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RESEARCH ARTICLE

Genomic signatures of past megafrugivore-mediated dispersal in Malagasy palms

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Abstract

1. Seed dispersal affects gene flow and hence genetic differentiation of plant populations. During the Late Quaternary, most fruit-eating and seed-dispersing megafauna went extinct, but whether these animals have left signatures in the population genetics of their food plants, particularly those with large, 'megafaunal' fruits (i.e. >4 cm—megafruits), remains unclear.

2. Here, we assessed the population history, genetic differentiation and recent migration among populations of four animal-dispersed palm (Arecaceae) species with large (*Borassus madagascariensis*), medium-sized (*Hyphaene coriacea*, *Bismarckia nobilis*) and small (*Chrysalidocarpus madagascariensis*) fruits on Madagascar. We integrated double-digest restriction-site-associated DNA sequencing (ddRAD) of 167 individuals from 25 populations with (past) distribution ranges for extinct (e.g., giant lemurs and elephant birds) and extant seed-dispersing animals, landscape and human impact data, and applied linear mixed-effects models to explore the drivers of genetic variation in Malagasy palms.

3. Palm populations that shared more megafrugivore species in the past had lower genetic differentiation than populations that shared fewer megafrugivore species. This suggests that megafrugivore-mediated seed dispersal in the past may have led to frequent gene flow among populations. In comparison, extant frugivore diversity only decreased genetic differentiation in the small-fruited palm. Furthermore, genetic differentiation of all palm species decreased with landscape connectivity (i.e. environmental suitability, forest cover and river density) and human impact (i.e. road density), while migration rates of the small-fruit palm increased with road density.

4. *Synthesis*. Our results suggest that the legacy of megafrugivores regularly achieving long dispersal distances is still reflected in the population genetics of palms that were formerly dispersed by such animals. Furthermore, low genetic differentiation was possibly maintained after the megafauna extinctions through alternative dispersal (e.g. human- or river-mediated), long generation times and long lifespans of these megafruit palms. Our study illustrates how species interactions that happened >1000 years ago can leave imprints in their population genetics.

KEYWORDS

Arecaceae, ddRAD, genetic differentiation, megafauna extinction, population genomics, recent migration rate, seed dispersal

1 | INTRODUCTION

More than 90% of woody plant species in tropical rainforests rely on frugivores (i.e., fruit-eating and seed-dispersing animals) for their seed dispersal (Jordano, 2000). For these plants, most long-distance dispersal events are provided by large-bodied frugivores (i.e. megafrugivores—the subset of largest animal species that have fruit as the main part of their diet in a given ecosystem; Moleón et al., 2020), that are able to ingest large fruits and seeds (e.g. 'megafaunal' fruits, larger than 4 cm in diameter; Guimarães et al., 2008) and move them across long distances given their large home ranges (Pires et al., 2018). Megafrugivores, such as the African elephant (*Loxodonta africana*), thereby connect their food plant populations, leading to increased gene flow and low speciation rates of plants with megafaunal fruits (McConkey et al., 2018; Onstein et al., 2017). By influencing population connectivity and gene flow, seed dispersal is crucial for the regional-scale genetic structure of plant populations (Browne et al., 2018).

During the last 125,000 years, extinction rates of large-bodied vertebrates have increased globally due to human impact (Smith et al., 2018). The extirpation of megafrugivores from ecosystems may have had cascading effects on the seed dispersal and thus connectivity of plant populations, especially for plants carrying large fruits that cannot be dispersed by the remaining smaller-bodied frugivores in the ecosystem (Janzen & Martin, 1982). Frugivore extinctions may therefore limit plant seed dispersal and gene flow, leading to high genetic differentiation (Giombini et al., 2017; Pérez-Méndez et al., 2016), with major consequences for the capacity of vertebrate-dispersed plants to track climate change (Fricke et al., 2022). Such historical interactions with now extinct megafrugivores may also have left imprints on current species and populations. For example, the turnover (i.e. beta diversity) of palm species across assemblages in Madagascar was shaped—at least partly—by historical co-occurrences with now extinct megafrugivores, predominantly in the western region of the island (Méndez, Viana, et al., 2022a). Similarly, the Neotropical distribution of palm fruit sizes was best explained by considering past—now extinct—megafrugivores (Lim et al., 2020). However, whether historical interactions with now extinct megafrugivores have also affected plant population genetics remains largely unknown.

In Madagascar, the decline of megafauna began approximately 1000 years ago, primarily due to increasing human impact (Crowley, 2010). Hunting and a transition to herding and farming, changed landscapes and megafauna habitats, ultimately leading to the extirpation of all megafauna (Godfrey et al., 2019; Li et al., 2020). Some of these extinct megafaunal species, such as giant lemurs (e.g. *Pachylemur* spp. and *Archaeolemur* spp.), elephant birds (e.g. *Aepyornis* spp. and *Vorombe* spp.) and giant tortoises (*Aldabrachelys* spp.), were probably fruit-eaters (Godfrey et al., 2004; Pedrono et al., 2013). Interestingly, fossil pollen suggests that the extinction of endemic megafauna in Madagascar coincided with a gradual decline in abundance of trees relying on megafrugivores for seed dispersal (Domic et al., 2021). However, there is no direct evidence of any plant species extinction in response to the extinction of its megafrugivore interaction partners. Instead, it has been suggested that several plant species that were adapted to seed dispersal by megafrugivores have persisted in today's ecosystems due to domestication by humans (Kistler et al., 2015), secondary seed dispersal by smaller-bodied frugivores (Blanco et al., 2019), or other, non-biotic forms of dispersal (e.g. via rivers; Guimarães et al., 2008).

Environmental and landscape-related factors may have shaped plant population genetics through non-frugivory-related dispersal processes (Jiang et al., 2019; Sexton et al., 2014; Siepielski et al., 2017). Environmental suitability (i.e. the extent to which a specific geographic area provides the appropriate conditions for a species to survive and thrive) can act as either a barrier or a corridor for dispersal (McRae, 2006; Wang & Bradburd, 2014), thereby influencing genetic differentiation of plant populations. For example, rivers in Madagascar acted as barriers to dispersal, leading to high genetic structure in leafless vanilla orchids (*Vanilla* spp.; Andriamihaja et al., 2021), while they acted as dispersal corridors for a megafruited tree, *Eligmocarpus cynometroides* (Fabaceae), allowing its spread across several biomes (Devey et al., 2013). Furthermore, forest cover, particularly of riparian forests, played an important role in connecting forest patches and structuring genetic diversity of an endemic tree in Madagascar (i.e. *Noronhia spinifolia*, Salmona et al., 2022).

Human activities have also been a major determinant of present-day genetics of plant populations (Smith et al., 2020). For example, fragmentation of ecosystems by human-made infrastructures, such

as settlements or road networks, may limit the movement of large vertebrates (e.g. frugivorous mammals) across the landscape, leading to dispersal limitation of plants with large vertebrate-dispersed fruits (Tucker et al., 2021). However, by moving plant seeds, humans may also facilitate long-distance dispersal events (Wichmann et al., 2008), potentially decreasing genetic differentiation among plant populations (Arredondo et al., 2018; Bullock et al., 2018). Additionally, unpaved roads can function as seed corridors for plants, as some terrestrial mammals select them for faecal marking, facilitating seed dispersal along road verges (Suárez-Esteban et al., 2013). Human activities may therefore either increase gene flow among plant populations or disrupt gene flow, depending on the context.

Here, we assessed whether patterns resulting from dispersal services provided by Madagascar's past megafrugivores can still be detected in the genomes of current palm (Arecaceae) populations. We focussed on palms because several species have been identified as 'anachronisms' in Madagascar's ecosystems due to their megafaunal fruit sizes (>4 cm in length, hereafter referred to as megafruits) that seem maladapted to dispersal by the current frugivore pool (Albert-Daviaud et al., 2020). We selected species from the western part of the island, where megafrugivorous animals were possibly most abundant in the past (Crowley et al., 2011). These savanna vertebrate-dispersed palm species can be classified in three fruit size classes: large megafruits (30 cm in average length—*Borassus madagascariensis*), medium-sized megafruits (5.5 cm—*Hyphaene coriacea*, 4.4 cm—*Bismarckia nobilis*) and small fruits (<4 cm; 1.3 cm—*Chrysalidocarpus madagascariensis* [previously *Dypsis madagascariensis*, Eiserhardt et al., 2022]; Table 1). The relatively small fruits of *C. madagascariensis* can still be dispersed by extant frugivores (e.g. *Eulemur macaco*; Adany et al., 1994), whereas the megafruits of the other three species are too large to be swallowed and dispersed by any native extant frugivore on Madagascar (Perry & Hartstone-Rose, 2010). Furthermore, the palms with medium-sized megafruits (*H. coriacea* and *B. nobilis*) are highly used by humans for house construction, basketry or food (Rakotoarinivo et al., 2020). Human-mediated dispersal may thus have contributed to the persistence and genetics of these species during the last 1000 years. Finally, the three megafruit species belong to the tribe Borasseae (*B. madagascariensis*, *H. coriacea* and *B. nobilis*) and are dioecious, with male and female individuals separated, while *C. madagascariensis*

is monoecious (male and female flowers on the same individual). Little is known about the pollinators of these palms, but flowers are relatively small and inconspicuous, suggesting a wide range of insect pollinators (Henderson, 1986).

We hypothesize (H1) that historical long-distance dispersal events by megafrugivores facilitated gene flow between populations, thereby leaving imprints on the current genetics of megafruit palm populations. Specifically, we expect that palm populations that historically shared more megafrugivore species were more connected through frequent megafrugivore-mediated seed dispersal and gene flow, leading to greater genetic similarity (i.e. lower genetic differentiation) than populations that shared fewer megafrugivore species. In contrast, genetic differentiation of populations of the small-fruited palm are expected to have been primarily shaped by past and ongoing dispersal by extant smaller-bodied frugivores, with a higher number of shared extant frugivores leading to lower among-population genetic differentiation. Furthermore, we hypothesize that landscape-related features such as forests, rivers and environmental suitability (H2) and humans (H3) have contributed to the genetic differentiation and recent migration rates of palm populations, possibly by replacing seed dispersal services of megafrugivores after their extinction. Specifically, we expect that forest cover, environmental suitability and river density have served as corridors for gene flow, thereby reducing genetic differentiation and increasing migration rates among populations. Similarly, human population density and road density may have increased population connectivity by facilitating dispersal among plant populations, either directly through movement of plant material and/or indirectly, by providing corridors for extant frugivores that move along road verges. Alternatively, humans and roads may have fragmented landscapes and reduced population connectivity, thereby increasing population differentiation and decreasing migration rates among populations.

To test these hypotheses, we integrated palm population-level genomic data with past inferences of megafrugivore distributions, current frugivore distributions, landscape and human impact variables. We applied linear mixed effect models to disentangle the frugivory-related, landscape-related and human-related drivers of palm population genetic differentiation and recent migration rates. Our results provide novel insights into how past interactions with megafrugivores may have influenced the genetics of plants on Madagascar.

TABLE 1 Average fruit length in cm, number of sampled populations and mean (± standard deviation, SD) population-level historical genetic differentiation (*Fst*) and recent among (M-among-pops) and within (M-within-pops) population migration rates for four palm species. For more detailed information and values calculated per population, see Tables S5 and S6 in Supporting Information.

	Fruit length (cm)	Number of populations	Mean *Fst* (SD)	Mean M-among-pops (SD)	Mean M-within-pops (SD)
Borassus madagascariensis	30	3	0.192 (0.035)	0.031 (0.007)	0.938 (0.015)
Hyphaene coriacea	5.5	7	0.129 (0.034)	0.025 (0.001)	0.847 (0.008)
Bismarckia nobilis	4.4	8	0.125 (0.039)	0.029 (0.019)	0.798 (0.047)
Chrysalidocarpus madagascariensis	1.3	7	0.188 (0.061)	0.025 (0.004)	0.847 (0.015)

2 | MATERIALS AND METHODS

2.1 | Sample collection and library preparation

We sampled leaf tissue from 25 natural populations of four palm species that differ in fruit sizes (large megafruits: *B. madagascariensis*; medium-sized megafruits: *H. coriacea*, *B. nobilis*; small fruits: *C. madagascariensis*) throughout their distribution in the western part of Madagascar during July, August and September of 2019 (Figure 1, Table 1, Table S1). More details on sampling for each species are provided in Table S1. The uneven number of sampled populations across the species mirrors the natural distribution and abundance of each species in Madagascar, with *B. nobilis*, *H. coriacea* and *C. madagascariensis* having wider distributions than *B. madagascariensis*. The latter is restricted and more fragmented in its distribution, and hence endangered according to the International Union for Conservation of Nature Red List of Threatened Species (IUCN, 2012). Fieldwork was organized in collaboration with Kew Madagascar Conservation Centre and the Botanical and Zoological Garden of Tsimbazaza, with collecting permits 158/19/MEDD/SG/DGEF/DGRNE and 172/19/MEDD / SG/DGEF/DGRNE.

Genomic DNA was extracted using the DNeasy Plant Kit (Qiagen) following the manufacturer's instructions. DNA was quantified using a Qubit 4.0 fluorometer (Invitrogen), and five to seven individuals per population per species that contained the highest DNA concentration were selected for genomic library preparation. DNA samples were processed following a modified version of the double-digest restriction-site-associated DNA sequencing (ddRAD) protocol (Peterson et al., 2012). For each individual, 200 ng of DNA was used and digested with two restriction enzymes, EcoRI and MspI. Then, barcodes were ligated and 48 individuals were pooled per library and purified with the Promega-Wizard Kit. This was followed by size selection on a 2% agarose gel using Pippin Prep electrophoresis (Sage Science) to obtain fragment lengths between 350 and 450 bp. Subsequently, polymerase chain reaction (PCR) was used to increase the number of fragments by performing 24 individual PCRs for each pool (12 cycles each), which were later combined and purified using Promega–Wizard Kit and AMPure beads. The quality of each library was controlled on a Bioanalyzer 2100 (Agilent Technologies). The libraries were sequenced with a unique barcode and a unique index per individual in paired-end (PE 150 bp) using an Illumina HiSeq 2000 at the Helmholtz Centre for Environmental Research—UFZ, Department Computational Biology, Leipzig, Germany.

2.2 | ddRAD data filtering and SNP calling

Raw sequence demultiplexing, quality filtering and genotyping were performed using the software Stacks v2.53 (Catchen et al., 2011; Rochette et al., 2019), on the EVE High-Performance Computing platform at the Helmholz Centre for Environmental Research, Leipzig. Demultiplexing was performed by running *process_radtags*,

removing reads with uncalled bases or low-quality scores, and rescuing barcodes and RAD-tags (−clean, −quality, −rescue). From this step onwards, all analyses were carried out for each species independently. Stacks' parameters (−M, −n, −m) were optimized per species following the r80 method by Paris et al. (2017) with the de-novo_map.pl program from Stacks v2.53. More detailed information on parameter settings for each species can be found in Appendix S1 from Supporting Information.

We then used the *populations* program with the population map, which provides information on the natural populations (i.e. sampling sites) of individuals (Figure 1, Figure S1, Table S1). To control for false-positive loci, we used -r 0.75 (a locus must be found in 75% of individuals of a single population to be processed), −min-maf 0.05 (a variable site must possess a minimum minor allele frequency of 5% to be included) --max-obs-het 0.7 (a variable site must have observed heterozygosity <0.7 to be included) and --write-single-snp (restricting data analysis to the first SNP per locus). We used VCFtools/0.1.16 to explore the data for variant mean depth (--site-mean-depth) and missing data per individual (--missing-indv). To mitigate missing data and recover a higher number of loci, we followed the protocol of Cerca et al. (2021) and deleted 'bad apples' (i.e. individuals with more than 90% missing data) from the population map, and reran *populations*. Then, we filtered the data with VCFtools to remove indels (--remove-indels), retaining only sites with less than 50% missing data (--max-missing 0.5), retaining only biallelic SNPs (--min-alleles 2, --max-alleles 2), with a minimum mean read depth of 5 and maximum mean depth of 100 (--min-meanDP 5, --max-meanDP 100, --minDP 5, --maxDP 100). This approach allowed us to be more selective about the retained loci and SNPs required for model assumptions in downstream analyses, and also to recover a higher number of loci after the removal of the 'bad apples' (Table S2).

2.3 | Population structure and evolutionary relationships of palm populations

We assessed population structure within species using ADMIXTURE v.1.3.0 (Alexander et al., 2009). To run ADMIXTURE, we modified the vcf file created by *populations* (by transforming chromosome from numeric to non-numeric), and used PLINK v.1.90 (Purcell et al., 2007) to obtain a bed file, which was used in ADMIXTURE. We assumed 1–10 genetic clusters (K), with a total of 20 replicates for each K, and we determined the best K by estimating the 10-fold cross-validation error.

To explore evolutionary relationships between all individuals and populations of each species, we reconstructed phylogenetic trees for each species. Phylogenetic reconstruction was based on the maximum likelihood (ML) method using RAxML v.8.2.12 (Stamatakis, 2014). A de novo alignment was created with the *populations* program, using a population map as input file with each individual assigned to a different population (as many populations as individuals), and with the option --phylip-var. This created an alignment including only variable sites, which was the input file for
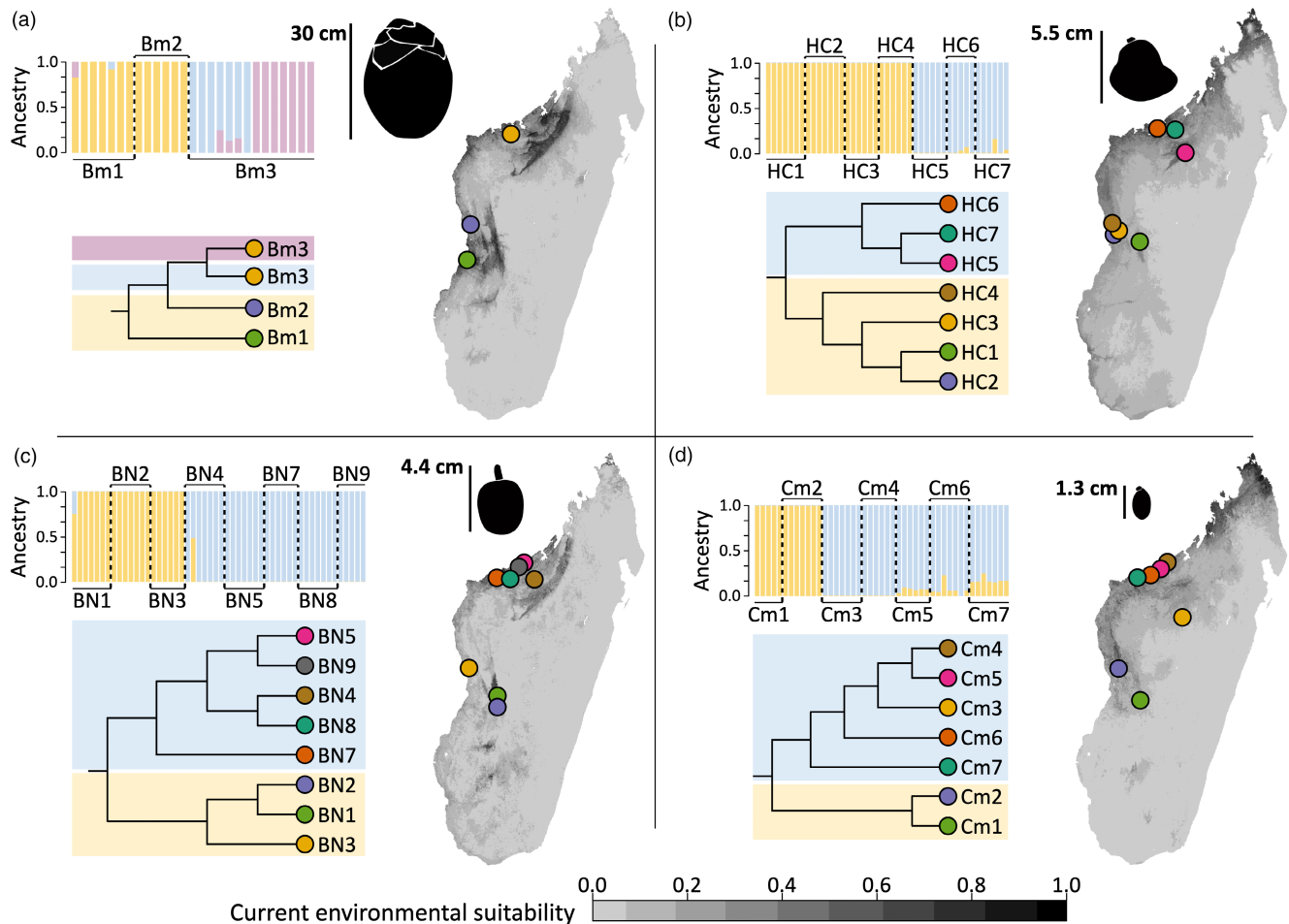
FIGURE 1 Palm population distribution, evolution and genetic structure. The sampled palm populations for each of the four species are depicted against the environmental suitability maps resulting from the ensemble species distribution models. Darker colours indicate more suitable areas (legend shown at the bottom of the figure). The reconstructed phylogenetic trees illustrate phylogenetic relationships among populations for each species. Each population has a unique colour that matches both the phylogeny (tip) and sampling locality on the map. The background colours in the phylogenetic trees refer to the two genetic clusters recovered with the ADMIXTURE analysis, illustrated by the admixture bar plots above the phylogeny for each species. Each horizontal bar represents a different individual, with the identified admixture proportions among the clusters. Clusters reflect the north/south separation of populations: the 'southern' group of populations in yellow and the 'northern' group of populations in blue. Average fruit length for each species is also indicated. (a) *Borassus madagascariensis* (Bm), (b) *Hyphaene coriacea* (HC), (c) *Bismarckia nobilis* (BN) and (d) *Chrysalidocarpus madagascariensis* (Cm).

the RAxML software. We used the '-f a' and the 'autoMRE' options, which determine when enough bootstrap analyses have been performed to reach convergence, using the majority-rule consensus tree criterion. We used the GTRGAMMA model of rate heterogeneity and the Lewis correction for SNP ascertainment bias (Tamuri & Goldman, 2017). The best tree for each species was visualized in R and rooted with function *midpoint.root* from the 'phytools' R package (Revell, 2012).

2.4 | Species distribution models for palms

To identify population connectivity through environmental suitability for each species, we predicted the potential current distributions using ensemble species distribution models (SDMs; Araújo et al., 2019) with the 'sdm' v1.2–32 R package (Naimi & Araujo,

2016). We followed the workflow from Barratt et al. (2021), and the assembled present-day palm species occurrence data from Méndez, Viana, et al. (2022a), complemented with occurrence records from Global Biodiversity Information Facility (GBIF; https://www.gbif.org/). The GBIF data were added to improve accuracy of the niche models by maximizing the number of unique occurrences. Since species occurrence data tend to be spatially clustered due to sampling biases, we thinned the data by removing occurrences that were closer than 5 km from each other, using the 'spThin' v0.2.0 R package (Aiello-Lammens et al., 2015). After thinning the data, the final data set included 37 occurrence records for *B. madagascariensis*, 48 records for *B. nobilis*, 64 records for *H. coriacea* and 80 records for *C. madagascariensis* (Figure S2).

We made an a priori selection of 21 environmental predictors that we considered important for palm distributions (see Appendix S2 in Supporting Information). To avoid collinearity

between predictors, we used function *vifstep* from R package 'usdm' v2.1–7 (Naimi et al., 2014), which calculates the variance inflation factor (VIF) for the group of predictors, and excludes strongly correlated variables (VIF > 10) in a stepwise procedure. This procedure was repeated for each species, resulting in 10 predictors for *B. madagascariensis*, nine predictors for *B. nobilis*, 13 for *H. coriacea* and 14 for *C. madagascariensis* (for specific predictors per species see Appendix S2 in Supporting Information). To generate environmental suitability maps, we constructed an ensemble model for each species based on five commonly used species distribution modelling methods: generalized linear models (Nelder & Wedderburn, 1972), generalized additive models (Hastie & Tibshirani, 1990; Wood, 2011), boosted regression trees (Friedman, 2001; Miller et al., 2016), random forests (Breiman, 2001; Liaw & Wiener, 2002) and maximum-entropy (Hijmans et al., 2023; Phillips et al., 2006) implemented in the 'sdm' R package, with function *sdm*. To generate background points (pseudo-absences) that sufficiently represent the available environmental and geographic space for each species, we created a 100-km buffer around presence points with function *background.buffer* from package 'ENMTools' v1.1.2 (Warren & Dinnage, 2024), and then generated 1000 random points inside that buffer (Figure S2; VanDerWal et al., 2009) with function *randomPoints* implemented in package 'dismo' v1.3–14 (Hijmans et al., 2023). Models were calibrated on 30% of the data, and the remaining 70% was used to evaluate model accuracy, using the 'subsampling' partitioning method, and by repeating each model five times. The final ensemble models were built by weighted-averaging the individual models proportionally to their area under the curve (AUC), only including models that performed adequately (AUC >0.7 and TSS >0.5; Swets, 1988) with function *ensemble*.

2.5 | Genetic differentiation and recent migration rates

Historical pairwise population genetic differentiation (*Fst*) was calculated using *populations* in Stacks v2.53 with the --fstats option. Recent migration rates (i.e. gene flow) between populations (M-among-pops) and within populations (M-within-pops) were calculated using BayesAss3 (Wilson & Rannala, 2003), implemented in BA3-SNPS V.3.0.4 (Mussmann et al., 2019). BayesAss3 uses allele frequency data and Markov chain Monte Carlo (MCMC) simulations to estimate migration rates, defined as the proportion of immigrants from a donor to a sink population. Optimal final mixing parameters for migration rates (m), allele frequencies (a) and inbreeding coefficients (f) were determined by conducting 10 replicates with the *BA3-SNPS-autotune.py* script as recommended by Mussmann et al. (2019) for each species (for more information about optimizing final mixing parameters see Appendix S3 in Supporting Information). Subsequently, BA3-SNPS was run for 10 million generations, discarding the first million generations as burn-in and sampling every 100th generation.

We estimated the 95% credible intervals on migration rates by calculating the mean ± 1.96 × standard deviation. Migration rates that included zero within their 95% credible intervals were considered non-significant. Chain convergence was verified using Tracer V 1.7.2 (Rambaut et al., 2018). Output matrices from BA3-SNPS were converted into gene flow plots with the use of the R package 'circlize' v. 0.4.6 (Gu et al., 2014).

As BayesAss3 cannot handle missing data, we conducted missing data imputation for each species prior to running BA3-SNPS. This was done using LinkImputeR v1.2.4 (Money et al., 2017) by prioritizing to retain the maximum number of individuals and loci, and optimizing for accuracy. For more detailed information about imputation of genetic data see Appendix S4 in Supporting Information. The imputed datasets were exclusively used for BayesAss3 analyses.

2.6 | Explanatory variables for palm population genetics

We assembled data for the potential drivers of palm population connectivity, including extant and extinct frugivore distributions, landscape-related (forest cover, rivers) and human-related (road density, human population density) variables. Furthermore, average fruit length for each species was extracted from the PalmTraits 1.0 database (Kissling et al., 2019). We identified frugivore distributions by assembling polygon ranges from the IUCN, ver. 2020–2 (IUCN, 2020) for all 93 extant Malagasy frugivore species identified in Méndez, Viana, et al. (2022a). Similarly, for the 14 extinct megafrugivore species (giant lemurs and elephant birds), we used the reconstructed historical distribution maps from Méndez, Viana, et al. (2022a), which were modelled using co-occurrence of extant and extinct taxa across fossil sites. For the two extinct giant tortoises, we used the historical ranges inferred by Pedrono et al. (2013). Climatic variables were retrieved from https://www.worldclim.org/ (1 km resolution), percentage of forest cover for 2010 and other environmental variables were extracted from https://madaclim.cirad.fr/, rivers from http://landscapeportal.org/, and human-related variables (i.e. road density and human population density from 2010) from Venter et al. (2016) (at 1 km resolution).

To estimate connectivity between populations due to seed dispersal, we calculated the number of shared extinct and extant (mega)frugivore species between population pairs for each species (n = 73 pairs of populations). Furthermore, we measured the forest cover, river density, human population density, road density and environmental suitability (resulting from the suitability maps created by the species distribution models) among all population pairs. To achieve this, we used the coordinates of each population to generate a straight spatial line connecting each population pair, with function *SpatialLines* from R package 'sp' v2.1 (Pebesma & Bivand, 2005). Then, we obtained raster values along each line with function *extract* from R package 'raster' v3.6–26 (Hijmans, 2023), and averaged the raster-based environmental suitability, landscape- and human-related variables that connected each population pair.

2.7 | Determinants of historical genetic differentiation and recent migration rates

We used linear mixed-effects models to identify the main (fixed) frugivory-related (extant frugivores, extinct frugivores), landscape-related (forest cover, rivers, environmental suitability) and human-related (road density, human population density) effects on population genetic differentiation (Fst) and recent migration rates (M-among-pops) between palm populations. We included the palm species identity as a random effect to account for interspecific variability, thereby acknowledging the unique genetic background and evolutionary history inherent to each species. Furthermore, our data set is structured around pairwise comparisons of populations, where each population of each species is involved in multiple comparisons, leading to the non-independence among the data points. To address this, we incorporated a maximum-likelihood population effect (MLPE) correlation structure to correct for the non-independence in pairwise distance matrices (Clarke et al., 2002). Linear mixed effect models were fitted with function *lme* from the 'nlme' v3.1 R package (Pinheiro et al., 2017), and the MLPE correlation structure was included with R package 'corMLPE' v0.0.3 (https://github.com/ns-pope/corMLPE).

Furthermore, to assess differences in drivers of genetic differentiation and migration rates between megafruit and small-fruited palms, we repeated the linear mixed effects models for subsets of populations with megafruits (*n* = 52 population pairs) and small fruits (*n* = 21 population pairs).

Correlations between variables were assessed before modelling (cor <0.7; Figure S3), and we standardized all predictor variables using function *scale* from the 'base' R package to compare their effect sizes in the models.

3 | RESULTS

3.1 | ddRAD data filtering and SNP calling

We obtained paired-end Illumina reads for 28 individuals of *B. madagascariensis* (228,464 retained reads), 47 of *H. coriacea* (513,399 retained reads), 63 of *B. nobilis* (866,044 retained reads) and 46 of *C. madagascariensis* (665,801 retained reads, Table S2). Stacks output haplotype files contained between 5091 (*B. madagascariensis*) and 26,435 (*C. madagascariensis*) variant sites (Table S2). After filtering for 'bad apples' (i.e. removing individuals with more than 90% missing data), one individual of *B. madagascariensis*, two of *H. coriacea*, 11 of *B. nobilis* and one of *C. madagascariensis* were removed. After excluding non-bi-allelic loci, filtering for minor allele frequencies, maximum observed heterozygosity, and excluding sites with more than 50% missing data, final numbers of SNPs per species used for subsequent analyses (Table S2) were as follows: 1926 (*B. madagascariensis*), 12,898 (*H. coriacea*), 5519 (*B. nobilis*) and 11,589 (*C. madagascariensis*). The demultiplexed ddRAD sequences are available from ENA (European Nucleotide Archive) project PRJEB56299.

3.2 | Population structure and evolutionary relationships of palm populations

ADMIXTURE analyses evidenced low intraspecific genetic structure across all four species (Figure 1), with K = 2 or K = 3 genetic clusters for each species. The two clusters were generally consistent with the geographical separation of northern and southern populations (Figure 1). In *B. madagascariensis* and *C. madagascariensis*, results of cross-validation error between K = 2 and K = 3 were very similar (*B. madagascariensis*: CV-error-K2 = 0.686, CV-error-K3 = 0.679; *C. madagascariensis*: CV-error-K2 = 0.586, CV-error-K3 = 0.588; see Figures S4 and S5), and it was therefore difficult to conclude the most appropriate K value for these two species.

The phylogenetic reconstruction showed additional information on relationships between individuals and populations into clades, generally supporting clades of individuals sampled from the same 'natural' population (Figure 1, see Figure S6 for the full phylogenetic trees). For *B. madagascariensis*, northern populations were derived from the south, and the phylogeny supported divergence within the northern Bm3 population, even though individuals were only 2 km apart (Table S1, Figure 1 and Figure S6a). This is consistent with the ADMIXTURE clusters (i.e. K = 3 separates population Bm3 into two clusters, Figure 1). For the other species, the northern and southern populations were recovered as sister clades in the phylogeny. For *B. nobilis*, not all individuals clustered within a clade that reflected their natural population, suggesting high admixture between populations (especially for BN1-BN2 and BN4-BN8, Figure S6c). For the small-fruited *C. madagascariensis*, population Cm3 was a separate cluster when considering K = 3 (Figure S5). This population is located geographically between the northern and southern populations, but is not sister to the northern or southern clade; instead, it is sister to two (out of four) northern populations (Figure 1, Figures S5 and S6d).

3.3 | Species distribution models for palms

Species distribution models generally showed good performance (AUC >0.8 and TSS >0.5; Table S3). A mix of temperature- and precipitation-related variables were the most important for the species distributions and environmental suitability of these palms (Figure 1, Table S4). Specifically, maximum temperature of the warmest month, precipitation seasonality, precipitation of the driest month and temperature seasonality, were consistently important for shaping distributions across species (Table S4).

3.4 | Genetic differentiation and recent migration of palm populations

We revealed that the small-fruited palm species displayed the highest value of genetic differentiation among populations (Cm1-Cm4: Fst = 0.287; Figure S7, Table S5), and showed high variance in genetic differentiation (Table 1 and Table S5, Figures S7 and S8).

However, the highest average genetic differentiation was found in the large-sized megafruit palm *B. madagascariensis* (Fst = 0.192; Table 1, Figure S8), while the medium-sized megafruit species, *B. nobilis* and *H. coriacea*, exhibited the lowest average Fst (Fst = 0.125 and Fst = 0.129, respectively; Table 1, Figure S8). We detected overall low migration rates between populations for all species (mean M-among-pops across all four species = 0.027; Table 1 and Table S6, Figure S9). The only statistically significant migration rate between populations was detected from population BN8 to population BN4 in *B. nobilis* (M-among-pops [BN8-BN4] = 0.154; Table S6, Figure S9). Most gene flow occurred within populations, with the large-sized megafruit *B. madagascariensis* showing the highest average within population migration (M-within-pops = 0.956; Table 1 and Table S6, Figure S9).

3.5 | Determinants of genetic differentiation and recent migration among palm populations

The model predictors accounted for a considerable proportion of the variance in historical genetic differentiation (Fst) of palm populations (all species: conditional $R^2 = 0.49$, marginal $R^2 = 0.24$; megafruit-only: conditional $R^2 = 0.34$, marginal $R^2 = 0.34$; small fruit-only: Adjusted $R^2 = 0.83$; Table S7). In contrast, model predictors explained less variation in recent among-population migration rates (all species: conditional $R^2 = 0.19$, marginal $R^2 = 0.19$; megafruit-only: conditional $R^2 = 0.23$, marginal $R^2 = 0.21$; small fruit-only: Adjusted $R^2 = 0.43$; Table S8). We found support for the hypothesis (H1) that historical long-distance dispersal events by megafrugivores have left imprints on the genetic differentiation of palm populations (Figures 2 and 3). Specifically, the model including all palm species indicated that populations that shared more past (now extinct) megafrugivore species had lower genetic differentiation (Fst) ($\beta = -0.17$, $p = 0.042$; Figures 2 and 3, Table S8). When only including megafruit palm species, this trend was visible but not significant ($\beta = -0.15$, $p = 0.195$; Figures 2 and 3, Table S8), while for the small-fruited palm the effect was reversed ($\beta = 0.54$, $p = 0.030$; Figure 2, Table S8). In comparison, small-fruited populations that shared more extant frugivore species also had lower genetic differentiation (Fst) ($\beta = -0.38$, $p = 0.043$; Figures 2 and 3, Table S8), but genetic differentiation increased rather than decreased with shared extant frugivores for megafruit palms or when all species were combined (all palms: $\beta = 0.19$, $p = 0.042$; megafruit-only: $\beta = 0.28$, $p = 0.040$; Figures 2 and 3, Table S8).

We also found support for the hypotheses that landscape-related features (H2) and human impact (H3) have shaped the genetics of palm populations on Madagascar. Specifically, populations that were connected through higher environmental suitability had lower genetic differentiation (Fst) for all models (all species: $\beta = -0.44$, $p < 0.001$; megafruit-only: $\beta = -0.57$, $p < 0.001$; small fruit-only: $\beta = -1.40$, $p < 0.001$; Figure 2, Table S7). Similarly, forest cover and river density led to decreased genetic differentiation for all palms and megafruit-only palms (all species: forest cover: $\beta = -0.14$,

$p = 0.016$, river density: $\beta = -0.14$, $p < 0.001$; megafruit-only: forest cover: $\beta = -0.21$, $p = 0.005$, river density: $\beta = -0.17$, $p = 0.005$; Figure 2, Table S7), but not for the small-fruited palm (Figure 2, Table S7). Finally, populations connected through higher road densities had lower genetic differentiation (all palms: $\beta = -0.10$, $p = 0.033$; Figure 2, Table S7), but human population density had no significant effect on genetic differentiation (Figure 2, Table S7). For recent migration (M-among-pops) the only significant predictor was road density, with higher road densities increasing migration rates in the small-fruited palm ($\beta = 1.09$, $p = 0.048$; Figure 2, Table S8). However, there were tendencies for river density and human population density to increase migration among palm populations (all species combined; Figure 2, Table S8).

4 | DISCUSSION

We disentangled the determinants of present-day western Malagasy palm population genetics, including frugivory-related (H1), landscape-related (H2) and human-related (H3) predictors. The combined effect of these variables probably contributed to the broad-scale north/south genetic structuring among all western Malagasy palms (Figure 1). We found support for the hypothesis (H1) that the legacy of megafrugivores regularly achieving long dispersal distances is still reflected in the genetics of palms that were once dispersed by such large animals (Figures 2 and 3). Specifically, we found that a higher number of shared megafrugivore species among palm populations in the past led to decreases in genetic differentiation (Fst), thus suggesting higher levels of historical gene flow. In comparison, shared extant frugivores among populations were associated with lower Fst values in the small-fruited palm species only (Figures 2 and 3). Additionally, forest cover, river density and environmental suitability increased historical connectivity among populations, reflected by lower Fst values (H2, Figure 2), possibly by facilitating dispersal through connected habitats (i.e. 'stepping stones') or via rivers. Finally, higher road densities among populations decreased genetic differentiation and increased migration rates among populations (H3, Figure 2), emphasizing the potential role of human-mediated seed dispersal in shaping palm population genetics.

4.1 | Study limitations and considerations

Although our results illustrate the importance of frugivory- and landscape-related processes in shaping palm genetics, our sample size (four species, 25 populations, 73 population pairs) is limited. Furthermore, it is difficult to exclude the possibility that other co-varying or confounding variables, such as pollination systems, reproductive strategies (dioecy versus monoecy), secondary seed dispersal, range size, historical demography, and life history traits (e.g. lifespan, resistance to fire) may have additionally influenced gene flow, migration rates, and genetic differentiation among
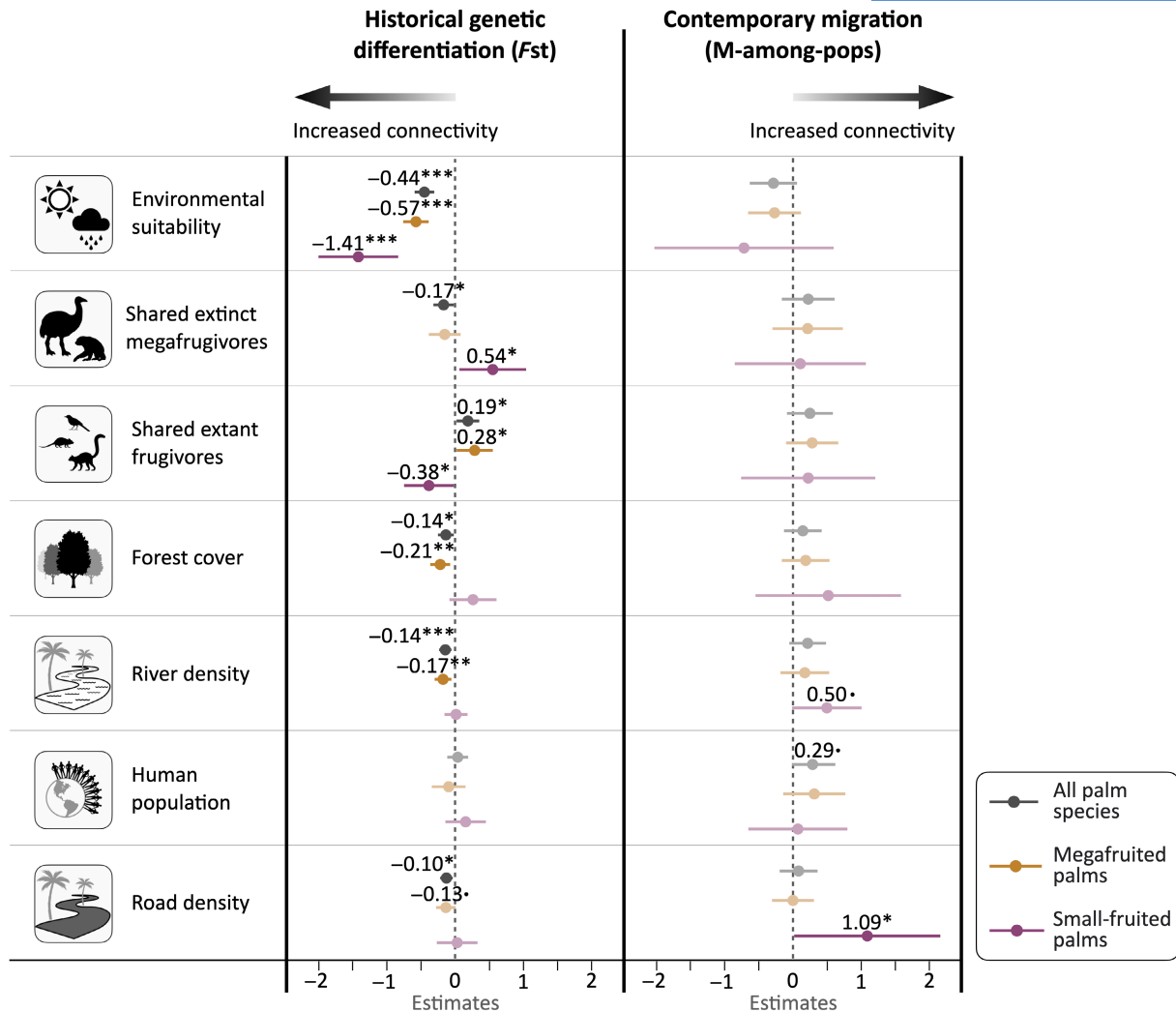
FIGURE 2 Determinants of historical genetic differentiation and recent migration rates among Malagasy palm populations. Mean effect estimates (circles) of fixed effects from linear mixed effect models are shown with the 95% confidence intervals (bars) on historical among population genetic differentiation (F_{st}; left panel) and on recent among population migration rates (M-among-pops; right panel) for all populations combined ($n = 73$ population pairs; in grey), for megafruit palm populations (three species, $n = 52$ population pairs; in yellow) and for small-fruited palm populations (one species, $n = 21$ population pairs; in purple). Environmental suitability, forest cover, river density, human population density and road density were calculated as average values along a straight-line connecting population pairs for each species. Extinct and extant (mega-)frugivores were calculated as the number of shared species between population pairs. The significance of each predictor variable is given ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ·$p < 0.1$. Non-significant effects (p-values >0.05) show higher transparency. See Tables S7 and S8 for detailed results of the models.

populations (Ellegren & Galtier, 2016; Hamrick et al., 1992). For example, the small-fruited palm is monoecious, whereas the three megafruit palms are dioecious, and monoicous species are expected to show higher genetic differentiation than dioecious species because monoecious plants are more likely to self-pollinate, while dioecious species are obligated out-crossers (Ballesteros-Mejia et al., 2016). However, as this trait does not show within-species variation, we could not evaluate its effect in our models. Thus, expanding this research to include other plant families within Madagascar with varied reproductive strategies and pollination systems would provide valuable comparative data to further understand how past and ongoing interactions with frugivores, traits and evolutionary history have shaped the genetics of

Malagasy plants. Nevertheless, our study provides a solid foundation to evaluate questions at the interface of population genomics and ecology, due to the use of genome-wide data, a comparative framework to evaluate our hypotheses, and a robust statistical analysis.

4.2 | Long-distance dispersal events by megafrugivores have increased historical gene flow in Malagasy palms

Our results support the hypothesis (H1) that long-distance dispersal events provided by megafrugivores in the past may have increased
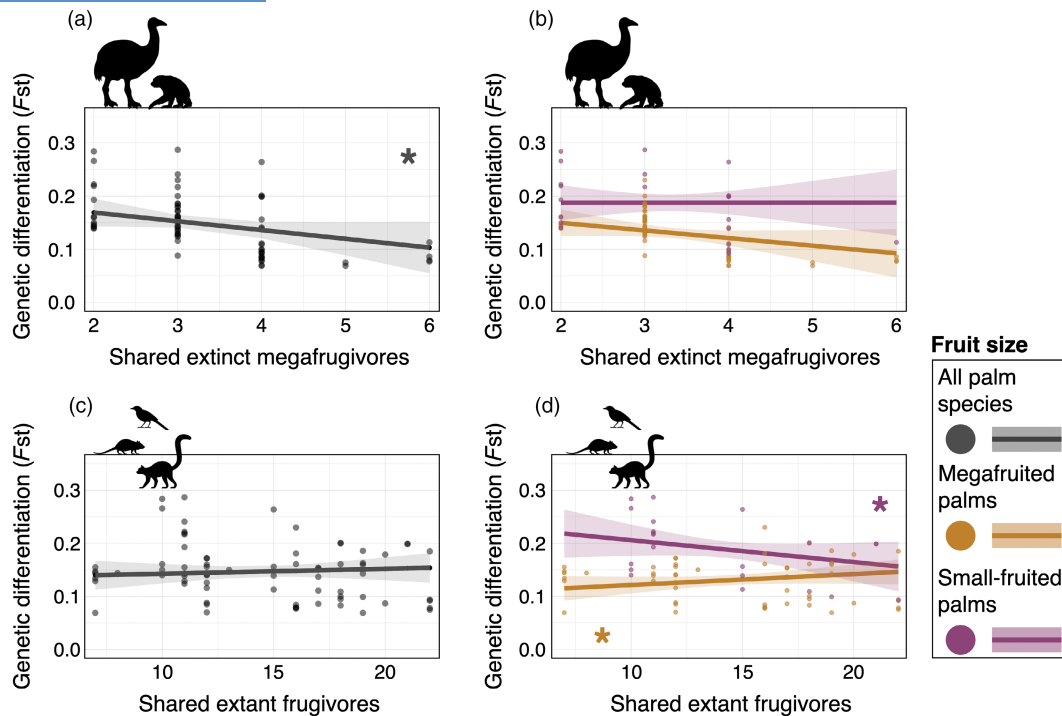
FIGURE 3 Extinct megafrugivores have shaped genetic differentiation in Malagasy palms. Linear relationship between historical genetic differentiation (F_{st}) and the number of shared extinct (a, b) or extant (c, d) frugivore species between palm population pairs for all four species ($n = 73$ population pairs, grey line in a, c), for megafruit palms (three species, $n = 52$ population pairs, purple line in b, d) and for the small-fruited palm (one species, $n = 21$ population pairs, orange line in b, d). Genetic differentiation decreases with more shared extinct frugivores between populations (a), mostly driven by megafruited palms (b), while extant frugivores only decrease genetic differentiation in the small-fruited palm (d). Significance of the relationship is shown: $* = p < 0.05$.

gene flow, and therefore decreased genetic differentiation of palm populations (Figures 1–3). This is evidenced by the negative correlation between the number of shared megafrugivore species (i.e., giant lemurs, elephant birds and giant tortoises) between population pairs and genetic differentiation (F_{st} values; Figures 2 and 3). Additionally, our results highlight the role of current Malagasy frugivores in increasing gene flow among populations, particularly for the small-fruited palm (Figures 2 and 3). While the correlation between shared megafauna and genetic differentiation is striking, it is possible that past megafaunal distributions—which were inferred from fossil co-occurrences with extant species and their current distributions (Méndez, Viana, et al., 2022a)—co-vary with distance between population pairs, environmental suitability and human impact, that is, factors that also directly interact with palm genetics (Figure 2 and Figure S3). Although correlation coefficients between geographical distance and our pairwise model predictors were generally low (cor <0.7, except between distance and environmental suitability with cor = 0.8; Figure S3), it remains challenging to disentangle causation from correlation. Nevertheless, the comparison with extant frugivores (Figure 3) is valuable in this respect, because it is subjected to similar distance-based correlations, but only showed the expected negative relationship with genetic differentiation in the palm species with small fruits. Indeed, fruits of this species are dispersed by extant frugivores such as *Eulemur macaco* (Adany et al., 1994). Although previous studies have illustrated the important role of

frugivory for seed shadows and gene flow among plant populations (Jordano et al., 2007), our findings suggest that past dispersal events by now extinct frugivores may have left imprints in plant genetic population structure as well.

4.3 | Landscape-related drivers of Malagasy palm genetic differentiation

Genetic differentiation of Malagasy palms was mostly influenced by environmental suitability, with higher suitability leading to more connectivity, gene flow, and hence lower genetic differentiation (Figure 2). Temperature- and precipitation-related variables may have shaped the suitability landscape for palms, as these came out as the most important variables in the species distribution models (Table S4). Furthermore, forest cover and rivers were identified as corridors for gene flow for the megafruit palm species (Figure 2). Whereas forests may serve as 'stepping stones' for gene flow, rivers may have provided an alternative dispersal mechanism for large-fruited palms (Devey et al., 2013; Gallaher et al., 2015; Guimarães et al., 2008). Stepping stones, defined as small, isolated habitat patches that facilitate the movement of organisms across fragmented landscapes, are crucial for species' long-distance dispersal and range expansion (Saura et al., 2014). Forest corridors may also influence frugivore distributions due to their habitat requirements, with continuous forests

harbouring the largest (past and present) frugivores (Chiarello, 1999; Magioli et al., 2021). Such large-bodied frugivores are able to disperse the largest fruits, whereas smaller-bodied or volant frugivores, such as *Alectroneas madagascariensis* or *Coracopsis nigra*, which are known seed dispersers of small-fruited palm species (Bollen et al., 2004a, 2004b; Méndez, Viana, et al., 2022a), may dominate the more fragmented habitats in Madagascar.

4.4 | Maintenance of gene flow in Malagasy palms after the megafrugivore extinctions

Our results suggest that the loss of dispersal services by megafrugivores since their extinctions ca. 1000 years ago has not yet manifested itself in population genetic differentiation. This contrast with observed patterns in the Canary Islands, where defaunation and downsizing of frugivorous lizards led to decreased plant population connectivity (Pérez-Méndez et al., 2018). There are two possible explanations for this.

First, it is possible that gene flow between Malagasy palm populations has been maintained by alternative dispersal strategies, such as barochory, hydrochory, secondary seed dispersal (Blanco et al., 2019), or pollen dispersal (Giombini et al., 2017). For instance, we found that river density decreased genetic differentiation between megafruit palm populations, indicating the potential role of waterways in seed dispersal (Figure 2). Furthermore, secondary seed dispersal by animals such as rodents, although less documented in Madagascar (but see Méndez, Viana, et al., 2022a; Razafindratsima, 2014), could be contributing to maintaining gene flow, as seen in Neotropical megafruit species (Collevatti et al., 2019). This may also apply to Malagasy plant species with megafruits, although we did not find that extant frugivore diversity (including secondary seed dispersers) increased connectivity among megafruited palm populations (Figures 2 and 3).

However, human use, and thereby (occasional) dispersal of fruits by humans, may have facilitated gene flow between palm populations (H3, Figure 2). Indeed, higher human population density showed an increasing trend with recent migration between palm populations, and higher road density increased recent migration (and hence connectivity) among populations of the small-fruited palm (Figure 2). Particularly, palms essential to local livelihoods in western Madagascar, such as *B. nobilis* and *H. coriacea* (Rakotoarinivo et al., 2020), showed the overall lowest genetic differentiation, highest recent migration rates and highest admixture (Figure 1, Figure S6, Table 1), suggesting enhanced gene flow due to human utilization. The level of human use of the palms included in our study negatively correlated with their average *F*st values (i.e. higher level of human use, lower genetic differentiation; see Rakotoarinivo et al., 2020 for level of utilization; Figure S10). To illustrate, *B. madagascariensis* (i.e. species bearing the largest fruits within the palm family in Madagascar) is the least used by humans and showed the highest genetic differentiation (Table 1, Figure 1 and Figure S8). This species may experience reduced gene flow

due to limited human-mediated dispersal, smaller number of individuals, and therefore, more fragmented populations. In line with our results, human-mediated dispersal (based on diet) has been shown to be the most important dispersal mode for a large proportion of large-fruited Neotropical species (that used to rely on now extinct megafrugivore-mediated dispersal), leading to larger geographical range sizes of large-seeded megafruit species used by humans than of species not used by humans (Van Zonneveld et al., 2018). Similarly, human use and cattle were probably important for the propagation, gene flow, and long-distance dispersal of a Mediterranean palm (i.e. *Chamaerops humilis*; Delibes et al., 2019; Guzmán et al., 2017).

Second, it is possible that the relatively recent timing of the megafauna extinctions (ca. 1000 years; Crowley, 2010), did not provide enough time for significant genetic differentiation to emerge among palm populations (Crowley, 2010). The concept of 'extinction debt' (Tilman et al., 1994) suggests that long-lived species may show delayed genetic responses to reduced gene flow. Given an estimated palm generation time of 40 years (Gaut et al., 1992), the 25 generations since the disappearance of the megafauna may not have been sufficient for detectable genome-wide changes. Similarly, a meta-analysis of 102 Neotropical plants revealed that, despite the significant megafauna loss during the Late Quaternary extinction wave (more than 10,000 years ago), there was no difference in genetic differentiation of species formerly dispersed by megafrugivores compared to non-megafrugivore dispersed species (Collevatti et al., 2019). Instead, pollination mode, mating and breeding systems explained population genetic variation. The detrimental effect of hindered gene flow may therefore be context dependent, or only become evident over time.

5 | CONCLUSIONS

We show that long-distance dispersal events provided by megafrugivores in the past may have increased gene flow, and therefore decreased genetic differentiation and genetic structure among palm populations on Madagascar. These results underscore that historical events can leave imprints in the genetics of populations and species today. Our population genetic findings align with macroecological and macroevolutionary evidence at broad spatial and temporal scales that illustrate how pre-Quaternary climate or megafrugivores have influenced current plant assemblage composition (e.g. beta diversity) and trait (e.g. fruit size) distributions (Blach-Overgaard et al., 2013; Lim et al., 2020; Méndez, Viana, et al., 2022a; Wölke et al., 2023; Xu et al., 2023). Nevertheless, the extinction of all megafrugivores on Madagascar may affect current and future gene flow among vertebrate-dependent plant populations, with potentially detrimental effects on the genetic diversity and adaptive potential of these species to climate change (Fricke et al., 2022). Monitoring their genetics and current dispersal mechanisms is therefore of uttermost concern (Frankham, 2022; Svenning et al., 2019).

AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST STATEMENT

Renske Onstein is an associate editor of *Journal of Ecology*, but took no part in the peer review and decision-making processes for this paper.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/1365-2745.14340.

DATA AVAILABILITY STATEMENT

Demultiplexed ddRAD sequences are available in ENA (European Nucleotide Archive) project PRJEB56299. Supporting information for this study and all data used, including (mega)frugivore species lists, climatic and human-related variables per population pair, are openly available at: https://doi.org/10.5061/dryad.pc866t1z6 (Méndez et al., 2024a). Scripts with code to reproduce all analyses and figures are available in Zenodo: https://doi.org/10.5281/zenodo.11064997 (Méndez et al., 2024b). The code to the pipeline to conduct all the bioinformatic analyses to the ddRAD data is published at Zenodo: https://doi.org/10.5281/zenodo.7362068 (Méndez, Barratt, et al., 2022b).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Parameter optimization in Stacks.

Appendix S2. Predictor variables selection for species distribution models.

Appendix S3. Optimization of final mixing parameters for BayesAss3.

Appendix S4. Imputation of genetic missing data.

Figure S1. Bioinformatics workflow.

Figure S2. Occurrences and background points for each species used for the SDMs.

Figure S3. Correlation matrices for pairwise predictors.

Figure S4. Cross validation error per species for ADMIXTURE.

Figure S5. Results from ADMIXTURE analyses showing K3.

Figure S6. Phylogenetic trees.

Figure S7. *F*st among populations.

Figure S8. *F*st, M-among-pops and M-within-pops per fruit size class.

Figure S9. Gene flow plots—BayesAss3.

Figure S10. Correlation between genetic differentiation of species and human use.

Table S1. Coordinates of the centroids of all sampled palm populations.

Table S2. Loci before and after cleaning for 'bad apples'.

Table S3. Species distribution models (SDMs) mean performance per species.

Table S4. Relative variable importance of predictors used to construct the SDMs.

Table S5. Pairwise genetic statistics and geographical distance between populations.

Table S6. Detailed results from BayesAss3 with contemporary migration.

Table S7. Results from linear mixed effect models for genetic differentiation (*F*st).

Table S8. Results from linear mixed effect models for contemporary among population migration (M-among-pops).