in 1980, comprise six treatments (no burn, annual, biennial, triennial, quadrennial and sexennial burns) replicated twice in a randomized design on 100 m x 50 m plots, each with a 5m mowed boundary (as described in 1). The area in which the plots are situated is semi-arid savanna and the soil at the site is a shallow silty loam of the Glenrosa series (2). Values represent monthly average daily minimum temperature, daily maximum temperature and rainfall for years spanning 1997 to 2015. The mean annual rainfall of 440mm, the majority of which falls in the Austral summer. Data was provided by the South African Weather Service (weather station 0078227A3; www.weathersa.co.za).

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Monthly rain (mm)	56.1	50.1	56.7	43.8	16.3	17.4	17.8	21.3	23.0	37.1	43.4	54.4
Daily Max. Temperature (°C)	30.1	30.0	28.6	25.6	23.7	21.4	21.3	22.8	24.4	25.7	27.0	28.5
Daily Minimum Temperature (°C)	16.8	17.2	15.4	12.3	9.3	6.7	6.1	7.4	9.0	11.6	13.4	15.5

Table S2. Details of the species used in this study and herbarium numbers of specimens of each species deposited at the Selmar Schonland Herbarium, Rhodes University.

Species	Subfamily	Herbarium number
<i>Cymbopogon pospischilii</i> (K. Schum.) C.E. Hubb.	Panicoideae (Andropogoneae)	GRA:Simpson s.n. 29
<i>Digitaria eriantha</i> Steud.	Panicoideae (Paniceae)	GRA:Simpson s.n. 27
<i>Melica racemosa</i> Thunb.	Pooideae	GRA:Simpson s.n. 28
<i>Themeda triandra</i> Forssk.	Panicoideae (Andropogoneae)	GRA:Simpson s.n. 26

Table S3. Details of reference chloroplast genomes used to separate chloroplast and nuclear data. Representative whole chloroplast genomes were downloaded from Genbank and cleaned reads were mapped to the respective reference genome using Bowtie2 v.2.2.3 (3) with default settings for paired end reads. All reads not mapping to the chloroplast were then retrieved using SAMtools v.1.2 (4) and BEDtools v.2.19.1 (5) and used for further analyses

Study species	Reference plastid species	GenBank reference
<i>Cymbopogon pospischilii</i>	<i>Cymbopogon flexuosus</i>	NC_035040.1
<i>Digitaria eriantha</i>	<i>Digitaria exilis</i>	NC_024176.1
	<i>Digitaria exilis</i>	KJ513091.1
<i>Themeda triandra</i>	<i>Themeda</i> sp	KU291484.1

Table S4. Mixed model coefficients for the two initial (field-state) plant traits (plant height and total dry biomass). The intercept represents the species *Cymbopogon pospischilii* and the annual burn treatment. Dig = *Digitaria eriantha*; Mel = *Melica racemosa*; NB = no burn treatment; SE = standard error; The = *Themeda triandra*.

	Plant height		Total biomass	
	Estimate ± SE	t value	Estimate ± SE	t value
(Intercept)	28.20 ± 2.77	10.18	2.56 ± 0.12	20.99
NB	8.36 ± 1.30	6.42	0.85 ± 0.09	9.17
Dig	-5.78 ± 1.68	-3.44	-0.93 ± 0.13	-7.09
Mel	5.22 ± 2.18	2.39	-1.69 ± 0.13	-12.83
The	-4.89 ± 1.50	-3.26	-0.54 ± 0.13	-4.07
log (Total dry mass)	2.31 ± 0.99	2.33		

Table S5. Mixed model coefficients for pre-fire plant traits (number of flowering tillers, fuel load, root biomass, proportion of total biomass in roots, total biomass and plant height). The intercept represents the species *Cymbopogon pospischilii* and the annual burn treatment. Dig = *Digitaria eriantha*; Mel = *Melica racemosa*; NB = no burn treatment; SE = standard error; The = *Themeda triandra*.

	Flowering tillers		Fuel load		Root biomass		Proportion root biomass		Total biomass		Plant height	
	Estimate ± SE	t value	Estimate ± SE	t value	Estimate ± SE	t value	Estimate ± SE	t value	Estimate ± SE	t value	Estimate ± SE	t value
Intercept	1.17 ± 0.17	7.05	1.35 ± 0.08	17.00	2.66 ± 0.16	17.16	0.58 ± 0.03	23.19	3.04 ± 0.10	31.28	3.53 ± 0.11	31.59
NB	0.07 ± 0.16	-0.42	-0.32 ± 0.12	-2.65	-0.49 ± 0.22	-2.22	-0.11 ± 0.02	-4.84	0.07 ± 0.09	0.79	0.01 ± 0.04	0.22
Dig	0.33 ± 0.14	2.27	-0.01 ± 0.11	-0.08	-0.20 ± 0.22	-0.91	-0.17 ± 0.03	-5.48	0.43 ± 0.12	3.48	-0.37 ± 0.06	-6.01
Med	0.48 ± 0.15	-3.22	-0.12 ± 0.12	-1.06	-0.64 ± 0.22	-2.94	-0.01 ± 0.03	-0.21	-0.46 ± 0.12	-3.73	0.02 ± 0.05	0.31
The	0.10 ± 0.15	0.15	-0.16 ± 0.11	-1.43	-0.34 ± 0.31	-1.54	0.07 ± 0.03	2.13	-0.25 ± 0.12	-2.03	-0.29 ± 0.05	-5.37
NB : Dig	-0.36 ± 0.21	-1.69	0.35 ± 0.16	2.16	0.41 ± 0.31	1.31						
NB : Med	-0.24 ± 0.21	-1.15	0.12 ± 0.16	0.73	0.34 ± 0.31	1.09						
NB : The	-0.12 ± 0.22	-0.55	0.20 ± 0.17	1.21	0.45 ± 0.31	1.47						
log(Total dry mass)	0.57 ± 0.10	5.91									0.05 ± 0.05	1.10

Table S6. Mixed model coefficients for post-fire regrowth traits (regrown dry biomass, regrown leaf area, regrown leaf SLA and regrowth rate). The intercept represents the species *Cymbopogon pospischilii* and the annual burn treatment. Dig = *Digitaria eriantha*; Mel = *Melica racemosa*; NB = no burn; SE = standard error; The = *Themeda triandra*.

	Regrown SLA		Regrown area		Regrown mass		Regrowth rate	
	Estimate ± SE	t value	Estimate ± SE	t value	Estimate ± SE	t value	Estimate ± SE	t value
(Intercept)	10305.8 ± 796.0	12.95	6.932 ± 0.289	23.96	-2.126 ± 0.261	-8.14	0.046 ± 0.001	34.17
NB treatment	793.3 ± 717.9	1.11	0.049 ± 0.148	0.34	-0.017 ± 0.138	-0.12	0.0007 ± 0.001	0.83
Dig	4462.6 ± 968.0	4.61	-0.222 ± 0.199	-1.12	-0.547 ± 0.180	-3.04	-0.025 ± 0.001	-27.36
Mel	7910.3 ± 1047.8	7.55	-1.179 ± 0.213	-5.32	-1.682 ± 0.193	-8.73	-0.029 ± 0.001	-28.99
The	6080.7 ± 1020.0	5.96	-0.335 ± 0.208	-1.61	-0.779 ± 0.187	-4.16	-0.040 ± 0.001	-41.50
log(Total pre- fire dry mass)			1.098 ± 0.191	5.75	0.969 ± 0.173	5.62	0.003 ± 0.001	4.21

Supporting Figures

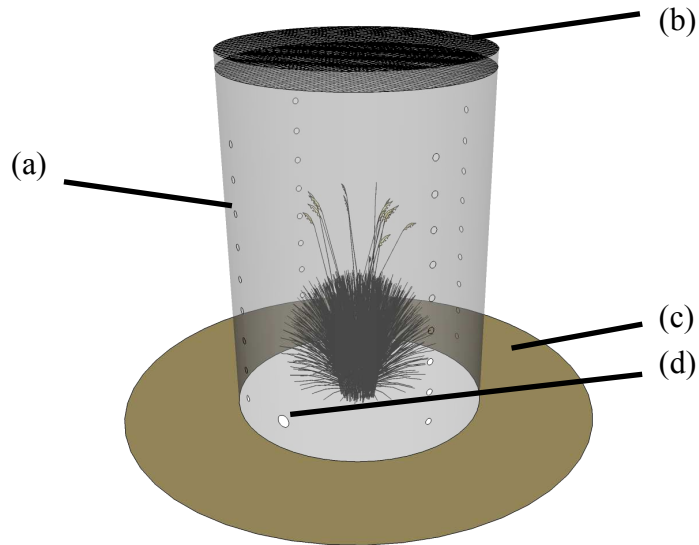


Fig. S1 Schematic drawing of the set-up used to burn plants. (a) Modified, cleaned 45-gallon oil drum (85 cm tall and 61cm diameter) with the top and base removed, and a series of holes drilled in four vertical lines down its side for ventilation; (b) double layer of fine mesh fitted into the top of the drum to act as a spark arrestor; (c) wet hessian sacking placed around the base of the barrel to minimize risk of the fire spreading; (d) the ignition hole through which a lit blowtorch was applied. Each plant was carefully removed from its pot to minimize root disturbance, and lowered into a hole (not shown). The depth of the hole was adjusted to ensure that the soil surface of all the plants was at the same height and thus burned evenly.

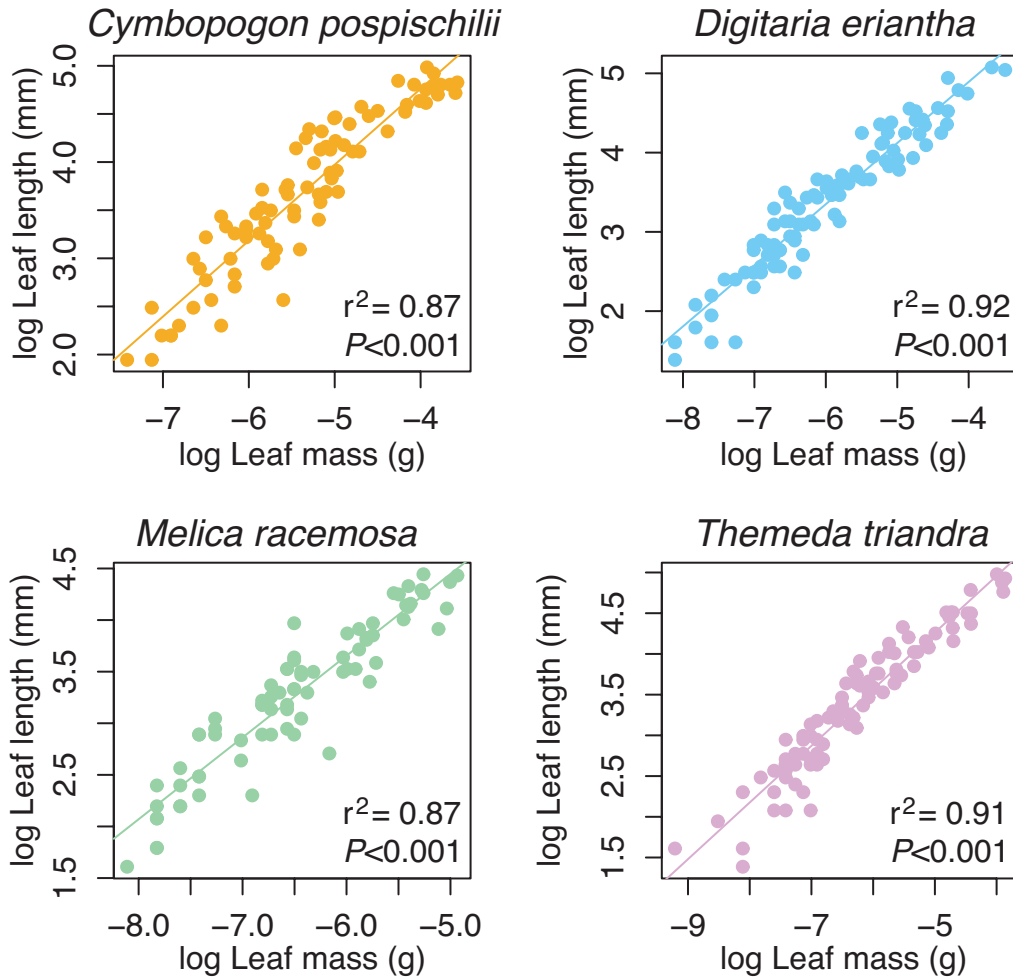


Figure S2. The relationship between leaf length and dry mass for the four studied grass species, as determined by fitting linear models to the log-transformed values of both variables. The fit of the models to the data was good (R^2 values >0.87 for all species) and the slope of these relationships was used to convert leaf length regrowth rate into leaf biomass regrowth (in mg GDD^{-1}).

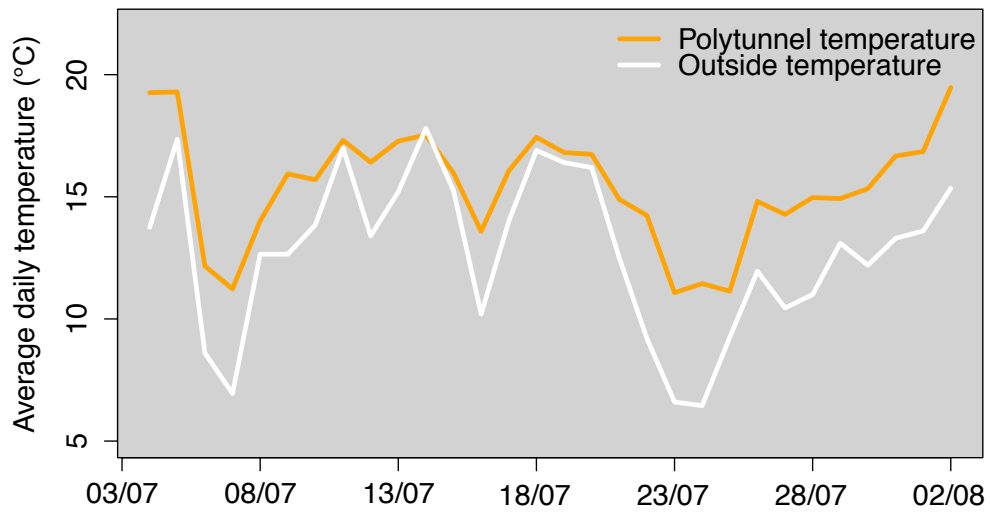


Figure S3. A comparison of temperatures inside and outside the polytunnel during the period of plant regrowth following the experimental burn. Daily average temperatures were slightly higher (2.7°C on average) in the polytunnel than outside, thus the plants experienced conditions similar to early spring without the complication of late season frosts. Outside temperatures were provided by the South African Weather Service (weather station 0056917 8; www.weathersa.co.za)

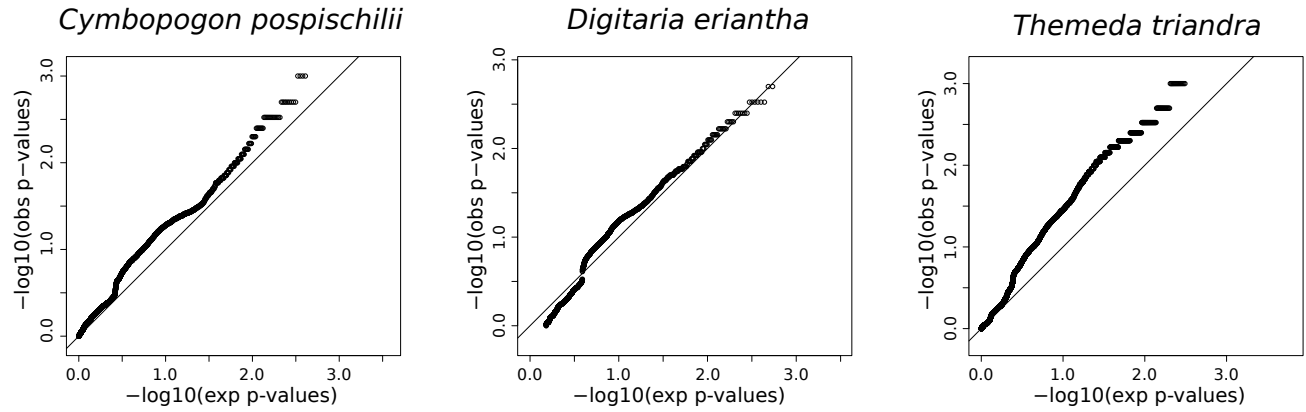


Figure S4. Relationships between P-values observed for each SNP and those expected under a null hypothesis (i.e. no differentiation) for three grass species. We see genome-wide departure from the null hypothesis, in the direction of having more genes with small p-values than expected, showing we have power to detect differentiation between treatments despite the low sample sizes. “Obs” = observed; “exp” = expected.

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