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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

Solomon Tamrat^{1*}, James S. Borrell^{2*†}, Eleni Shiferaw³, Simon Kallow^{4,5}, Rachael M. Davies⁴, John B. Dickie⁴, Gizachew W. Nuraga¹, Oliver White², Feleke Woldeyes³, Sebsebe Demissew¹, Paul Wilkin²

*Denotes joint first authors

†Corresponding author

¹Department of Plant Biology and Biodiversity Management, Addis Ababa University, Addis Ababa, P.O. Box 3293,5 Ethiopia.

²Royal Botanic Gardens, Kew, Richmond, Surrey, TW9 3AE, UK.

³Ethiopian Biodiversity Institute, Addis Ababa, P.O.Box: 30726, Ethiopia.

⁴Royal Botanic Gardens Kew, Millennium Seed Bank, Wakehurst, Ardingly, Sussex, RH17 6TN, UK.

⁵Katholieke Universiteit Leuven, Department of Biosystems, Willem de Croylaan 42, 3001, Leuven, Belgium.

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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)

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

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

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

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

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

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

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

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

MATERIALS AND METHODS

Observations, collection, processing and storage

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

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

Morphological characterisation and viability

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

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

Germination trials

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

- i) First, using RPS seed we screened a range of 15 constant and alternating temperature regimes to guide our experimental design for subsequent germination tests (Exp 1).
Constant conditions comprised six regimes: 10°C, 15°C, 20°C, 25°C, 30°C and 40°C.
Alternating conditions included 10-20°C of diurnal variation to simulate larger temperature shifts, comprising an additional nine regimes: 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.
- ii) Based on results from the initial screening, seeds from seven wild accessions and seven domesticated accessions (those with sufficient seeds available for fully replicated trials) were exposed to a refined range of 11 temperature regimes (Exp 2).
- iii) To evaluate the relative importance of an absolute shift in ambient temperature (e.g. warming due to disturbance) and regular diurnal temperature shifts (i.e. our alternating temperature regimes) we exposed a subset of domesticated seed to five constant temperatures for three months, and then moved them to the ‘optimum’ alternating temperature identified in earlier tests (Exp 3).
- iv) To evaluate the extent and influence of dormancy, a subset of domesticated accessions was stratified at 10°C for three months, and then transferred to five other temperature regimes (Exp 4).

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

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

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

Climate data for Ethiopia were sourced from WorldClim v2 (Fick and Hijmans, 2017) at 2.5 arc minute resolution (~10 km). In the first instance, we extracted climate values for our study accessions, and tested for significant differences in Annual Mean Temperature and Mean Diurnal Range using unpaired t-tests. We then collated 472 onset localities from GBIF (GBIF.org, 2018), publications (Borrell et al., 2019; Pironon et al., 2019) and personal observations, and subsampled these to a 10 km grid consistent with the environmental data layers, retaining 94 unique domesticated records and 19 unique wild records. We extracted climate data for these cells and aggregated them for domesticated and wild onset 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 inflorescence 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 Nechwé, 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 cm³ and seed weight from 0.20 g - 2.85 g in domesticated accessions, and 1.15 - 5.05 cm³ 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).

Germination trials

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

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

P_{ST} — F_{ST} comparison

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

Optimum enset germination conditions

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

CONCLUSIONS

In the future, development of enset seed germination protocols, underpinned by diverse seed collections, is a compelling strategy to safeguard the genetic diversity of a food security crop, with a lower risk of provenance information loss than living plants in a germplasm collection (Thormann 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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TABLES

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	-	-

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

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	-	-

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 '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.

768

769 **Figure 6.** Analysis of the influence of temperature shifts and dormancy on domesticated enset
770 germination (EXPs 3 and 4). A) The influence of absolute temperature change from constant to
771 alternating temperature, showing a positive relationship with increased germination response for
772 larger temperature shifts. B) Comparison of germination response for stratified seeds (3 months at
773 10°C), versus no stratification (immediate exposure to experimental conditions).