Genetic variation in *Loudetia simplex* supports the presence of ancient grasslands in Madagascar

George P. Tiley[1,†], Andrew A. Crowl[2], Tchana O. M. Almary[3], W. R. Quentin Luke[4], Cédrique L. Solofondranohatra[5], Guillaume Besnard[6], Caroline E.R. Lehmann[7,8], Anne D. Yoder[2], Maria S. Vorontsova[1]

[1]Royal Botanic Gardens Kew, Richmond, UK

[2]Department of Biology, Duke University, Durham, North Carolina, 27708 USA

[3]Kew Madagascar Conservation Centre, Antananarivo 101, Madagascar

[4]East African Herbarium, National Museums of Kenya, P.O. Box 45166, Nairobi, 00100, Kenya

[5]Madagascar Biodiversity Center, Parc Botanique et Zoologique de Tsimbazaza, Antananarivo 101, Madagascar

[6]CNRS-UPS-IRD, UMR5174, Laboratoire Évolution & Diversité Biologique, Université Paul Sabatier, Toulouse, France

[7]School of GeoSciences, University of Edinburgh, Edinburgh, UK

[8]Royal Botanic Garden Edinburgh, Edinburgh, UK

[†]Author for Correspondence: g.tiley@kew.org

Summary

1) Research Aims — The extent of Madagascar's grasslands prior to human colonization is unresolved. We used population genetic analyses of a broadly dominant C_4 fire-adapted grass, *Loudetia simplex*, as a proxy for estimating grassland change through time. We carefully examined the utility of target-enrichment data for population genetics to make recommendations for conservation genetics. We explored the potential of estimating individual ploidy levels from target-enrichment data and how assumptions about ploidy could affect analyses.
2) Methods — We developed a novel bioinformatic pipeline to estimate ploidy and genotypes from target-enrichment data. We estimated standard population genetic summary statistics in addition to species trees and population structure. Extended Bayesian skyline plots provided estimates of population size through time for empirical and simulated data.
3) Key Result — All Malagasy *Loudetia simplex* individuals sampled in this study formed a clade and possibly indicated an ancestral Central Highland distribution of 800m in altitude and above. Demographic models suggested grassland expansions occurred prior to the Last Interglacial Period and supported extensive grasslands prior to human colonization. Though there are limitations to target-enrichment data for population genetic studies, we find that analyses of population structure are reliable.
4) Key Point —Genetic variation in *Loudetia simplex* supports widespread grasslands in Madagascar prior to the more recent periods of notable paleoclimatic change. However, the methods explored here could not differentiate between paleoclimatic change near the Last Glacial Maximum and anthropogenic effects. Target-enrichment data can be a valuable tool for analyses of population structure in the absence a reference genome.

Keywords

Effective Population Size, Target-Enrichment, HybSeq, Polyploidy, Anthropogenic Climate Change, Conservation Genetics

Societal Impact Statement

Recognizing *Loudetia* dominated grasslands were widespread prior to human colonization highlights that open ecosystems were and continue to be an important component of Madagascar's biodiversity. Urgently required are biodiversity inventories and integrative taxonomic treatments of grassland flora and fauna to asses risks to understudied ecosystems historically regarded as wastelands. Substantial financial and logistical barriers exist to implementing conservation studies using contemporary genomic tools. We ameliorated some of the challenges for population genetic analyses of non-model polyploids lacking reference genomes by developing computational resources to leverage a cost-effective data generation strategy that requires no prior genetic knowledge of the target species.

Résumé

1) Les objectifs de la recherche — L'étendue des écosystèmes ouverts de Madagascar avant la colonisation humaine reste à éclaircir. Nous avons utilisé une analyse de la population génétique d'une graminée C_4 adaptée au feu, largement dominante, Loudetia simplex, comme référence pour estimer les changements au niveau de ces biomes au fil du temps. Nous avons examiné attentivement l'utilité des données d'enrichissement ciblé pour la génétique de population afin de formuler des recommandations pour la conservation génétique. Nous avons exploré le potentiel de l'estimation du niveau des ploidies individuelles à partir des données d'enrichissement ciblé et comment les hypothèses à propos de ces ploidies pourraient affecter les analyses.
2) Les méthodes — Nous avons développé un nouveau canal bioinformatique pour estimer les ploidies et les génotypes à partir des données d'enrichissement ciblé. Nous avons estimé les statistiques standard de la population génétique, en plus des arbres des espèces et de la structure de la population. L'utilisation des tracés étendus du ciel bayésien a fourni une estimation de la taille de la population au fil du temps pour des données empiriques et simulées.
3) Résultat clé — Tous les individus Malagasy de *Loudetia simplex* échantillonnés dans cette étude ont formé un clade, indiquant une éventuelle ancienne distribution dans les hauts plateaux. Les modèles démographiques suggèrent une expansion des prairies bien avant la dernière période interglaciaire et soutiennent l'existence d'une vaste distribution avant la colonisation humaine. Bien qu'il y ait des limites à l'enrichissement des données cibles pour l'étude de la génétique des populations, nous constatons que l'analyse des structures des populations est fiable.
4) Les points clés — La variation génétique de *Loudetia simplex* soutient l'existence de vastes prairies à Madagascar avant les périodes plus récentes de changements paléoclimatiques notables. Cependant, les méthodes explorées ici n'ont pas permis de faire la différence entre les changements paléoclimatiques près du dernier maximum glaciaire et les effets anthropogènes. Les données d'enrichissement ciblé peuvent être un outil précieux pour les analyses de la structure des populations en l'absence d'un génome de référence.

Déclaration d’impact societal

Reconnaître que les prairies dominées par Loudetia étaient répandues avant la colonisation humaine souligne que les écosystèmes ouverts étaient et continuent d'être un composant important de la biodiversité de Madagascar. Il est urgent de réaliser des inventaires de la biodiversité et une taxonomie intégrée pour le traitement de la flore et de la faune des écosystèmes ouverts afin d'évaluer les risques pour les écosystèmes sous-étudiés considérés historiquement comme des terres en friches. Des barrières financières et logistiques existent pour mettre en œuvre l'étude de la conservation en utilisant les outils génomiques contemporains. Nous avons amélioré certains des défis liés aux analyses génétiques de populations de polyploïdes non modèles, sans génomes de référence, en développant des ressources informatiques pour exploiter une stratégie pouvant générer des données rentables ne nécessitant aucune connaissance génétique préalable de l'espèce cible.

Famintinana

1) Ny tanjon'ny fikarohana — Mbola tsy fantatra mazava tsara ny fivelaran'ny hivoka teto Madagasikara talohan'ny fahatongava'ny olombelona. Mba ahafantarana ny fihovana nitranga nandritra ny fotoana naharitra teo amin'ireo hivoka ireo dia nanao famakafakahana ara-genetika amin'ny ahitra C_4 miompana amin'ny afo iray antsoina Loudetia simplex ara-tsiantifika na Berambo na Hara amin'ny teny malagasy izahay. Nandinika tsara ny maha-zava-dehibe ny fampitomboana ny antotan-kevitra mba ahafahana manolo-kevitra momba ny fiarovana ny fototarazo genetika. Nandinika ny mety mampiavaka ny fanombanana an'ny ploidy tsirairay amin'ny fampitomboana antotan-kevitra sy ny mety ho fiantraikan'ny fiheverana momba ireo ploidy ireo amin'ny fikarohana.
2) Fomba Fiasa — Namorona fantsona bioinformatika vaovao mba ahafahana manombana ny ploidy sy ny « genotypes » avy amin'ny antotan-kevitra nokendrena izahay. Notombanana ny antontan'isa famintinana ny fototarazo ara-genetikan'ireo vondron'ahitra ireo, miampy ny karazana hazo sy ny firafitry ny vondrona na koa hoe mponina. Nanome tombantombana ny haben'ny mponina amin'ny alàlan'ny fotoana ny antontan-kevitra voavinavina azo tamin'ny fikarohana. Fikarohana izay azo tamin'ny alalan'ny « Bayesina Skuline Plots ».
3) Vokam-pikarohana fototra — Ny vondrona *Loudetia simplex* eto Madagasikara izay niasana dia namorona « clade » na fikambanana iray, izay manondro ny mety maha ela netezana sy tranainy an'io ahitra io eny amin'ny faritra avo. Ny modely demografika dia manoro hevitra amin'ny naha be velarana ny hivoka izay efa ela talohan'ny vanim-potoana « interglacial » farany ary manohana ny fivelarana midadasika an'ireo kijana ireo alohan'ny fonenan'ny olombelona. Na dia misy fetrany aza ny fampitomboana ny antotan-kevitra kendrena amin'ny fandalinana ny fototarazo genetika momban'ny mponina, dia hita fa azo itokisana ny fikarohana natao momban'ny firafitry ny mponina.
4) Hevi-dehibe — Ny fahasamihafana ara-genetika ao amin'ny *Loudetia simplex* dia manohana ny fisian'ny hivoka na kijana midadasika eto Madagasikara talohan'ny vanim-potoanan'ny fiovana paleoclimatika nisongadina. Na izany aza, ny fombam-pikarohana nampiasana teto dia tsy nahavita nanavaka ny fiovan'ny paleoclimatika akaikin'ny vanim-potoana lehibe nangatsiaka farany sy ny vokatry ny fitrandrahana nataon'ny olombelona. Mety ho fitaovana manan-danja amin'ny famakafakana ny firafitry ny mponina ny antotan-kevitra nampitombona na dia tsy misy fitaovana genomika iangaina aza.

Fanambarana fiantraika ara-tsosialy

Ny fanekena fa niely patrana ny hivoka itoeran’ny *Loudetia* talohan'ny fanjanahan'ny olombelona dia manamarika fa ireo hivoka ireo dia singa manan-danja amin'ny zavamananaina eto Madagasikara. Ilaina maika ny fahafantarana ara biolojika sy taxononomique ny zavamaniry sy ny biby amin'ny hivoka mba hanombanana ny loza mety hitranga amin’ny hivoka izay tsy ananana fahalalana maro sady heverina ho tany maina. Misy sakana ara-bola sy ara-pitaovana amin'ny fampiharana ny fandalinana momba ny fiarovana izay nampiasana fitaovana génomika ankehitriny. Nohatsarainay ny sasany amin'ireo fanamby mifandraika amin'ny famakafakana ara-genetika ny mponina manana ploidy maro tsy modely, izay tsy misy fitaovana genomika iaingana , amin'ny alàlan'ny fampivoarana loharanon-kevitra kajy mba hitrandrahana paikady izay mety hiteraka angon-drakitra mahomby tsy mitaky fahalalana mahakasika ny fototarazo ara-genetika ny zava-maniry izay tiana karohina.

Introduction

The degree to which anthropogenic activities have transformed the ancestral vegetative composition of Madagascar's ecosystems is heavily debated. While the narrative that most of central Madagascar was once covered in closed-canopy forests has been rejected (Bond et al., 2008; Vorontsova et al., 2016; Hackel et al., 2018; Solofondranohatra et al., 2020; Bond et al., 2022; Lehmann et al., 2022), the degree to which a former mosaic of grassland, shrubland, and forests has become more dominated by grasses is contentious (reviewed in Joseph et al., 2021). Understanding population dynamics of individual ecologically important species and plant communities will have profound effects on conservation policy decisions and the economic productivity of grasslands for local communities. However, it is difficult to determine where grasslands, which we consider to be an ecosystem where the ground layer is a majority of tussock-forming grass with little tree cover that experiences a dry season with regular fire regimes (e.g. Dixon et al., 2014), were in the past and how extensive they were. Stratigraphic sediment records from Central Madagascar indicate an increased relative abundance of grasses and fire through the Holocene (Burney, 1987; Gasse & Van Campo, 1998; Virah-Swamy et al., 2010), especially near the expected age of human colonization [c. two thousand years ago (ka); Dewar & Wright, 1993; Dewar et al., 2013; Pierron et al., 2017]. Megafaunal isotope records also support Holocene expansions of C_4 grasslands but show a vegetative mosaic of C_4 grasses and C_3 vegetation was at least present (Crowley et al., 2021) near the last glacial maximum (LGM; c. 26.5-19 ka). Reconstructing spatio-temporal vegetative dynamics beyond the LGM begin to approach limitations of paleoecological data, but molecular evolutionary approaches can fill the gaps by providing probabilistic inferences through time. Population genetic approaches for reconstruct demographic histories has been successfully applied to lemurs to reconstruct patterns of past forest connectivity (Quéméré et al., 2012; Yoder et al., 2016; Salmona et al., 2017; Teixeira et al., 2021; Tiley et al., 2022) and may similarly provide insights into the Late Pleistocene dynamics of grasses between the LGM and Last Interglacial period (LIG; c. 132-112 ka) or older. This strategy has been used to reconstruct vegetative dynamics of temperate Japanese grasslands from forb species (Yamaura et al., 2019).

An array of tools now exists to estimate the effective population size (N_e) of species through time. As generating genomic data for non-model groups has become more accessible, population genomic methods that utilize precisely estimated site frequency spectra (SFS; Nielsen et al., 2000; Gutenkunst et al., 2009; Excoffier et al., 2013; Liu & Fu, 2020; Blischack et

al., 2022) have become a valuable tool for reconstructing the demographic history of natural populations. There are still barriers to generating population genomic data though, either due to the lack of suitable tissue, an appropriate reference genome, or costs. Target-enrichment libraries have been transformative for the analysis of plant phylogenetic relationships (e.g. Breinholt et al., 2021; Baker et al., 2022), but the population genetic utility of such data is not well-explored. A survey of taxa with low sample sizes suggested population genetic parameters estimated from target-enrichment data could be unreliable, in addition to violating assumptions of the neutral coalescent model that underpins most tests of selection and demographic change (Slimp et al., 2021). However, the relatively long loci recovered by target-enrichment (or HybSeq) methods could be appropriate for a demographic modeling approach, the extended Bayesian skyline plot (EBSP; Heled & Drummond, 2008), which has been successfully applied to a range of conservation genetic questions despite some model violations. We are interested in exploring the potential of target-enrichment data for population or conservation genetic insights from plant groups that may be lacking genomic resources.

Here, we examined the population genetics of the grass species *Loudetia simplex* (Tristachyideae: Panicoideae: Poaceae). It is largely dominant in Madagascar's Central Highlands (Koechlin, 1993; Hagl et al., 2021; Fig. 1a) and is notable for its fire- and grazing-adapted functional traits (Solofondranohatra et al., 2018). Population genetic analyses of microsatellites suggest Malagasy *L. simplex* has been isolated from mainland African populations with no detectable level of gene flow, and that there is additional population structure between the northern and southern extents of the species range across the Central Highlands of Madagascar (Hagl et al., 2021). Here, we use a subset of individuals analyzed by Hagl et al. (2021) along with a new sample from the center of the *L. simplex* distribution to explore the population genetic utility of target-enrichment data and its implications for the natural history of Madagascar's grasslands. Because Malagasy *L. simplex* are putative polyploids (tetraploids and hexaploids; Hagl et al., 2021), we developed novel bioinformatic tools for the processing and analysis of polyploid data, integrated into the PATÉ allele phasing pipeline (Tiley et al., 2021).

Materials and Methods

Sampling and Sequencing

Target-enrichment data was generated for 24 individuals of *Loudetia simplex* and one outgroup, *Tristachya nodiglumus* (Supplementary Table S1). Twenty individuals were selected to best

222 represent Madagascar from available collections as a single unstructured population and avoid
223 complications of population substructure and low sample sizes in downstream analyses (Fig.
224 1b). Genomic DNA samples were used from Hagl et al. (2021) while new extracts used a
225 modified CTAB protocol (Doyle & Doyle, 1987) from silica-dried leaf tissue. Genomic DNA was
226 then sent to Rapid Genomics (Gainesville, FL, USA) where sample enrichment and sequencing
227 was performed with a bait kit developed for angiosperms following Breinholt et al. (2021). All
228 sample metadata is provided in Supplementary Table S1.
229
230 *Sequence Assembly and Processing*
231 Initial contigs were assembled with HybPiper (Johnson et al., 2016) with default settings. We
232 then used HybPiper's supercontigs as reference sequences for PATÉ (Tiley et al., 2021; Crowl
233 et al., 2022) to genotype individuals, filter low-quality variants with GATK v4.2.0 (McKenna et
234 al., 2010; DePristo et al., 2011), and phase haplotype sequences using HPOP-G (Xie et al.,
235 2016). Ploidy information for individuals (Supplementary Table S1) was based on previous
236 microsatellite allele count data (Hagl et al., 2021); however, we also implemented a statistical
237 test to infer ploidy level in PATÉ based on mixtures of distributions from allele balance data,
238 similar to other approaches that use the number of reads supporting an alternate allele over
239 total reads to infer ploidy (Weiß et al., 2018). We extended the mixture models of Tiley et al.
240 (2018) to determine the ploidy of a sample with the model weights as a selection criterion
241 (Supplementary Methods: *Ploidy Test*). PATÉ control files with all filtering options for GATK are
242 provided in the Supplementary Data (https://doi.org/10.5061/dryad.905qfttqx). Because target-
243 enrichment probes may represent multiple exons from the same gene with overlapping flanking
244 regions, we were concerned that some assembled loci might overlap and violate some
245 independence assumptions in downstream analyses. Therefore, we performed an all-by-all
246 blastn search (Camacho et al., 2009) with an e-value of 1×10^{-5} among the supercontig
247 assemblies followed by single-linkage clustering (https://github.com/gtiley/HybSeq-Cleaning).
248 Only the longest locus per cluster was retained for downstream analyses.
249
250 To investigate nucleotide variation and the potential population genetic value of non-coding
251 versus coding regions of target-enrichment loci, we identified the following regions:
252 1) *Left Flank* – Non-coding sequence upstream of the 5' start codon or splice site.
253 2) *Core* – The coding region targeted by probes for sample enrichment.
254 3) *Right Flank* – Non-coding sequence downstream of the 3' stop codon or splice site.

Identification of individual regions was automated (https://github.com/gtiley/Locus-Splitter) based on the tblasx program from BLAST v2.10.0 (Camacho et al., 2009) to find in-frame alignments between our assembled sequences and the original transcriptome loci used for probe development. Individual regions were aligned with MAFFT v7.471 (Katoh & Standley, 2013). The aligned regions were then concatenated together to make whole-locus alignments.

Because we are interested in investigating the analytic impact of ploidy in sequence data, we created three datasets from the phased ploidy-aware data:
1) *One Haplotype (OH)* – Only one phased haplotype sequence was randomly selected from each individual for each locus.
2) *Ambiguous Genotype (AG)* – The genotypic information for an individual is represented as a single sequence with IUPAC ambiguity codes for biallelic sites. Analyses that treat IUPAC codes as missing data were randomly resolved into two sequences.
3) *Phased Haplotypes (PH)* – All phased haplotype sequences for an individual are used.

We expect the OH data to be representative of majority consensus sequences (e.g. HybPiper supercontigs) since our target-enrichment loci are anchored in single exons and we anticipate that any risk of assembling chimeric sequences from such data without paralog warnings is low. Any site genotyped as polyallelic, having more than one alternate allele, was assumed to be an error and treated as missing sequence across all data sets. All expected copies of a locus are represented even if they are invariant. For example, some individuals in the PH data may have six identical sequences at a locus if they are hexaploids and every site is homozygous. While the ploidy at an individual locus may vary due to differential gene loss among chromosomes, this is a simplifying assumption to preserve expected allele frequency information.

Phylogeny Estimation

A species tree was estimated for all *L. simplex* individuals using whole-locus sequences for the OH, AG, and PH datasets with BPP v4.6.2 (Flouri et al., 2020). We chose a full-likelihood multispecies coalescent (MSC; Rannala & Yang, 2003) model since it is robust to low-information gene tree estimates (Xu & Yang, 2016). Markov chain Monte Carlo (MCMC) analyses collected 10,000 posterior samples, saving every 10 samples after a 10,000 generation burnin. Model and prior specifications are provided in the Supplementary Material (Supplementary Methods: *BPP Prior Specification*). The starting tree for our Bayesian species tree analyses was based on a concatenated maximum likelihood (ML) estimate from the OH

data with IQTREE v2.1.2 (Minh et al., 2020). We used unpartitioned analyses with ModelFinderPlus (Kalyaanamoorthy et al., 2017) for the OH, AG, and PH data to estimate ML evolutionary distances between individuals, with random concatenation of haplotypes within individuals.

Population Structure

Potential population structure within our sample of *L. simplex* from Madagascar was explored with STRUCTURE v2.3.4 (Pritchard et al., 2000). For each dataset, we sampled only one biallelic SNP per locus, if present, and required a minimum minor allele count of three with no more than 25% missing data. To explore uncertainty due to sampling error of sites in a target-enrichment dataset that is relatively small compared to contemporary population genomic data, we generated 10 datasets with random SNP sampling. The number of clusters that best satisfy Hardy-Weinberg assumptions was determined with the ΔK method (Evanno et al., 2005). Principal component analyses (PCAs) were conducted on the OH, AG, and PH datasets with adegenet v2.1.3 (Jombart & Ahmed, 2011) by compressing allele counts to the same dimension as the lowest ploidy level present. For example, a hexaploid with three reference and three alternate alleles would be scored as a 2 on a scale from 0 to 4, to be comparable to tetraploids in the dataset. Additional details on allelic imbalances would be lost as, for example, hexaploids with either one or two alternate alleles would be represented as a 1 on the tetraploid scale. Scripts for generating PCA or other clustering-ready matrices from fasta files are available on GPT's GitHub (https://github.com/gtiley/fasta-conversion). Additional details about compression of PCA data matrices can be found in the Supplementary Material (Supplementary Methods: *PCA Input Data*). The presence of isolation-by-distance as an explanation for observed patterns of genetic structure and ordination was tested with the patristic distances from the ML analyses or the uncorrected pairwise distances (p-distances) regressed against the natural logarithm of the great circle distance between individuals. A Mantel test was performed for isolation-by-distance analyses in the ape v5.6.2 R package (Paradis & Schliep, 2019).

Population Genetic Summary Statistics

The pairwise nucleotide diversity (π), number of segregating sites (S), and Tajima's D were calculated across locus regions and datasets with the PopGenome v2.7.5 R package (Pfeifer et al., 2014). The significance of Tajima's D statistics was determined by 100 standard coalescent simulations with ms (Hudson, 2002). We generated 200 bootstrap replicates of each alignment to calculate a percentile bootstrap 95% confidence interval and compare the relative estimation

323 error between regions and datasets using the root mean square error (RMSE) from the
324 bootstrap samples. The scaling and interpretation of parameters are described in the
325 Supplementary Material (Supplementary Methods: *Summary Statistics*). We tested for
326 differences in the distribution of summary statistics among locus regions (left flanks, cores, and
327 right flanks) and data types (OH, AG, and PH) with Wilcoxon Rank-Sum tests. We tested for
328 biases in Tajima's *D* results among locus regions and data types with X^2 tests.
329
330 *Demographic History*
331 We chose the extended Bayesian skyline plot (EBSP, Heled & Drummond, 2008) as the most
332 appropriate model of N_e change through time for our sample of target enrichment data. Here we
333 assumed a strict molecular clock underlying all lineages but allowed rates of evolution to vary
334 among loci. One locus was fixed to the clock rate and all other locus rates were relative. Rates
335 of molecular evolution among plants are especially complicated, as there can be life histories
336 with variable reproductive strategies, unknown generation times, and overlapping generations.
337 Thus, we relied on an estimate of the neutral substitution rate at third-position synonymous sites
338 from grass genomes (Christin et al., 2014) and constructed a vague prior around this mean rate,
339 such that any biologically plausible uncertainty in the rate of evolution is reflected in our
340 posterior sample. It is a tenuous rate estimate at best, since it remains unknown whether
341 polyploid *L. simplex* largely propagates clonally, through apomixis, or through sexual
342 reproduction. The MCMC analyses for the OH, AG, and PH data collected 20,000 samples after
343 a burnin of 50,000,000 and sample interval of 10,000. Two replicates were run for each analysis
344 to evaluate convergence and mixing was assessed with Tracer v1.7 (Rambaut et al., 2017). All
345 model and prior specifications are provided in the Supplementary Material (Supplementary
346 Methods: *EBSP Prior Specification*) and are replicable from XML files on Dryad
347 (https://doi.org/10.5061/dryad.905qfttqx).
348
349 *Adequacy of Demographic Models*
350 To explore if EBSP models could reliably differentiate between Late Pleistocene versus
351 Holocene population size increases, we evaluated the accuracy of EBSP inferences with
352 simulations that captured some features of our target-enrichment data. We simulated 57 loci
353 that were 1000 bp in length for 20 haploid individuals 100 times with ms (Hudson, 2002) and
354 seq-gen (Rambaut & Grass, 1997) for four scenarios: 1) a constant N_e of 100,000, 2) an N_e
355 increase from 10,000 to 100,000 individuals roughly coinciding with the LGM at 20,000 years in
356 the past, 3) the same ten-fold increase but at 2,000 years in the past to reflect anthropogenic

357 expansion, and 4) a scenario combining both LGM and anthropogenic effects by exponentially
358 increasing N_e from 10,000 to 100,000 between 20,000 years ago and the present. Each
359 simulated dataset was then evaluated with the same EBSP model using a fixed clock used for
360 our empirical analyses, except the MCMC algorithm collected 10,000 posterior samples with a
361 sample interval of 1,000 after a 100,000 sample burnin. Commands for simulations are given in
362 the Supplementary Material (Supplementary Methods: *Simulating Demographic Histories*).
363
364 **Results**
365 *Sequencing Statistics*
366 The probe set targeted 408 loci from which an average of 327 loci were assembled by HybPiper
367 (Supplementary Table S2). These were supported by an average of 217 read pairs per locus.
368 Although there was variable read depth across loci and individuals, the number of assembled
369 loci was significantly correlated with read depth (Pearson's correlation coefficient = 0.57, *p*-
370 value = 0.002). After joint-genotyping all individuals as diploids, normal mixture models
371 suggested all individuals were hexaploids, with the exception of two individuals from South
372 Africa (Supplementary Table S3). Some individuals were inferred as pentaploids, but since this
373 was unexpected based on previous observations from microsatellites (Supplementary Table S1)
374 these cases were assumed to be hexaploids. Although available evidence leaves the ploidy of
375 some individuals ambiguous and raises skepticism of the inference methods, genotyping and
376 phasing of individuals proceeded with the ploidy suggested by our mixture models. We inferred
377 an average of 247 loci with allelic variation, which had a heterozygosity of 0.012 variants per
378 base. After reducing the number of loci with BLAST clustering to avoid tight linkage, a final 301
379 loci were retained from the OH, AG, and PH datasets for downstream analyses. The average
380 number of haplotype blocks across all loci was 1.005, meaning that in very few cases there was
381 insufficient data to phase across the entire locus (Supplementary Table S2). In cases where
382 multiple variants within a locus could not be phased, only the variants from the longest block
383 were retained and variants on the shorter block were replaced with missing data.
384
385 *Phylogeny Estimation*
386 Species trees revealed all Malagasy *L. simplex* form a well-supported clade (Supplementary
387 Fig. S1), but there was disagreement in the biogeographic history within Madagascar based on
388 the treatment of ploidy. Individuals in northern Madagascar formed a clade with a posterior
389 probability of 0.9 or greater across the OH, AG, and PH analyses. However, the individuals from
390 central and southern Madagascar were paraphyletic in the OH and AG analyses

391 (Supplementary Figs. S1a and S1b). A clade of central and southern individuals was recovered
392 in the PH analysis, but with a weak posterior probability of 0.64 (Fig. 1c; Supplementary Fig.
393 S1c). Individuals from the center of the *L. simplex* distribution appear to be the earliest diverging
394 lineages in the OH and AG analyses (Supplementary Figs. S1a and S1b), or with respect to the
395 sample of central and southern individuals in the PH analysis (Fig. 1c; Supplementary Fig. S1c).
396
397 *Population Sub-Structure in Madagascar*
398 STRUCTURE analyses recovered a variable number of Hardy-Weinberg groups across the ten
399 datasets that randomly sampled one non-singleton biallelic SNP per locus (Supplementary
400 Table S4). For the OH data, we found a *K* of two, three, or four in eight, one, and one of the
401 respective replicates. For AG data, the optimal number of clusters was more ambiguous with a
402 *K* of two, three, and four recovered for four, five, and one replicates. PH data found *K* of two and
403 three for eight and two replicates. We note that the method of Evanno et al. (2005) cannot infer
404 if one panmictic population is the best model, and while there is reasonable geographic
405 structure at *K* of two weighted across biological and technical replicates (Fig. 2a) the non-zero
406 admixture between individuals at the most extreme ends could indicate a single population with
407 a cline of genetic variation. This is further supported by the lack of distinct cluster assignments
408 at higher values of *K* despite some evidence for additional population structure (Supplementary
409 Fig. S2). A weak signal of isolation-by-distance was detected when regressing genetic distance
410 against spatial distance (Fig. 2b). Notably, the AG data inferred much higher pairwise distances
411 between individuals; although, the relative differences and overall trend were similar. The same
412 patterns can be observed when using patristic distances from ML phylogenetic estimates,
413 although the disparity between AG versus OH or PH distances is reduced, as is the percent of
414 genetic variation explained by spatial distances (Supplementary Fig. S3).
415
416 PCAs reflected findings from STRUCTURE analyses and reinforce the presence of a genetic
417 cline (Fig. 3). For OH data, where only one reference or alternate allele was observed per
418 individual, Malagasy *L. simplex* is clearly distinguished from mainland African individuals (Fig.
419 3a), but there is no clear separation of individuals based on geographic location (Fig. 3b). PCAs
420 of data that accounts for heterozygosity but not dosage recovered a single Malagasy versus
421 mainland African pattern (Fig. 3c), PCAs reflect a north-to-south gradient of genetic variation
422 regardless of whether allelic dosage was treated as hexaploid (Fig. 3d), tetraploid (Fig. 3e), or
423 diploid (Fig. 3f). The total percent of variance explained by principal component axis 1 (PC1)

424 and axis 2 (PC2) increased as allelic dosage was compressed from a hexaploid to a diploid
425 state.
426
427 *Variation in Summary Statistics*
428 There were significant differences in the distributions of π, *S*, and Tajima's *D* among locus
429 regions and between the OH, AG, and PH data (Fig. 4). For π, there was always a difference
430 between flanking regions and the exon core (Supplementary Table S5), with the cores
431 surprisingly having more variation. Differences were also observed between the flanking regions
432 and cores for *S* and Tajima's *D*, except when comparing the left flanks and cores for OH data
433 (Supplementary Table S5). The only case of a difference between the left and right flanks was
434 when estimating π for AG data (Supplementary Table S5). The OH and PH data resulted in
435 similar estimates of π; however, the AG and PH data had similar distributions of *S*
436 (Supplementary Table S5). Any differences in Tajima's *D* statistics were largely explained by an
437 over-representation of values less than negative two, indicating positive selection or population
438 size expansion, in the OH data and among core regions across datasets (Table 1).
439
440 We attempted to explore biases in summary statistic estimates due to sampling error of sites
441 through bootstrapping the OH (Supplementary Fig. S4), AG (Supplementary Fig. S5), and PH
442 data (Supplementary Fig. S6). Our observed summary statistics typically fell outside of their
443 percentile bootstrap 95% confidence intervals, but other confidence interval approximation
444 techniques (e.g. Efron & Narasimhan, 2020) may be more appropriate for population genetic
445 summary statistics (Leblois et al., 2003). However, we used the bias, difference between the
446 bootstrap mean and observed estimate, and the RMSE of bootstrap replicates with respect to
447 the observed estimate, to gain some general insights. Notably, the bias of π is larger and much
448 noisier in the AG data (Supplementary Fig. S5) compared to the OH (Supplementary Fig. S4)
449 and PH (Supplementary Fig. S6) data. Even if the bias for a whole-locus is low, there can be
450 high variability between regions. For the observed values, estimates are not always proportional
451 among regions either. The RMSE of an estimate tends to decrease along with genetic variation,
452 implying some role of genotyping error or rare variants and singletons inflating values at the
453 higher end of the spectrum. Loci with very low levels of genetic variation can also be
454 problematic though, as the RMSE for flanking regions increases towards the tail of the
455 distribution, creating opportunity for sampling error to wildly swing estimates regardless of ploidy
456 (Supplementary Figs. S4-S6). The number of segregating sites increased with ploidy of the

data, but π was relatively stable. However, differences between π and S will affect estimates of Tajima's D, and this could affect interpretations of demography or selection acting on loci; although, the majority of loci and their regions were not significantly different from neutral expectations given the simulation-based test statistics (Supplementary Table S6).

Population Size Change Through Time

After filtering for longer loci with low amounts of missing data, 57 loci were retained for EBSP analyses. Reducing our data to a subset of more informative loci was done for computational convenience, because EBSP models are parameter-rich and require long MCMC runs for convergence. Runs converged for each data type for both analyses with a fixed rate of molecular evolution (Fig. 5) and variable rate (Supplementary Fig. S7). All analyses suggested the *L. simplex* N_e increased through the Late Pleistocene, well before the LIG, approximately one million years ago. Between the LGM and period of potential human colonization of Madagascar in the Holocene, N_e had already reached present-day estimates. N_e estimates through the Holocene were only possible for the PH data, as accounting for the within-individual variation should have captured more recent coalescences. Simulations cast doubt on the seriousness with which EBSP results can be interpreted through the Holocene though. For example, when N_e was truly constant through time, some individual EBSP analyses would suggest recent N_e increases; although, the mean estimate across simulations recovered the constant population scenario (Fig. 6a). This problem was exaggerated in the presence of model over-fitting (Supplementary Fig. S8). In scenarios with recent N_e increases, the contemporary N_e was underestimated but a trend indicating a recent N_e increase was detectable (Figs. 6b-6d). However, simulations indicated it would not be possible to differentiate between N_e increases that occurred during the LGM (Fig. 6b) versus the Late Holocene (Fig. 6c), let alone some combination of paleoclimate and anthropogenic effects (Fig. 6d). Interpreting these recent N_e increases from EBSP analyses becomes impossible with model over-fitting (Supplementary Fig. S8).

Discussion

Robustness of Analyses Across Loci and Ploidy

Although target-enrichment data has been transformative for species-level phylogenomic studies (Breinholt et al., 2021; Baker et al., 2022), the robustness of population genetic estimates for a sample of closely related individuals was largely unexplored (but see Slimp et al., 2021). Some of the most important measures of genetic variation for populations were

susceptible to sampling error, especially in the flanking intron regions (Supplementary Figs. S4-S6). While estimates from cores showed less error, they will lead to some violations of population genetic methods that assume evolution consistent with neutral theory, given potential signatures of purifying selection (Table 1). This creates some conflict, since most of the information is contained within the exon cores of target-enrichment loci (Fig. 4), presumably due to read coverage. Because Tajima's *D* alone cannot disentangle demography from selection, some judgment calls and tolerance for downstream model violations are necessary. The majority of loci examined were consistent with neutral expectations based on simulations (Supplementary Table S6), and the proportion of loci with significant Tajima's *D* statistics were exaggerated when ignoring heterozygosity (Table 1). Although, the estimates of nucleotide diversity and simulations under the standard neutral coalescent used here should be reasonable for polyploids in cases of perfect multisomic inheritance in outcrossers (Arnold et al., 2012; Blischak et al., 2023), we note that the presence of subgenome structure and selfing could cause deviations in expectations of allele frequencies (e.g. Ronfort et al., 1998; Meirmans & Van Tienderen, 2013; Blischak et al., 2023). The mechanism of polyploidization is unknown in *L. simplex*, but our results do provide some expectations of how genotyping choices for polyploids can impact standard summary statistic estimates.

Predicting the effects of assumptions about genotype on estimates of genetic variation and downstream analyses are not always straightforward. For example, the pairwise distances (Fig. 2b) or evolutionary distances (Supplementary Fig. S3) between individuals was higher when assuming all individuals were diploid (AG) compared to assuming all individuals were haploid (OH) or genotyped at their anticipated ploidy level (PH). If we were willing to ignore the absolute estimates of genetic distance, the interpretation of isolation-by-distance analyses was the same across the OH, AG, and PH data. This implies that biologically meaningful conclusions can arise even with uncertainty in ploidy and phasing. Interestingly, treating all individuals as diploid was sufficient for STRUCTURE to recover northern and southern clusters with some admixed central individuals of varying proportions (Fig. 2a; Supplementary Fig. S2). Similarly, PCAs reflected the geographic distribution of our *L. simplex* samples in the AG and PH data with, surprisingly, the AG data explaining more of the observed genetic variance (Fig. 3f) than the PH data (Fig. 3d). This may be due to genotyping error at higher ploidy levels caused by read stochasticity and a flat genotype likelihood surface (Blischak et al., 2016) or caused by incorrect ploidy inferences. A similar mixture model approach to the one implemented here (Weiß et al., 2018) has been shown to have good performance based on comparisons of allele balance distributions from

target-enrichment data and flow cytometry-based ploidy estimates (Viruel et al., 2019). We are cautiously optimistic about the prospects of direct ploidy estimation from sequence data, because likelihood-based criteria can be prone to overfitting (Tiley et al., 2018). However, our ploidy estimates are generally consistent with the previous inferences from microsatellite allele counts (Hagl et al., 2021; Supplementary Table S1; Supplementary Table S3). A positive insight from our comparisons across ploidy levels is that if unknown individuals are genotyped as diploid, there will be sufficient information about population structure, and that ploidy estimation should not be a barrier to investigations of natural plant populations with little *a priori* cytological or genetic knowledge.

Limits to Differentiating Anthropogenic and Paleoclimate N_e Change

Large population genomic studies in plants that can leverage demographic estimates from SFS (e.g. Excoffier et al., 2013; Liu & Fu, 2020; Blischak et al., 2023) are commonly focused on temperate regions (e.g. Yamaura et al., 2019) and species exploited for energy or agriculture (e.g. Huang et al., 2014; Wang et al., 2017). Thus, it is tempting to leverage models that use gene trees as data such as the EBSP (Heled & Drummond, 2008) or other extensions of skyline plots (Pybus et al., 2000), since they could be paired with relatively low-cost target-enrichment strategies that yield multi-locus nuclear data while stretching material quality.

While our analyses provide valuable insights into the ancient origins of *L. simplex* dominated grasslands, both our empirical and simulated analyses show we cannot differentiate between N_e increases that occurred during human colonization or paleoclimate change near the LGM. The change in vegetative composition towards more C_4 grass species in Central Madagascar is well-documented (Burney, 1987; Gasse & Van Campo, 1998; Virah-Swamy et al., 2010; Crowley et al., 2021). It is likely that these increases in C_4 grasses included *L. simplex*; although, an increase in census size may not be reflected by N_e (Fig. 5; Supplementary Fig. S7), the expected population size under a neutral coalescent model. Our simulations showed that detecting these recent increases is difficult, and that we cannot differentiate between a ten-fold increase that occurred 200 (2 ka) or 2000 generations ago (20 ka; Fig. 6). Diagnosing a true N_e change becomes impossible in the presence of model overfitting (Supplementary Fig. S8) and diagnosing these problems in empirical data can be difficult aside from convergence assessments.

558 These limitations do not necessarily disagree with an assessment of EBSP models from a larger
559 genomic dataset of endemic palm species in Madagascar (Helmstetter et al., 2021). Helmstetter
560 et al. (2021) found that the estimated N_e trends were valuable predictors of IUCN risk
561 assessments, and that population decreases could be detected at anthropogenic time scales.
562 However, it should be easier to detect population declines than population increases in a
563 system that already had a large degree of standing genetic variation, such as *L. simplex*. This is
564 because the time until the most recent common ancestor of a genealogy will be much more
565 recent in a small population than a large one (e.g. see equation 2.13 from Gillespie, 2004).
566 Analyses of demographic models with genomic SFS should be able to increase resolution at
567 recent anthropogenic time scales (Patton et al., 2019) and differentiate anthropogenic from
568 paleoclimatic demographic effects (Tiley et al., 2022), but this would require more extensive
569 sampling and sequencing. Additionally, our analyses treat all individuals as a single population,
570 and since we detected some level of population structure, we cannot rule out their confounding
571 effects on analyses of N_e through time (Heller et al., 2013; Mazet et al., 2016) or other
572 confounding affects such as clonal growth and overlapping generations. Population-level
573 sampling of multiple sites throughout a species range should be used to control for population
574 structure, while accounting for localized effects such as agriculture and grazing versus regional
575 climate effects.
576
577 *New Insights into* Loudetia simplex *Evolution and Policy Implications*
578 Our analyses yielded new findings about the evolution of *L. simplex* that are potentially
579 consequential for the broader understanding of Malagasy grasslands. First, our species trees
580 (Supplementary Fig. S1) indicate that individuals from what we call Central Madagascar – a
581 loose term meant only to indicate individuals from the center of the sampled *L. simplex*
582 distribution – represent the ancestral distribution. It is likely that *L. simplex* dominated
583 grasslands have origins in some of the higher elevation regions of the Central Highlands. For
584 example, MSV1564 comes from the Itremo protected area (Fig. 1b; Supplementary Table S1),
585 and further investigations of this region should be productive for characterizing natural
586 grasslands in Madagascar. The hypothesis of Central Highland origins, which we consider
587 800m in altitude and above, is also supported by our analyses of population structure (Fig. 2a;
588 Supplementary Fig. S2) and the weak but significant pattern of isolation-by-distance (Fig. 2b;
589 Supplementary Fig. S3).
590

591 Our models of N_e change through time suggests that *L. simplex* grasslands were already
592 extensive (Fig. 5; Supplementary Fig. S7) well before any hypothesized human presence in
593 Madagascar (c. 10 ka; Hansford et al., 2018) let alone human colonization (c. 2 ka; Dewar and
594 Wright, 1993; Dewar et al., 2013; Pierron et al., 2017). We found an approximate ten-fold
595 increase between one MA and the LIG which is beyond the limitations of available
596 paleoecological records (Burney, 1987; Gasse & Van Campo, 1998; Virah-Swamy et al., 2010;
597 Railsback et al., 2020; Crowley et al., 2021). Strong assumptions about the mutation rate and
598 generation time to calibrate analyses to absolute time and N_e were necessary, but our
599 interpretations of results are within the biologically plausible uncertainty of these parameters.
600 Even if the per-year substitution rate was overestimated by a factor of ten, the N_e increase
601 would still be constrained to the Late Pleistocene, occurring between the LIG and LGM. These
602 analyses, especially in the context of a previous investigation of population structure (Hagl et al.,
603 2021), suggest that *L. simplex* dominated grasslands have pre-human origins and likely
604 experienced some N_e increase coincident with Pleistocene climate change. Tree planting
605 programs have been deployed to combat the devastating habitat loss and deforestation in
606 Madagascar since the 1950s (Green & Sussman, 1990; Harper et al., 2007; Vieilledent et al.,
607 2018; Schüßler et al., 2018), with country-wide forest loss from 1953 to 2014 at 44% (Vieilledent
608 et al., 2018). The results of our study strongly imply that tree planting efforts should be cautious
609 of treating *L. simplex* dominated grasslands as degraded forests, and we suggest that focusing
610 resources on forest edges with native species will be more productive (Miandrimanana et al.,
611 2019).
612
613 *Potential Use Cases of Target-Enrichment Data for Conservation Genetics*
614 One application of target-enrichment data going forward could be analyses of population
615 structure and population assignment of unknown individuals. For example, many conservation
616 programs performing long-term biodiversity monitoring rely on reduced-SNP panels to
617 determine provenance of an individual or sample (e.g. Bourgeois et al., 2018; Stronen et al.,
618 2022). Developing SNP panels requires at least some *a priori* genomic resources, so target-
619 enrichment libraries could provide feasible approaches in the absence of such references and
620 practical improvements over existing barcoding strategies for identifying, for example,
621 Madagascar's rosewoods (Hassold et al., 2016); although acquiring viable DNA samples from
622 woody tissue would remain a barrier (Jiao et al., 2018). We anticipate limited use cases of
623 target-enrichment data in population genetics going forward as reference genome assembly is
624 becoming more accessible through decreasing long-read costs, and less complicated library

625 preparation protocols for whole-genome resequencing will become preferential to any target-
626 enrichment strategy. However, the population genetic inferences from target-enrichment data
627 should be biologically meaningful when available.
628
629 *A Practical Need for Plant Population Genomics in Madagascar*
630 There are few studies of plant population genetics from the Central Highlands and those are
631 limited to AFLP (Gardiner et al., 2017) or microsatellite data (Salmona et al., 2020; Hagl et al.,
632 2021). Demographic analyses from the endemic olive tree *Noronhia lowryi* suggested recent
633 population declines of the woody species, but it is unclear if these declines represent
634 anthropogenic or paleoclimatic change near the LGM (Salmona et al., 2020). While our
635 analyses of *L. simplex* are also ambiguous regarding this window of anthropogenic effects on
636 the extent of grasslands, we can at least conclude that the *L. simplex* N_e was quite large.
637 Interpreting what a large N_e means in the context of the *L. simplex* species distribution is difficult
638 though; it could be a single large distribution or multiple smaller isolated distributions that yield
639 the expected measures of genetic variation. Contemporary population genomic analyses will be
640 necessary to provide statistical power to differentiate between competing demographic
641 hypotheses, not only for grasses such as *L. simplex* but also species representing plant
642 diversity across families and life-history. A better reconstruction of the natural history of
643 Madagascar's grasslands is not only necessary for understanding diversity within and outside of
644 the Central Highlands (Yoder et al., 2016; Burbrink et al., 2019; Everson et al., 2020), but also
645 to make effective land management decisions for tree planting programs.
646
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Author Contributions

GPT, ADY, and MSV conceived the study. WRQL, CLS, CERL, GB and MSV collected samples. GPT and TOMA curated data. GPT and AAC analyzed data. GPT wrote the first draft. TOMA translated the abstract to French and Malagasy. All authors revised the manuscript and approved the final version.

Data Availability Statement

Scripts for analyses and input files are available through the Dryad Digital Repository: https://doi.org/10.5061/dryad.905qfttqx. Additional scripts are available on GPT's GitHub for identifying exon-intron boundaries from target enrichment data (https://github.com/gtiley/Locus-Splitter) generating matrices for PCAs from fasta files (https://github.com/gtiley/fasta-conversion) and the ploidy test implemented through PATÉ (https://github.com/gtiley/Phasing). Raw sequence data is available via the NCBI short read archive (SRA) database, with individual SRA and BioSample identifiers in Supplementary Table S1. Raw sequence data is associated with NCBI BioProject PRJNA952819.

Conflict of Interest Statement

The authors declare no conflict of interest.

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Figures

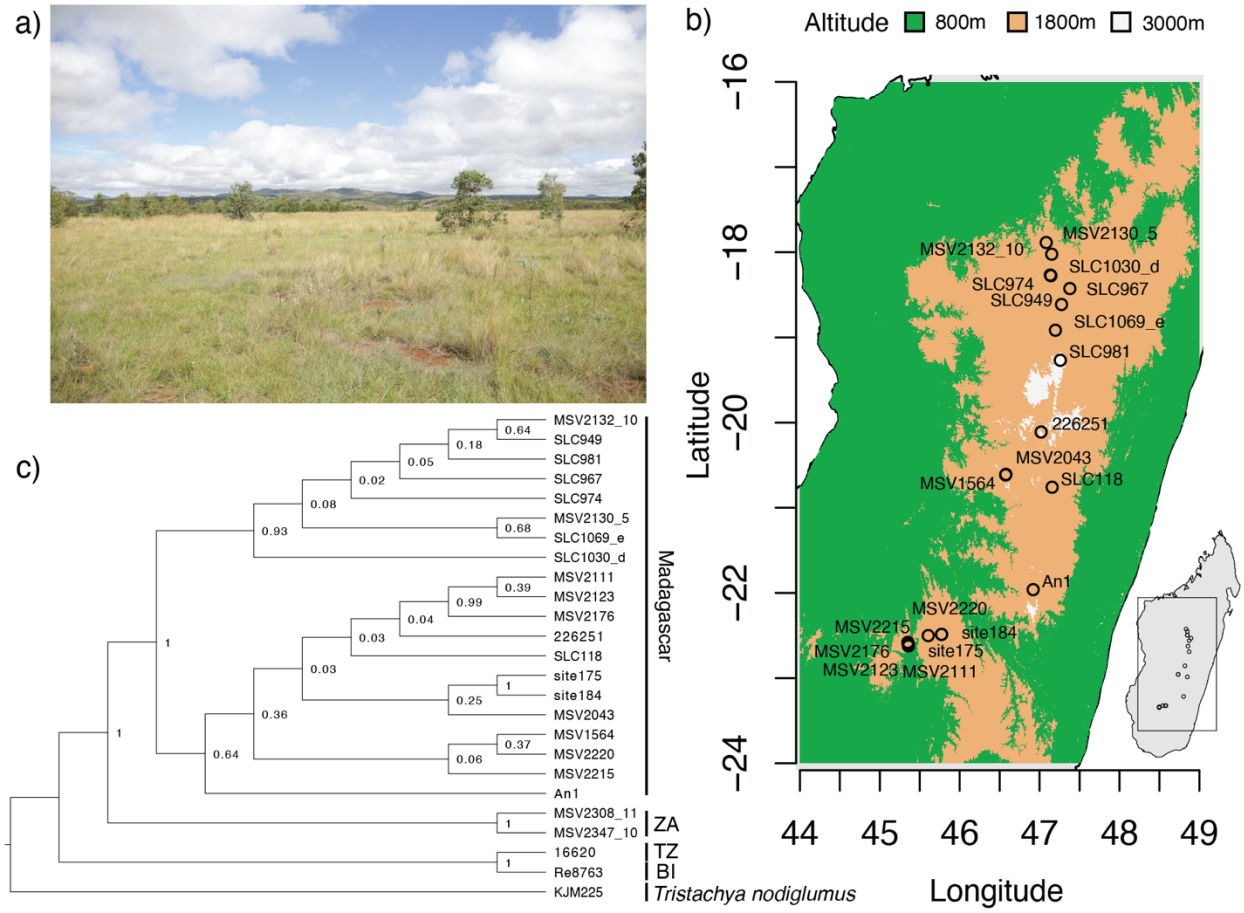

Figure 1 — *Loudetia simplex* distribution and single Madagascar origin. a) A field of *L. simplex* near Antsirabe Madagascar planted with eucalyptus (photo credit: George P. Tiley). b) Our sampling of *L. simplex* was based on collections made throughout Madagascar's Central Highland Plateau, which is typically considered an altitude between 800 and 1800 meters. c) Phylogenetic relationships of individuals based on the phased (PH) data. Numbers next to nodes are posterior probabilities. ZA = South Africa, TZ = Tanzania, BI = Burundi.

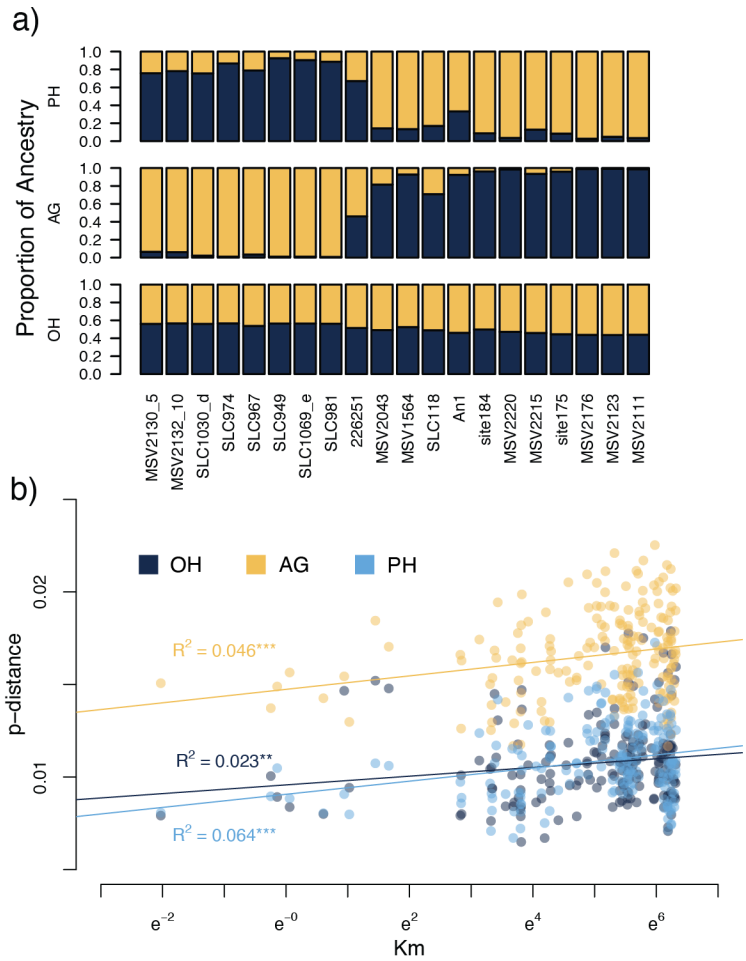

Figure 2 — Patterns of genetic structure and variation in Madagascar. a) Aggregated STRUCTURE results across runs for an *a priori* number of clusters of two (K = 2) for the different data types. c) A plot of natural-log-scaled geographic distance against the pairwise distance (p-distance). Lines are from a least-squares linear regression with the coefficient of determination (R^2) shown for each data type. Statistical significance was determined with a Mantel test using 9999 permutations. Significance levels are p-value < 0.05 (*), p-value < 0.01 (**), and p-value < 0.001 (***). Colors represent different data types: one haplotype (OH), ambiguous genotypes (AG), and phased haplotypes (PH).

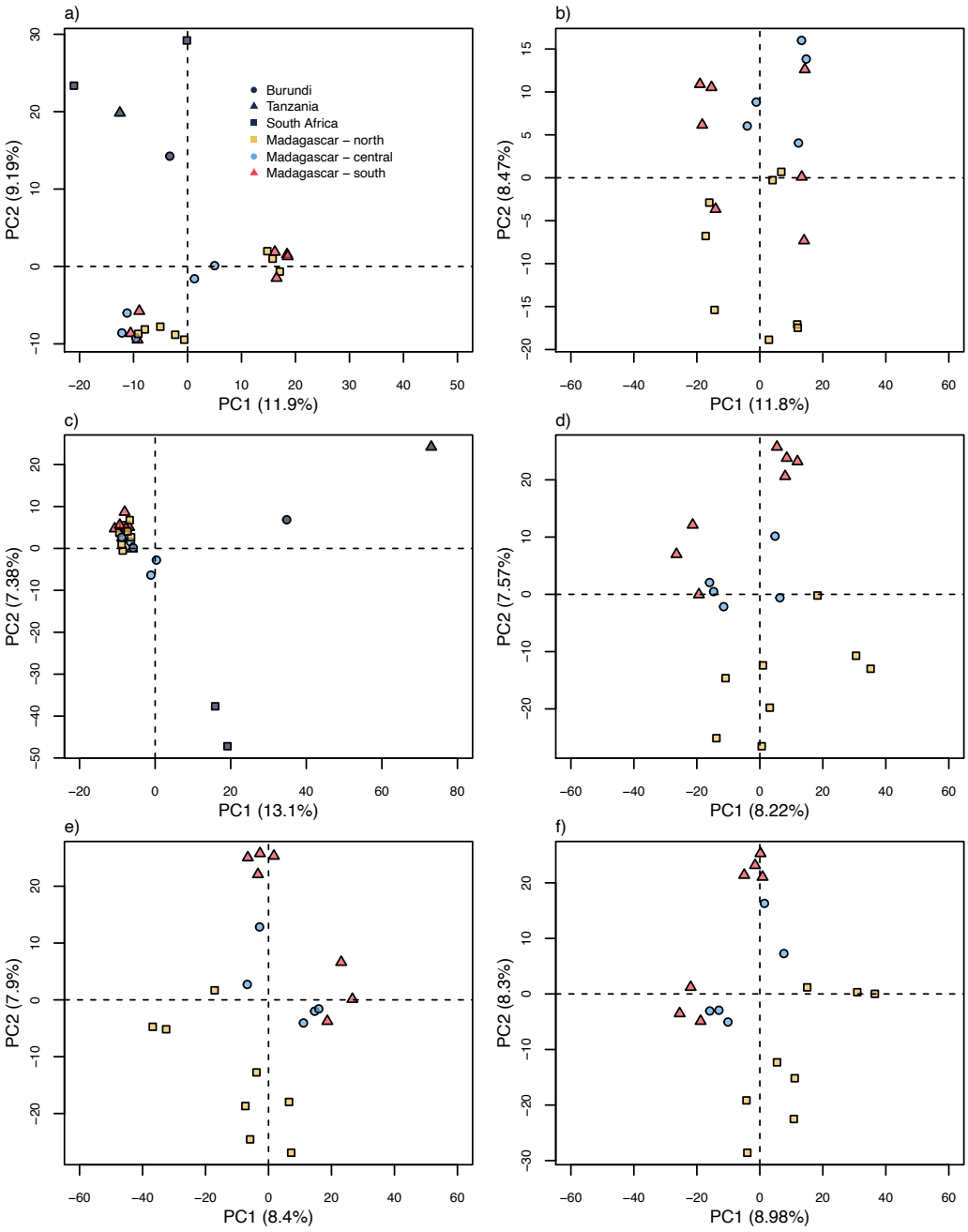

Figure 3 — Principal component analyses across data types. a) All individuals using haploid (OH) data. b) Only Malagasy individuals using OH data. c) All individuals treating all sites as diploid. d) Only Malagasy individuals treating all sites as hexaploid. e) Only Malagasy individuals compressing unbalanced allelic dosage to single categories. f) Only Malagasy individuals treating all sites as diploid.

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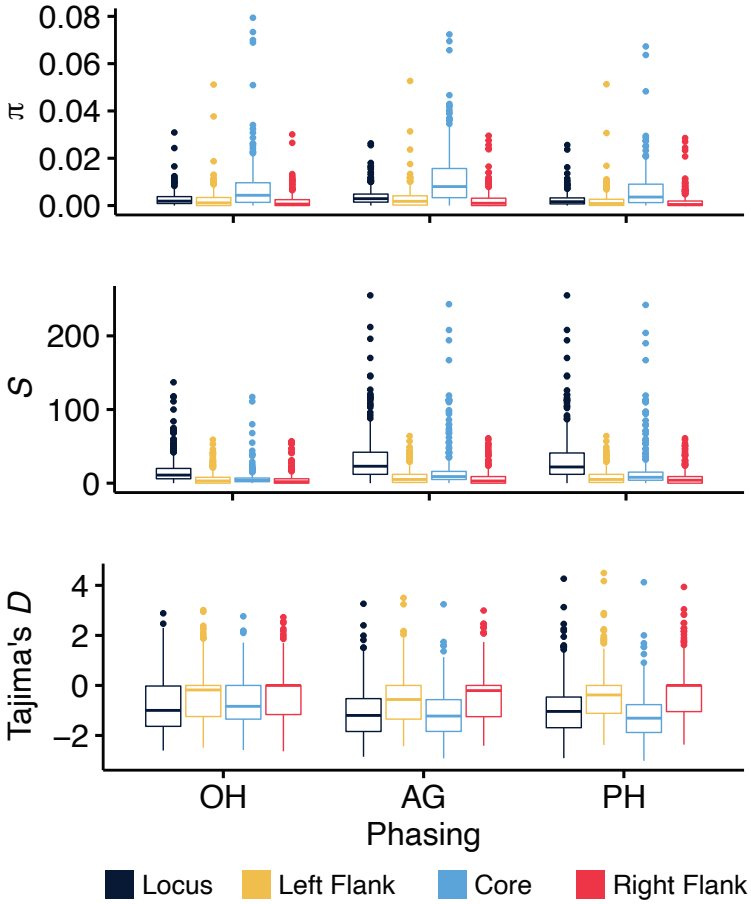

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1049 **Figure 4 — Distributions of summary statistics across locus regions.** Box-and-whisker
1050 plots show distribution medians and quartiles for the expected pairwise nucleotide divergence
1051 (π), the number of segregating sites (S), and a normalized measure of the difference between
1052 two estimators of nucleotide divergence, indicating deviations from neutral expectations
1053 (Tajima's D).

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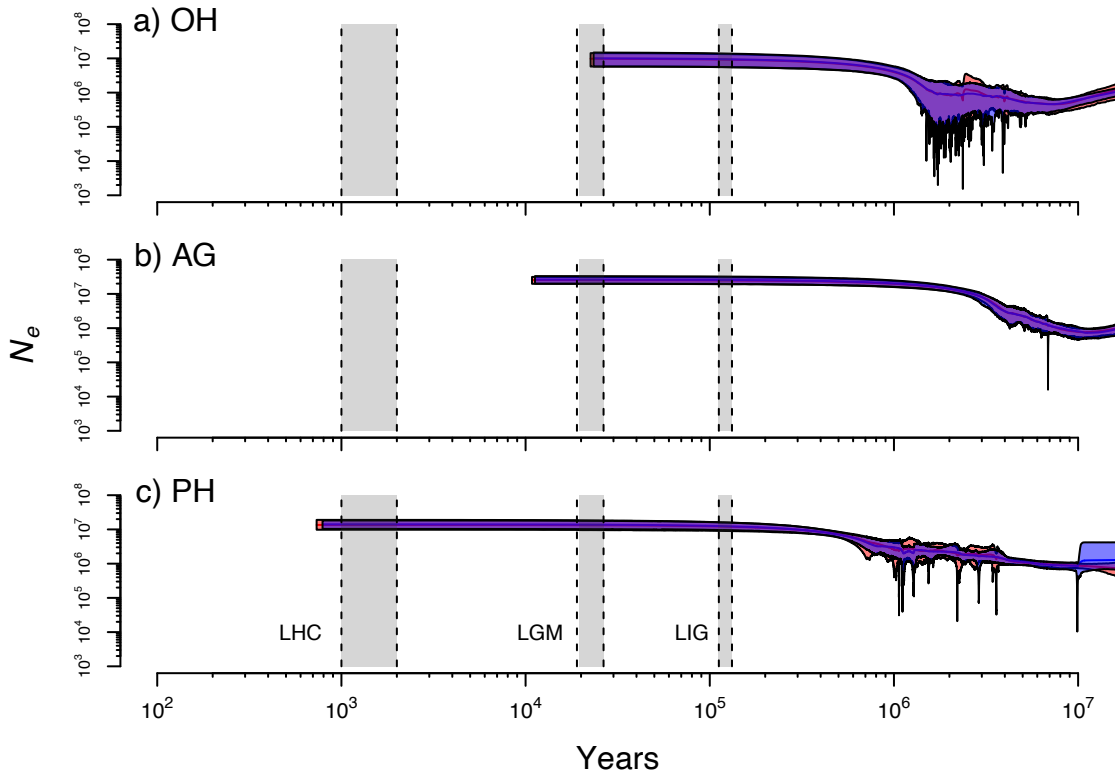

Figure 5 — Extended Bayesian Skyline Plot results with fixed clock rates. Two independent runs are shown with mean population sizes shown by solid lines. Polygons show the 95% HPD intervals with the overlap between runs in purple. Results are shown for the a) OH, b) AG, and c) PH data. Relevant time periods are shown in gray, which includes likely human colonization (LHC), the last glacial maximum (LGM), and last interglacial period (LIG).

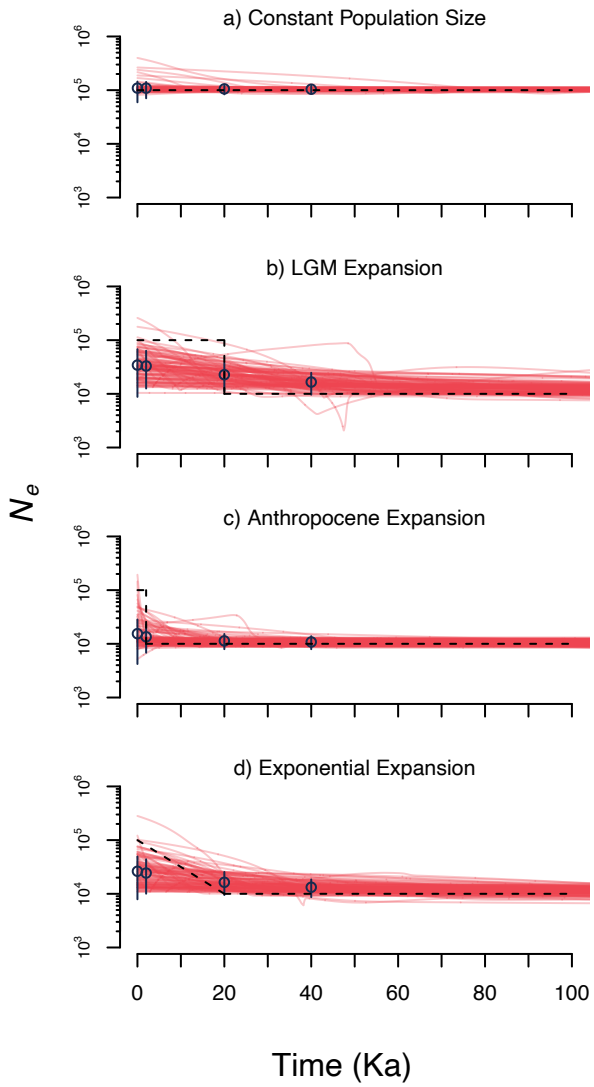

Figure 6 — Simulation results from EBSP analyses for an appropriate number of change points. Individual red lines are the mean N_e estimates from 100 simulated data sets. Blue points and vertical lines are the mean of mean N_e estimates and their 95% HPD intervals. The dashed black line is the true demographic history of the population.

1073 **Tables**

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Table 1 — X^2 test result from Tajima's *D* statistics across datasets and locus regions

Tested Effect	Fixed Effect	X^2	*p*-value	Largest Residual
Region	OH	7.72	0.27	Under-representation of Tajima's *D* < -2 in core regions
	AG	37.77	0.0001	Over-representation of Tajima's *D* < -2 in core regions
	PH	83.47	0.0001	Over-representation of Tajima's *D* < -2 in core regions
Dataset	Left Flank	12.65	0.046	Over-representation of Tajima's *D* < -2 in OH dataset
	Right Flank	8.61	0.19	Over-representation of Tajima's *D* < -2 in OH dataset
	Core	57.15	0.0001	Over-representation of Tajima's *D* < -2 in OH dataset

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