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Germination ecology of wild and domesticated *Ensete ventricosum*: Evidence for maintenance of sexual reproductive capacity in a vegetatively propagated perennial crop

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ABSTRACT

PREMISE: Loss of sexual reproductive capacity has been proposed as a syndrome of domestication in vegetatively propagated crops, but there are relatively few examples from agricultural systems. Compared to sexually propagated crops banked as seeds, vegetative crop diversity is typically conserved in living collections that are more costly and insecure. This vulnerability may disproportionately impact tropical crop diversity where vegetative reproduction predominates. In this study we assess sexual reproductive capacity in wild (sexual) and domesticated (vegetative) populations of enset (*Ensete ventricosum* (Welw.) Cheesman), a giant tropical banana relative and Ethiopian food security crop.

METHODS: We harvested seeds from 20 wild and domesticated enset to survey variation in seed weight, viability, external and internal morphology. We germinated seeds across a range of constant and alternating temperature regimes to characterise optimum germination requirements, and evaluate differences in performance.

KEY RESULTS: We found no significant difference between wild and domesticated enset seed viability, days to germination and internal morphological traits. However, we report a significant and unexpected shift in seed weight and germination behaviour, with seed from domesticated plants responding to cooler temperatures with greater diurnal range. Shifts in germination behaviour appear concordant with a climatic envelope shift in the domesticated distribution.

CONCLUSIONS: Our findings suggest sexual reproductive capacity has been maintained despite long-term near-exclusive vegetative propagation in domesticated enset. Furthermore, certain traits such as germination behaviour, may be under continued selection through rare sexually reproductive events. Improved understanding of sexual propagation in vegetative crops may have applications in germplasm conservation and plant breeding.

Keywords: clonal reproduction; Crop Wild Relative; domestication; Enset; Ethiopia; germination biology; Perennial crops; Seed banking; vegetative propagation

INTRODUCTION

The erosion of plant genetic resources poses a substantial threat to food security and the diverse benefits we derive from useful plants (Borrell et al., 2019; Powell et al., 2018). To address this challenge a significant effort has been made to conserve >2 million unique accessions, representing >16,500 plant species in 1750 genebanks worldwide (Commission for genetic resources on food and agriculture, 2010; Fu, 2017). However an emphasis on conventional seed crops may overlook the majority of perennial fruit crops for which vegetative propagation is the predominant means of replication and seed generation may be rare or absent (Miller and Gross, 2011; Castañeda-álvarez et al., 2016; Migicovsky and Myles, 2017a). Vegetatively propagated crops are especially important in the tropics (Denham et al., 2020), where *ex situ* or *in vitro* germplasm collections are currently the only viable approach for conserving genetic resources (Thormann and Dulloo, 2006). Maintaining such collections is often logistically challenging and prohibitively expensive, particularly for developing countries (Dulloo et al., 2013). Nevertheless, the majority of vegetatively propagated crops have sexually reproducing wild progenitors (Miller and Gross, 2011). Improving our understanding of reproductive biology in vegetative propagated perennial crops has potential to address these challenges by enabling a broader range of useful plants to be banked as seed and used in breeding programmes, enhancing conservation and use of genetic diversity (even if this does not conserve specific vegetative genotypes) (McKey et al., 2010; Borrell, Dodsworth, et al., 2019; Pironon et al., 2019; Denham et al., 2020).

Clonally propagated food crops encompass at least 34 families and a wide variety of morphological diversity (McKey et al., 2010). This diversity has hindered attempts to define a domestication syndrome in clonally reproduced crops, but a number of commonalities have been proposed (McKey et al., 2010; Miller and Gross, 2011; Denham et al., 2020). Foremost is the suggestion that prolonged vegetative reproduction can lead to the loss of sexual reproductive capacity (Eckert, 2002; McKey et al., 2010; Barrett, 2015; Denham et al., 2020). For example, cultivated bananas (*Musa*) differ from their wild relatives by being seedless and parthenocarpic (Heslop-Harrison and Schwarzacher, 2007)

74 whilst in *Citrus*, fertile varieties co-exist with and asexually propagated cultivars that reproduce via
75 nucellar embryony (Wu et al., 2018). Domestication of Pineapple (*Annas*) was also associated with a
76 combination of sexual and asexual selection, together with reduced seed production through lower
77 fertility and self-incompatibility (Chen et al., 2019). Loss of sexual reproductive capacity, if
78 widespread across domesticated perennial crops, could significantly hinder future perennial crop
79 breeding programmes and integration of diversity from crop wild relatives (Dempewolf et al., 2014;
80 Migicovsky and Myles, 2017a). Importantly, sexual reproductive capacity does not have to be
81 completely lost – reduced fertility, viability, altered germination behaviour or other changes as a
82 result of deleterious mutations and genetic drift in reproductive traits could still significantly hinder
83 programmes that seek to recombine or conserve diversity as seeds (e.g. McClure, Sawler, Gardner,
84 Money, & Myles, 2014; Migicovsky & Myles, 2017). However, surprisingly few studies have
85 attempted to survey the sexual reproductive capacity of vegetatively propagated crops in
86 agricultural systems (Elias et al., 2001; Scarcelli et al., 2006).

87 Here, we investigate the impact of domestication on seed and germination biology of the perennial
88 food security crop enset (*Ensete ventricosum* (Welw) Cheesman) (Figure 1). Enset is a giant
89 monocarpic herb, in the sister genus to the more widely known bananas (*Musa),* which provides a
90 staple starch source for 20 million people of South and South-west Ethiopia (Janssens et al., 2016;
91 Borrell, Biswas, et al., 2019). Wild enset reproduces sexually, generating inedible fruits that contain
92 hundreds to thousands of seeds (Ssali and Sheil, 2019), but unlike bananas, enset does not produce
93 vegetative suckers. However, during domestication (a process which also improved palatability and
94 diversified uses (Tamrat et al., 2020)), sophisticated agronomic practices were developed to enable
95 exclusively vegetative propagation (Borrell et al., 2020) (Figure 1B, 1C). Farmers achieve this by
96 removing the apical meristem and meristematic tissue of a two to three year old corm, resulting in
97 the generation of adventitious buds (Karlsson, Dalbato, Tamado, & Mikias, 2014). Currently, up to
98 1500 named clonal landraces have been recorded and are maintained by millions of rural farmers
99 (Borrell, Biswas, et al., 2019). Domesticated enset is restricted to Ethiopia and has been increasingly

100 overlooked in favour of introduced species (Borrell, Biswas, et al., 2019), whilst permanent
101 international transfer of enset plant material out of Ethiopia is not permitted. As a result,
102 domesticated enset is not banked as seed in international (or national) collections (Guzzon and
103 Müller, 2016), and only a handful of local institutes maintain field germplasm collections
104 (specifically; Areka, Yerefezy, Angacha and Hawassa, situated in southern Ethiopia). With increasing
105 pressures due to climate change (Conway and Schipper, 2011) and emerging pests and pathogens
106 (Blomme et al., 2017), this represents a significant risk for the future sustainability of enset
107 agriculture.

108 Conserving domesticated enset diversity as seeds has been considered challenging for several
109 reasons. Firstly, enset is monocarpic and is harvested before flowering (unlike banana), to avoid
110 reallocation of resources from edible storage organs to the inedible inflorescence (Borrell et al.,
111 2020). This means that developed fruits are rarely encountered in cultivation. Secondly, in clonally
112 propagated crops generally, reduction of sexual fertility has been identified as a characteristic of the
113 domestication syndrome (Denham et al., 2020). Indeed a recent genomic analysis concluded that
114 accumulation of mutations in genes associated with sexual reproduction may have contributed to
115 enset's domestication (Tesfamicael et al., 2020). However, there has been no systematic study of
116 whether the majority of domesticated enset landraces retain the ability to produce viable seeds.
117 Thirdly, enset is thought to be an obligate out-crosser, yet the pollinator(s) of enset in Ethiopia are
118 unknown (though see recent reports of the Nectar bat *Megaloglossus woermanni* visiting flowers
119 from Ssali & Sheil (2019) in Uganda), and significant conversion of wild habitat to agriculture has
120 taken place, meaning the pollinator may not be present in an agricultural setting. Finally, unlike
121 other tuberous perennials, such as Yam (*Dioscorea*) (Mengesha et al., 2013) or Cassava (*Manhiot*)
122 (Rival and Mckey, 2008), wild or sexually reproduced enset seedlings are not (knowingly)
123 incorporated into cultivated populations (Borrell et al., 2020), except for the localised harvest of
124 leaves from wild plants (see Discussion). Therefore, little to no indigenous knowledge pertains to
125 enset seed germination.

126 Several previous studies have found enset germination to be highly variable (0-90% success)
127 (Tesfaye, 1992; Messele, 1994; Diro et al., 2003). The most detailed study to date by Karlsson et al
128 (2013) found 5-55% germination depending on the accession. They found that exposure to sulphuric
129 acid, sodium hydroxide, ammonium nitrate, sodium hypochlorite or hot water had no significant
130 positive effect on germination; and indeed, scarification and 70% ethanol had significant negative
131 effects. Whilst seed germination ecology is also poorly understood in *Musa,* hindering conservation
132 and use in breeding (Laliberté, 2016), it has long been know that an alternating temperature regime
133 is a key requirement for germination in *M. balbisiana* (Stotzky et al., 1962). Separately, Simmonds
134 (1962) reported that immediately after clearing of tropical forest, banana seedlings will readily
135 emerge. This is thought to be due to exposure of the seeds on the soil surface to alternating day and
136 night temperatures. Using alternating temperatures as a germination cue may be a strategy
137 conserved in genus *Ensete*, and is therefore a useful starting point for investigating enset
138 germination behaviour.

139 Here, we present the largest survey to date of wild and domesticated enset seed morphology and
140 germination behaviour, aimed at evaluating evidence for reduced sexual reproductive potential in
141 this domesticated vegetative crop. We attempt to distinguish between multiple plausible scenarios.
142 Specifically, if sexual reproductive capacity has been conserved through domestication, we
143 hypothesise that we should observe comparable seed morphology, viability and germination
144 behaviour between wild and cultivated enset plants (see for example maintenance of silicon
145 herbivore-defence through domestication in grasses, Simpson et al., 2017), or potentially, continued
146 selection for the domesticated environmental niche (Meyer et al., 2012). An alternative hypothesis
147 would be that, after being released from selection pressure, enset reproductive traits would be
148 subject to deleterious somatic mutations and genetic drift. Therefore, traits may be expected to
149 display higher variance in domesticated individuals than wild plants. A third potential scenario
150 entails a bottleneck (though these are likely weaker in perennial vegetative crops (Gaut et al., 2015))

151 in which domesticated enset represents a subset of wild diversity, and therefore a subset of the
152 morphological or behavioural diversity in reproductive traits.

153 To address these scenarios, we frame our analysis with three questions; i) Is there evidence for
154 changes in seed morphology or viability in domesticated compared to wild enset? ii) Does seed
155 germination behaviour differ between wild and domesticated enset, and how do these germination
156 requirements relate to the climate of wild and domesticated enset distributions? And iii) do
157 domesticated enset seed traits exhibit higher variance than under neutral expectations. Finally, we
158 conclude by outlining the most favourable enset seed germination protocol to support germplasm
159 conservation of a regionally important food security crop.

160 MATERIALS AND METHODS

161 Observations, collection, processing and storage

162 Enset in Ethiopia is readily distinguished in the field as the only member of its genus and may be
163 subdivided into i) domesticated clonal landraces under farmers' vernacular names, and ii) wild
164 occurring, sexually reproducing populations. In this study we collected a total of 20 seed accessions
165 from seven wild and 13 domesticated individuals, in Spring 2018 (Table 1). Domesticated accessions
166 were collected with permission from farmers' fields, and wild collections were made in river valleys
167 at least 1 km from settlements cultivating enset, to mitigate the risk of feral or recently introgressed
168 individuals. Where possible domesticated and wild accessions were collected in the same localities
169 to mitigate differences arising from the climate the parent plants were exposed to. Through our
170 extensive fieldwork for this, and related studies, we maintained a database of a) vernacular names
171 of all observed and documented landraces, and b) those observed flowering, to help resolve the
172 question of whether all landraces produce flowers. We ranked both lists by the frequency at which
173 landraces were observed and compared ranks using a Wilcoxon rank sum test implemented in R
174 software (R Core Team, 2017) to ascertain whether landraces observed flowering are a random
175 sample of all observed landraces.

176 After collection, seeds were extracted from ripe fruits by hand and the pulp containing seeds was
177 washed thoroughly until all the flesh was removed. After extraction and cleaning, seeds were air
178 dried at room temperature (~20°C), packaged and transferred to Kew where they were stored at a
179 constant 60% relative humidity for 1-2 weeks (approximate humidity of collection region) in
180 preparation for germination trials. In addition to field collected enset seed, we also used non-
181 Ethiopian enset seeds legally sourced from the online horticultural distributor Rare Palm Seeds
182 (https://www.rarepalmseeds.com/) which are available in large quantities (hereafter RPS),
183 permitting us to screen a wider range of experimental conditions and act, to a certain extent, as a
184 control across all experiments.

185 **Morphological characterisation and viability**

186 For each accession, 20-50 seeds were weighed individually, using a balance accurate to 0.001g. Seed
187 size (diameter on an x, y, and z axis) was recorded for 10-20 seeds per accession using a digital
188 caliper accurate to 0.01mm. Due to the highly non-uniform shape of enset seeds, with no consistent
189 long or short axis, seed volume (estimated as the cube of the three measured axes) was used for
190 subsequent analyses. We used a Faxitron Ultrafocus x-ray (Faxitron Bioptics, LLC), to measure total
191 seed area (cross section), endosperm area and testa thickness (outer integument) in five seeds per
192 accession (Figure 2). Seeds were positioned with the proximal end facing upwards, and results
193 averaged by accession. We tested each dataset for homogeneity of variances and normality before
194 applying the appropriate unpaired t-test to evaluate differences in population means between
195 domesticated and wild seeds. All analyses were conducted in R software (R Core Team, 2017).

196 Tetrazolium tests were used to detect living tissue and viable seeds. Briefly, seeds were imbibed for
197 24 hours, chipped to expose the endosperm and placed in 1% buffered 2,3,5-triphenyl tetrazolium
198 chloride for two days in the dark at 30°C. Subsequently, seeds were carefully dissected, and the
199 embryo staining pattern recorded. Germination proportions were corrected for viability during
200 subsequent analysis.

201 **Germination trials**

202 We performed four germination experiments (Table 2), with temperature ranges selected based on
203 WorldClimV2 values for the region (Fick and Hijmans, 2017), previous reports from *Musa* (Stotzky et
204 al., 1962; Messele, 1994) and our knowledge of enset ecology in Ethiopia.

205 i) First, using RPS seed we screened a range of 15 constant and alternating temperature
206 regimes to guide our experimental design for subsequent germination tests (Exp 1).
207 Constant conditions comprised six regimes: 10°C, 15°C, 20°C, 25°C, 30°C and 40°C.
208 Alternating conditions included 10-20°C of diurnal variation to simulate larger
209 temperature shifts, comprising an additional nine regimes: 20/10°C, 25/10°C, 25/15°C,
210 30/10°C, 30/15°C, 30/20°C, 35/20°C, 40/20°C, 40/25°C.

211 ii) Based on results from the initial screening, seeds from seven wild accessions and seven
212 domesticated accessions (those with sufficient seeds available for fully replicated trials)
213 were exposed to a refined range of 11 temperature regimes (Exp 2).

214 iii) To evaluate the relative importance of an absolute shift in ambient temperature (e.g.
215 warming due to disturbance) and regular diurnal temperature shifts (i.e. our alternating
216 temperature regimes) we exposed a subset of domesticated seed to five constant
217 temperatures for three months, and then moved them to the 'optimum' alternating
218 temperature identified in earlier tests (Exp 3).

219 iv) To evaluate the extent and influence of dormancy, a subset of domesticated accessions
220 was stratified at 10°C for three months, and then transferred to five other temperature
221 regimes (Exp 4).

222 In all germination tests seeds were placed on moist sand (300g sand, 42ml de-ionised water) and
223 sealed in clear plastic boxes (120 x 180 mm). Boxes were then sealed in plastic bags to further
224 minimise moisture loss and contamination. Each box was considered a replicate and contained 60
225 seeds (except where specified), with a total of 235 replicates evaluated in this study. Seed boxes

226 were placed in the corresponding incubators with either constant temperature or 12 hour
227 alternating temperature cycles. All treatments included a 12-hour photoperiod, although Musaceae
228 seeds are unlikely to be photosensitive (data not shown; pers. comm. S. Kallow 2019). Germination
229 was defined by radicle emergence ≥2 mm, and tests were scored every 3-18 days depending on
230 activity for 120 days. We calculated the mean days to germination retrospectively as the number of
231 days required for 50% of the final germination count in each experimental replicate. We aggregated
232 these data by individual, excluding any replicate with zero germination.

233 To test for a significant difference between wild and domesticated germination behaviour (EXP2),
234 we fitted polynomial regression models for the logit transformed germination proportion against a)
235 the daily temperature change the replicate was exposed to and b) the mean temperature the
236 replicate was exposed to. For each variable we fitted two models, the first with all accessions and
237 the second with an additional variable grouping the data by type (wild v domesticated). We then
238 used ANOVA to test whether grouping produced a significantly better model fit. To evaluate the role
239 of stratification and dormancy (EXP3), we plotted the absolute temperature change between the
240 first temperature and the mean of the second temperature of the treatments, against germination
241 proportion and applied linear regression. The effect of a 10°C stratification treatment was compared
242 to non-stratification using an unpaired t-test (EXP4).

243 Climate data for Ethiopia were sourced from WorldClim v2 (Fick and Hijmans, 2017) at 2.5 arc
244 minute resolution (~10 km). In the first instance, we extracted climate values for our study
245 accessions, and tested for significant differences in Annual Mean Temperaure and Mean Diurnal
246 Range using unpaired t-tests. We then collated 472 enset localities from GBIF (GBIF.org, 2018),
247 publications (Borrell et al., 2019; Pironon et al., 2019) and personal observations, and subsampled
248 these to a 10 km grid consistent with the environmental data layers, retaining 94 unique
249 domesticated records and 19 unique wild records. We extracted climate data for these cells and
250 aggregated them for domesticated and wild enset separately.

P_{ST} — F_{ST} comparison

To test whether divergence of quantitative seed and germination traits are putatively as a result of natural selection or genetic drift we compared variance in trait values with neutral genetic differentiation. Genetic samples were collected for ongoing study (White et al. *in prep*) and are not identical individuals to those used for seed and germination traits due to difficulties extracting DNA from fully mature and senescing plants. Instead we selected a larger dataset of 21 domesticated individuals matched to the same named landraces surveyed for seed and germination traits, and 14 wild individuals for comparison. DNA was extracted, prepared as libraries and sequenced following the protocol of Ott et al., (2017). SNPs were called following the method of (Borrell et al., 2018) (see Data Accessibility for scripts). We calculated neutral F_{ST} between wild and domesticated enset populations based on 11,412 putatively neutral SNPs, using the R packages Adegenet and Genepop (Jombart, 2008; Rousset, 2012). Population differentiation for quantitative traits was estimated as P_{ST} (an approximation of Q_{ST} where the relative contribution of environmental variation is difficult to ascertain, see Silva and Silva, 2018) in the package Pstat (Silva and Silva, 2018). Due to limited knowledge, we generated estimates for a range of heritability values (0.2, 0.4 and 0.6) based on Visscher et al., (2008).

RESULTS

Observations, collection, processing and storage

We observed domesticated enset flowering in all enset cultivating zones, and across a wide range of environmental conditions (albeit at a very low frequency), but only a very small number were un-harvested and therefore able to mature and produce ripe fruits (Table S1). Location and altitude data for the 20 collected enset seed accessions is provided in Table 1. In our collected accessions, the number of harvested seeds per plant ranged from 19 to 3200 (mean 600). It was not possible to ascertain reproductive effort (through, for example, total seed production) due to the longevity of the infloresence and continued predation by frugivores. We note, however, that on several

occasions throughout our fieldwork in Ethiopia apparently ripe fruits were observed on domesticated enset plants that had developed infructescences, but that seeds were absent, undeveloped or very few in number (observed in landraces Nechwe, Kiticho and Badadet). We believe this is most likely attributed to lack of pollinators. During field surveys, we documented 1864 observations of 453 named landraces from across the enset growing region. In addition, we recorded 39 flowering individuals of 26 landraces. After ranking by frequency of observation we found no significant difference in rank order (W = 6251.5, P = 0.60).

Morphological characterisation and viability

The basic structure of enset seeds is similar to that of *Musa* (McGahan, 1961), though enset seeds are larger. At the proximal end is a micropyle forming a plug, behind which is a capitate embryo, the base of which is embedded within the endosperm (Figure 2). At the opposite end, in a separate chamber is the chalazal mass which forms a cavity. We observed that enset seeds appear highly variable in overall surface texture and the degree of striations.

Seed volume ranged from 1.26 - 6.29 cm^3 and seed weight from 0.20 g - 2.85 g in domesticated accessions, and 1.15 - 5.05 cm^3 and 0.78 g - 2.66 g respectively in wild accessions (Figure 3). Overall, wild enset accessions had significantly higher mean weight than domesticated enset (t = -2.52, df = 12.63, P = 0.04), but we found no significant difference in mean seed volume between domesticated and wild (t = 0.07, df = 19, p = 0.95). Similarly, we found non-significant differences for mean testa thickness (t = 0.29, df = 14, p = 0.78), mean total seed area (t = -1.57, df = 14, p = 0.14) and mean endosperm area (t= -1.85, df = 14, p = 0.08) (Figure 3). These patterns of (non)significance were consistent even if poorly germinating accessions are removed (*i.e.* Deri'ea, Suitiya landraces). Full morphological data are available in Appendix S1 and S2. Tetrazolium tests showed high variation in viability across accessions, with both wild and domesticated accessions ranging from 0-100% viability. Mean viability was 55% and 49.5% for wild and domesticated respectively with no significant difference (t = 0.12, df = 12.9, p = 0.84) (Table 1).

302 **Germination trials**

303 Mean time to germination was 36 days (sd = 15.7) for domesticated enset and 35 days (sd = 8.6) for
304 wild enset, with no significant difference detected ($t = 0.51$, $df = 11.89$, $p = 0.62$). In Exp 1.
305 alternating temperature regimes outperformed constant temperatures, with the exception of
306 constant 25°C (Figure 4). Based on these data we reduced our suite of temperature regimes in
307 subsequent experiments (Table 2.). ANOVA analysis of polynomial regression models for
308 germination behaviour in domesticated and wild enset were significantly different (i.e. grouping by
309 accession type resulted in significantly better model fit) for both the alternating temperature range
310 ($F_{136,132} = 4.32$, $p = 0.003$) and the mean experimental temperature ($F_{136,132} = 2.71$, $p = 0.033$)
311 (Figure 5A). Specifically, we found that domesticated accessions had an improved germination
312 response in cooler mean temperatures with higher alternating temperature amplitude. Comparison
313 of germination requirements to regional climatic conditions for wild and domesticated enset found
314 that domesticated enset is found in local climates that have significantly cooler Annual Mean
315 Temperatures (AMT) ($t = -5.52$, $df = 31$, $p = <0.001$), with significantly greater Mean Diurnal Range
316 (MDR) ($t = 3.42$, $df = 19.2$, $p = 0.003$), than wild enset (Figure 5B). Importantly, there was no
317 significant difference in the AMT ($t = -1.77$, $df = 12.56$, $p = 0.10$) or MDR ($t = -1.63$, $df = 13.96$, $p =$
318 0.13) of our collected accessions, therefore this is unlikely to be solely a maternal effect.

319 Analysis of Exp 3. found a significant positive relationship between germination proportion and the
320 absolute temperature change from a constant to alternating temperature regime ($F_{1,38} = 6.37$, $p =$
321 0.0159) (Figure 6A). Analysis of Exp 4. found that a three-month period of cold stratification at 10°C
322 prior to an experimental treatment also significantly improved germination compared to seeds
323 immediately exposed to the experimental treatment ($t = 5.22$, $df = 26.4$, $p = <0.001$) (Figure 6B). Full
324 germination data are available in Appendix S3 and S4.

325 **P_{ST} — F_{ST} comparison**

326 Estimated P_{ST} values varied substantially, with values for seed volume and testa thickness being
327 consistently close to zero, and seed weight, endosperm area and total seed area relatively high. Full
328 results across a range of assumed heritability values are reported in Table 3. Pairwise F_{ST} between
329 domesticated and wild enset populations was estimated at 0.188.

330 **Optimum enset germination conditions**

331 Based on a broad range of experimental conditions, we found an alternating temperature regime of
332 25/15°C, ideally with a period of moist cool stratification at 10°C for up to 3 months prior to be most
333 effective for domesticated enset. Most germination can be expected over 20-50 days, with a
334 germination proportion of ~30-50%. Considering that this germination success is still low, there may
335 be further opportunities to improve this protocol.

336 DISCUSSION

337 It has frequently been suggested that prolonged vegetative reproduction during domestication can
338 lead to the loss of sexual reproductive capacity (Eckert, 2002; Barrett, 2015; Denham et al., 2020;
339 Tesfamicael et al., 2020). In this study we show evidence that the indigenous Ethiopian vegetative
340 crop enset has retained viable sexual reproductive potential comparable to that of wild plants.
341 Currently, the duration over which enset has been domesticated, and the temporal advent of
342 vegetative reproduction is unclear (Borrell, Biswas, et al., 2019). However, if we consider the
343 extensive accumulation of indigenous knowledge associated with enset cultivation, particularly
344 clonal propagation (Garedew et al., 2017), and its origins in the Ethiopian Highlands, an important
345 and ancient center of crop domestication (Harlan, 1971), it is reasonable to conclude that enset has
346 a modest to long domestication history, and that vegetative propagation has been practiced for a
347 significant portion. This suggests that the potential for sexual reproduction has been maintained
348 despite prolonged vegetative propagation.

349 In our surveys, we found no evidence that certain landraces have lost the propensity to flower,
350 which appears consistent with farmer perceptions (pers. obs. J. Borrell). We also found no evidence

351 of differing seed viability rates between wild and domesticated enset. Though similar to other
352 authors (Karlsson et al., 2013), we did find high variability in seed viability across accessions. In a
353 comparison of wild and domesticated enset seed morphology, we found a significant difference only
354 for seed weight, with domesticated seeds generally being lighter. There was also a similar, but non-
355 significant trend for domesticated seeds to have a smaller endosperm area, whilst seed volume
356 showed no such pattern (Figure 3). Less dense seeds could be explained by reduced resource
357 provisioning, however this seems unlikely to be due to reduced maternal resource acquisition in an
358 agricultural context where enset receives substantial fertiliser (manure) input and cultural practices
359 aim to delay maturation to maximise energy accumulation (Borrell et al., 2020). An alternative
360 explanation is that highly fertile agricultural systems have relaxed selection on energy investment in
361 seeds, meaning that seeds with reduced resources may still be sufficiently fit for establishment,
362 though we are cautious against over interpreting these data.

363 Our germination trials indicate that not only has enset maintained the capacity for viable seed
364 production, but surprisingly, that the optimum germination requirements significantly differ
365 between domesticated and wild enset (Figure 5). Domesticated enset has an increased germination
366 response in cooler mean temperatures (~22°C) and with an increased amplitude of alternating
367 temperature (Figure 5A) compared to wild enset. Naturally, wild enset occupies consistently warm
368 moist tropical forest in Western Ethiopia, whereas the predominant region of contemporary enset
369 cultivation is a region with lower Annual Mean Temperature and higher Mean Diurnal Range (Figure
370 5B). This suggests, that wild and domesticated enset are potentially adapted to their respective
371 environments. Importantly, there was no significant difference between the Annual Mean
372 Temperature and Mean Diurnal Range of the wild and domesticated seed collection sites surveyed
373 here, suggesting that this observation is unlikely to be solely a maternal effect.

374 When we consider enset seed morphology and germination behaviour together, there are several
375 evolutionary explanations for these observations. First, a scenario where selection pressure has

376 been relaxed as a result of domestication, would culminate in domesticated enset reproductive
377 traits that have been subject to deleterious somatic mutations and genetic drift. In this situation we
378 may expect to see reduced seed viability, or at least consistently higher variance in domesticated
379 enset traits and $P_{ST} \approx F_{ST}$, which is not the case. If pervasive genetic drift is indeed in progress, then
380 potentially insufficient time or generations have passed for it to become apparent. Alternatively, in a
381 scenario where 'perfect' vegetative propagation has been maintained, seed morphology and
382 germination behaviour are expected to remain consistent between wild and domesticated enset, as
383 clonal lineages are essentially in stasis. Our data do not provide strong evidence for this, as we
384 detect significant differences in seed weight and germination behaviour. Instead, this could be
385 explained by a domestication bottleneck, whereby the progenitors of domesticated enset landraces
386 represent a subset of the total trait variation (i.e. domesticated enset arose from a population of
387 wild enset preferring cooler, more variable climes with lighter seeds). However this also seems
388 unlikely, given that i) in perennial crops we expect to observe a weak domestication bottleneck
389 (Gaut et al., 2015), ii) wild and domesticated appear adapted to their respective environmental
390 conditions and iii) based on climax vegetation maps of Ethiopia it is unlikely that wild populations
391 originated from further East in Ethiopia, in the cooler, more variable climates which enset cultivation
392 currently occupies (Friis et al., 2010).

393 Seeking a more parsimonious scenario, we suggest that despite virtually exclusive clonal propagation
394 in cultivation, it is possible that a small number of escaped or neglected domesticated plants are
395 continuing to reproduce sexually. Where this has occurred in a novel agricultural environment,
396 certain seed and germination traits have been subjected to directional selection, whilst others have
397 been maintained under balancing selection. This explanation is supported by both significantly
398 different germination behaviour concordant with local environment, evidence for local adaptation in
399 seed weight and endosperm area ($P_{ST} > F_{ST}$), and reduced variance in domestic versus wild indicating
400 putatively stabilising selection ($P_{ST} < F_{ST}$) in traits such as seed volume and testa thickness (or weak,
401 undetectable genetic drift). Previous work has shown that a comparatively low rate of sexual

402 reproduction would be sufficient to maintain this balancing selection (Rice and Chippindale, 2001;
403 Cutter, 2019). We conclude that this best describes the patterns we have observed, not only based
404 on the data report here, but also based on observation in other clonal crops that farmers actively
405 manage infrequent sexual reproduction. Examples include the practice of enoblement in Yams
406 (*Dioscorea*) (Cornet et al., 2010; Mengesha et al., 2013), or tolerance of volunteer manioc seedlings
407 in Cassava (*Manhiot*) (Rival and Mckey, 2008).

408 To further support this inference, we provide two qualitative observations of enset agronomic
409 practices that could contribute to the persistence of sexual reproduction. First, in Western regions
410 (e.g. Gesha and Bonga), small numbers of enset seedlings are collected from the wild and integrated
411 into the farm for the purpose of harvesting leaves (leaves are harvested from inedible wild plants,
412 where available, to avoid damaging and slowing the growth of edible domesticated plants). Similarly,
413 in a small number of cases, comparatively wealthy farmers occasionally allow a single domesticated
414 enset, often in a highly visible location near the front of their compound, to flower as a sign of
415 prosperity (pers. obs. J Borrell). Both these practices could increase the frequency of sexual
416 recombination, and the subsequent survival of seedlings, with associated impacts on the evolution
417 and domestication of enset.

418 More broadly, we note that enset germination requirements appear consistent with reports from
419 *Musa* (Stotzky et al., 1962, Kallow et al., in review). Specifically, in enset, alternating temperatures
420 elicit a stronger germination response than constant temperatures, though in *Musa* this is virtually
421 an absolute requirement. Using alternating temperatures as an environmental cue is hypothesised
422 to be a strategy for detecting disturbance and canopy gaps whereby solar radiation warms the seeds
423 in the day followed by a cooler ambient temperature at night. Enset is also reported to colonise
424 disturbed areas (Stotzky et al., 1962), and thus this trait appears to be conserved across the two
425 major branches of the Musaceae. Surprisingly, the magnitude of the transition from constant to
426 alternating temperature was also significantly associated with germination (Figure 6A). Kallow et al

427 (in review) hypothesise that in *Musa* seeds, alternating temperatures reduce the ratio of Abscisic
428 acid to Gibberellic acid (GA), reducing water potential and initiating elongation and cell growth. This
429 suggests that future approaches involving application of GA to seeds or the growing medium may
430 provide another mechanism for initiating germination. In addition, increased germination is also
431 observed where seeds were stratified at a constant temperature prior to germination. Climate data
432 indicates that the domesticated distribution of enset may reach a minimum of 8.2°C in the coolest
433 month, which coincides with higher rainfall. Whilst no seasonality has been reported in enset
434 flowering, this may be an additional, putatively conserved, mechanism for optimising germination
435 timing.

436 Despite the importance of understanding the germination biology of enset, this study was limited by
437 the fact that we were not able to determine total seed production of enset individuals, as
438 inflorescences ripen over a considerable time, and may be damaged by animals in the process,
439 though this would provide an important, comparative metric for sexual reproductive potential.
440 Similarly, we were also limited by the available sample size, in a rarely flowering crop, compounded
441 by significant variation associated with the accession. We anticipate that numerous factors such as
442 fruit maturity, length and type of storage, epigenetic and other factors may influence variability,
443 though we do not have sufficient power to resolve these in this study. To further evaluate
444 maintenance of enset sexual reproductive potential, we suggest that comparative analysis of the
445 floral structures of enset, to detect loss of function arising from genetic drift, may corroborate our
446 findings.

447 CONCLUSIONS

448 In the future, development of enset seed germination protocols, underpinned by diverse seed
449 collections, is a compelling strategy to safeguard the genetic diversity of a food security crop, with a
450 lower risk of provenance information loss than living plants in a germplasm collection (Thormann
451 and Dulloo, 2006). Moreover, we show that this is achievable, by demonstrating the maintenance of

sexual reproductive potential in enset, despite a significant period of near-exclusive vegetative propagation. Furthermore, we provide initial evidence that very low levels of sexual reproduction may be facilitating the adaptation of enset germination biology to domestication, and that enset cultural practices have unrecognised parallels with the agronomy of other clonal crops. An improved understanding of enset germination biology is a useful prerequisite for future crop development through sexual recombination of existing landraces, developing mapping populations and the breeding of novel genotypes, with significant reciprocal potential in bananas (*Musa*) a closely related and globally important group of clonally produced crops. In conclusion, we advocate for a broader effort in developing germination protocols for vegetatively propagated crops to provide an important alternative germplasm conservation strategy that is likely to disproportionately benefit tropical species and developing country agriculture in the global south.

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AUTHOR CONTRIBUTIONS

PW and SD conceived the project and acquired funding. JB, SK and RD designed the study. ST, JB, ES, GW and FW collected material. ST, JB, ES, SK and RD performed experiments. JB analysed data and wrote the first draft of the manuscript. All authors contributed to and approved the final version of the manuscript.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information section at the end of the article

Appendix S1 – Seed_morphology_(size).txt

Appendix S2 – Seed_morphology_(weight).txt

Appendix S3 – Germination_Exp_1-2.txt

Appendix S4 – Germination_Exp_3-4.txt

Genetic processing and SNP calling scripts are available here: https://github.com/o-william-white/Enset_tGBS.

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657 # TABLES

658 **Table 1.** Wild and domesticated enset accessions used for germination studies

Origin	Landrace name	Elevation (m a.s.l.)	Latitude (°N)	Longitude (°E)	Tetrazolium viability (%)	Annual Mean Temp. (°C)	Mean Diurnal Range (°C)
Domesticated	Addis Ababa	2430	9.03	38.76	84	16.02	13.25
Domesticated	Maze	2120	6.87	37.79	64	18.78	14.44
Domesticated	Hala	2120	6.87	37.79	66	18.78	14.44
Domesticated	Deri'ea	2700	7.93	37.90	0	15.37	12.97
Domesticated	Wanadiya	2125	6.86	37.79	48	18.78	14.44
Domesticated	Gefetano	2125	6.86	37.79	46	18.78	14.44
Domesticated	Gefetano	1930	6.83	37.75	75	20.24	14.72
Domesticated	Ganticho	1840	6.47	38.35	30	18.41	14.25
Domesticated	Suitiya	2120	6.87	37.79	0	18.78	14.44
Domesticated	Addis Ababa	2420	9.02	38.78	100	16.02	13.25
Domesticated	Midasho	2786	6.47	38.54	14	12.89	13.67
Domesticated	Kiticho	2787	6.48	38.54	50	12.89	13.67
Domesticated	Lemat	2053	8.45	38.03	67	17.97	13.49
Wild	W1	1880	7.17	36.22	0	18.78	14.35
Wild	W2	1850	7.16	36.20	38	18.47	14.30
Wild	W3	1936	7.16	36.20	54	18.47	14.30
Wild	W4	1936	7.16	36.20	68	18.47	14.30
Wild	W5	1927	7.16	36.20	78	18.47	14.30
Wild	W6	1936	7.19	36.20	52	18.11	14.14
Wild	W7	1930	7.29	36.14	100	17.97	13.99
Wild (non-Ethiopian)	'Rare Palm Seeds'	-	-	-	94	-	-

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667 **Table 2.** Overview of the four main germination experiments performed

Experiment	No. of conditions	Replicates	Temperature regimes
EXP1: Full range (RPS seeds) to test preferred temperature	6 constant, 9 alternating	15 replicates (900 seeds)	10°C, 15°C, 20°C, 25°C, 30°C, 40°C, 20/10°C, 25/10°C, 25/15°C, 30/10°C, 30/15°C, 30/20°C, 35/20°C, 40/20°C, 40/25°C
EXP2: Wild and domestic	5 constant, 6 alternating	77 replicates (3213) wild; 88 replicates (3844 seeds) domestic	10°C, 15°C, 20°C, 25°C, 30°C, 20/10°C, 25/10°C, 25/15°C, 30/10°C, 30/15°C, 30/20°C
EXP3: Domestic and RPS - testing shift from constant to alternating temperature	5 constant, moved to 1 alternating	40 replicates (1719 seeds)	10°C, 15°C, 20°C, 25°C, 30°C moved to 25/10°C
EXP4: Domestic and RPS - subset tested after cold stratification at 10°C	1 constant, 4 alternating	15 replicated (850 seeds)	10°C moved to 25°C, 25/10°C, 25/15°C, 30/20°C, 30/15°C

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680 **Table 3.** Phenotypic trait differentiation and trait variance in domesticated and wild enset.

Morphological trait	P_{ST} heritability estimates			Trait variance	
	0.2	0.4	0.6	Domesticated	Wild
Seed volume (mm^3)	0.00	0.01	0.01	578,127	324,302
Total seed area (mm^2)	0.20	0.33	0.42	1,678	679
Endosperm area (mm^2)	0.25	0.41	0.51	162	210
Seed weight (g)	0.36	0.53	0.62	0.13	0.22
Testa thickness (mm)	0.01	0.02	0.02	0.45	0.18
Mean F_{ST}	0.19	0.19	0.19	-	-

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FIGURES

Figure 1. Enset cultivation in Ethiopia. A) Mature enset flowering in a neglected field near Checha. B) A farmer removing the meristematic tissue of a two-year old enset, as part of processing for vegetative propagation. C) Numerous adventitious buds sprouting from prepared corm, near Bonga.

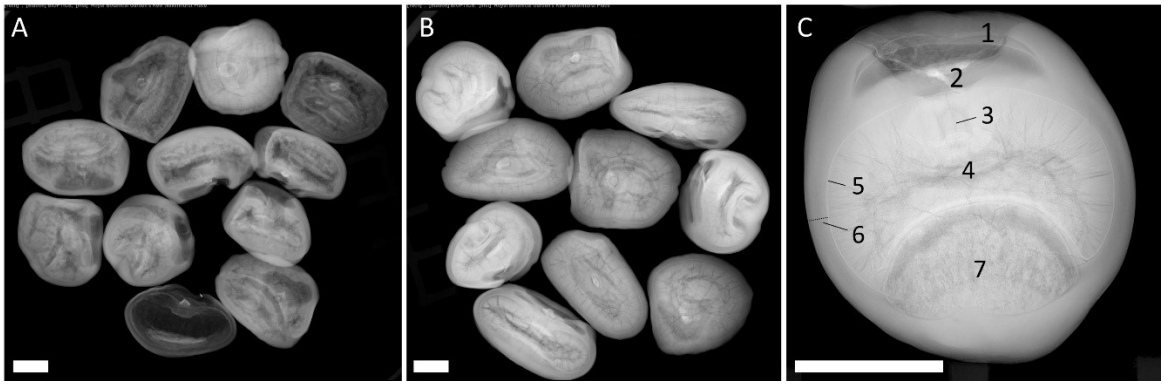

Figure 2. X-ray images of *Ensete ventricosum* seeds. Scale bar denotes 5mm. A) Domesticated accession 'Wanadiya'. A poorly filled seed can be observed in the lower center of the image. B) Wild accession six 'W6'. C) Major enset seed morphological traits referred to in this study: 1. Hilum. 2. Micropylar plug. 3. Embryo. 4. Endosperm surrounding embryo. 5. Inner integument. 6. Outer integument (testa) 7. Chalazal mass.

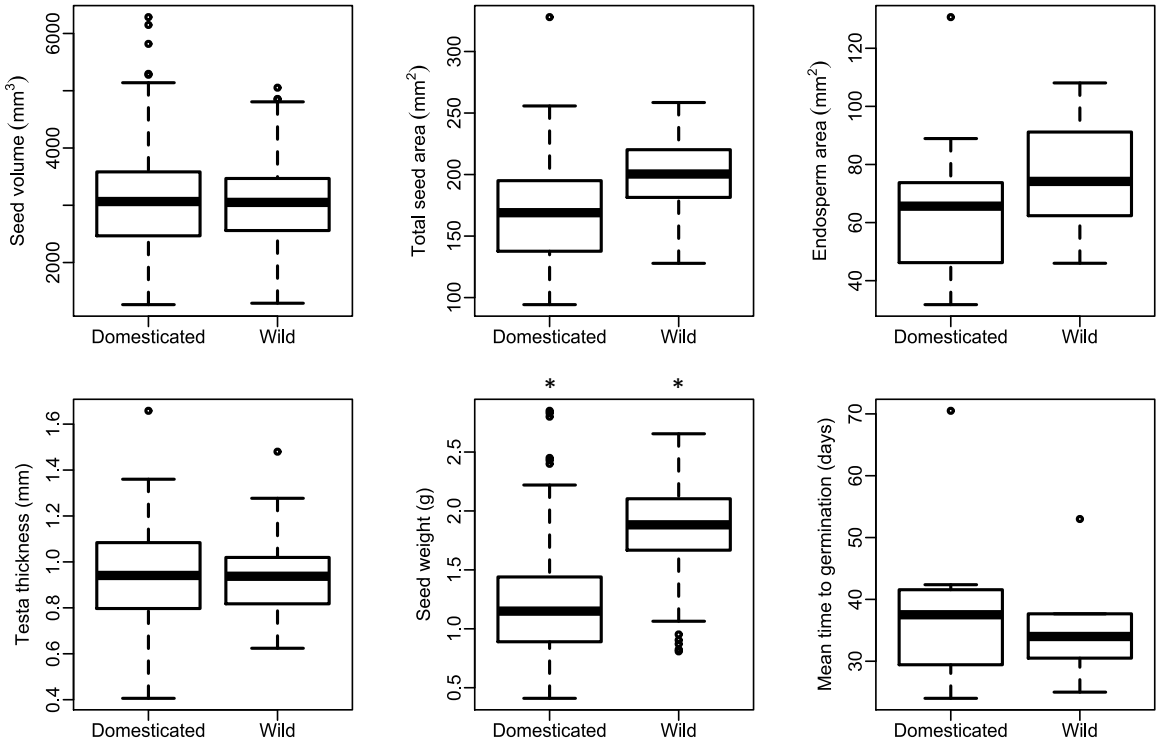

Figure3. Boxplots comparing seed traits in wild and domesticated enset. Asterisks denote traits in which we observed a significant difference.

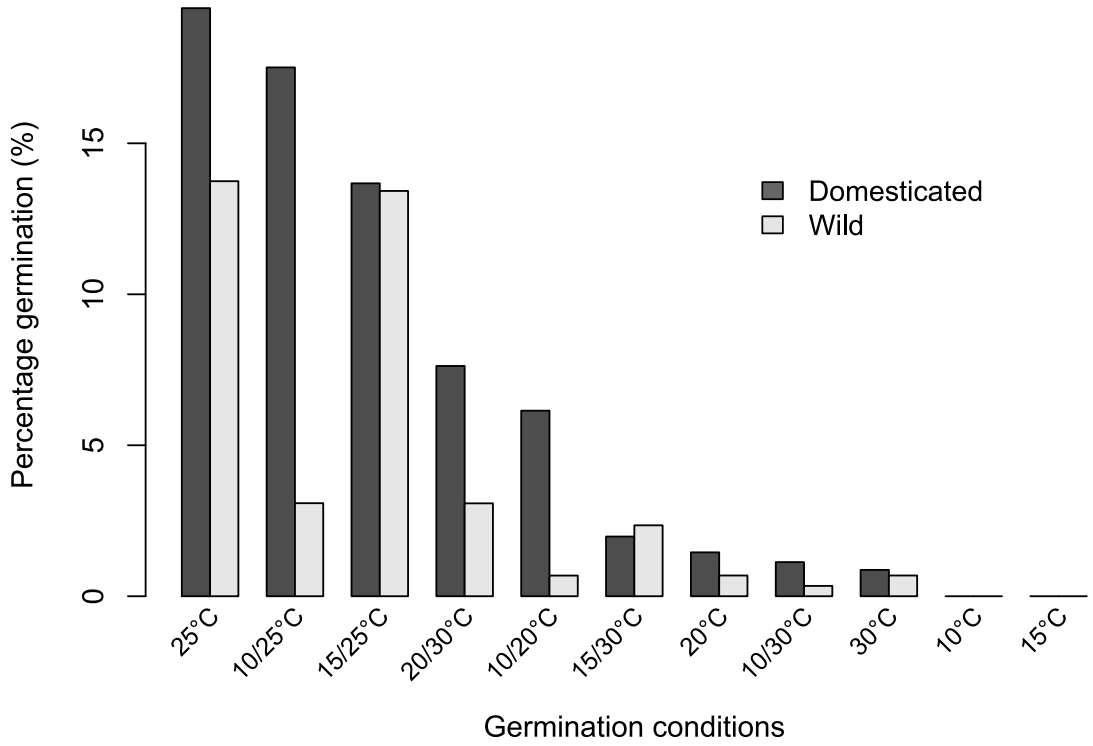

Figure 4. Percentage germination across a range of environmental conditions for wild and domesticated enset (Exp 2), after 120 days. Data are corrected for variation in seed viability.

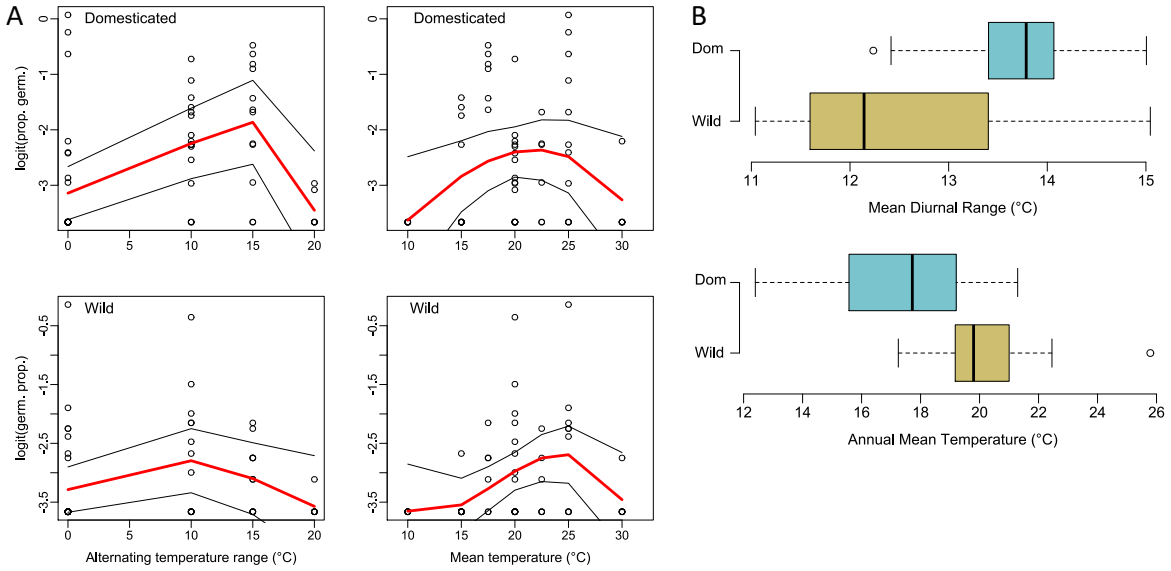

Figure 5. Germination behaviour and regional climate variables for wild and domesticated enset in Ethiopia. A) Polynomial regression of logit transformed germination proportion in wild and domestic accessions under varying mean temperature and alternating temperature regimes. Each point denotes a germination experimental replicate comprising 60 seeds of a single accession, corrected to account for variation in seed viability. B) Boxplots of regional climate for domestic and wild enset records in Ethiopia.

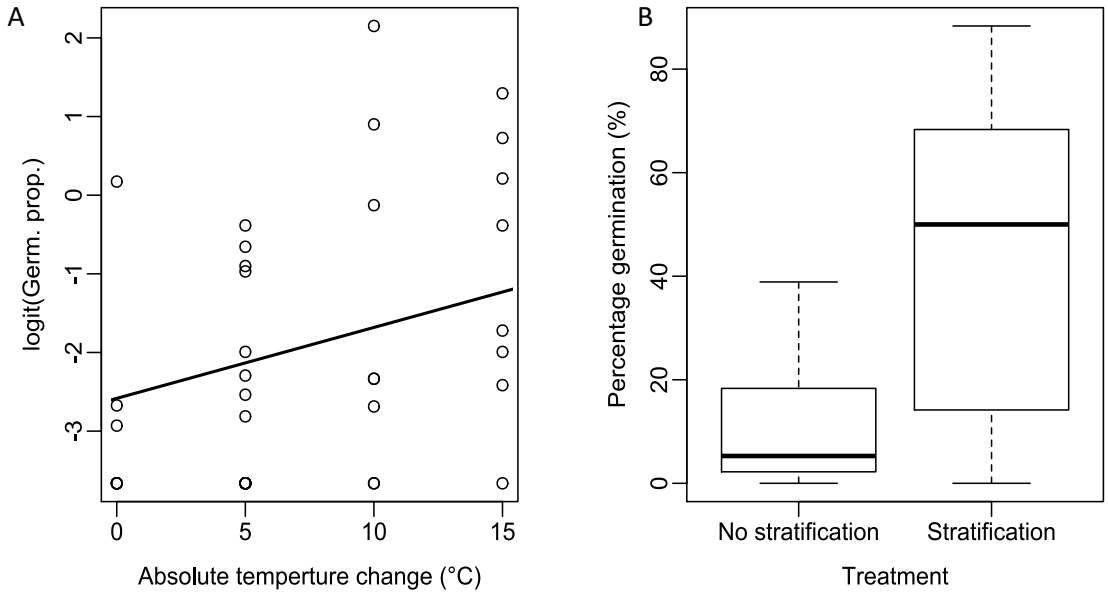

Figure 6. Analysis of the influence of temperature shifts and dormancy on domesticated enset germination (EXPs 3 and 4). A) The influence of absolute temperature change from constant to alternating temperature, showing a positive relationship with increased germination response for larger temperature shifts. B) Comparison of germination response for stratified seeds (3 months at 10°C), versus no stratification (immediate exposure to experimental conditions).