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Runs of homozygosity reveal contrasting histories of inbreeding across global lineages of the edible porcini mushroom, *Boletus edulis*

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Abstract

Inbreeding, the mating of individuals that are related through common ancestry, is of central importance in evolutionary and conservation biology due to its impacts on individual fitness and population dynamics. However, while advanced genomic approaches have revolutionised the study of inbreeding in animals, genomic studies of inbreeding are rare in plants and lacking in fungi. We investigated global patterns of inbreeding in the prized edible porcini mushroom *Boletus edulis* using 225 whole genomes from seven lineages distributed across the northern hemisphere. Genomic inbreeding was quantified using runs of homozygosity (ROHs). We found appreciable variation both among and within lineages, with some individuals having over 20% of their genomes in ROHs. Much of this variation could be explained by a combination of elevation and latitude, and to a lesser extent by predicted habitat suitability during the last glacial maximum. In line with this, the majority of ROHs were short, reflecting ancient common ancestry dating back approximately 200–1700 generations ago, while longer ROHs indicative of recent common ancestry (less than approximately 50 generations ago) were infrequent. Our study reveals the inbreeding legacy of major climatic events in a widely distributed forest mutualist, aligning with prevailing theories and empirical studies of the impacts of historical glaciation events on the dominant forest tree species of the northern hemisphere.

KEYWORDS
Boletus edulis, ectomycorrhizal fungi, inbreeding, last glacial maximum, porcini, runs of homozygosity

1 | INTRODUCTION

Since the time of Darwin, inbreeding, the increase in genome-wide homozygosity that occurs due to the inheritance of identical by descent (IBD) segments from a common ancestor, has been recognised as a major evolutionary force influencing mate choice, dispersal and individual fitness (Darwin, 1876; Wright, 1984). Inbreeding also shapes evolutionary dynamics at the population level, where the loss of fitness due to inbreeding depression can have profound negative demographic consequences (Charlesworth & Charlesworth, 1987; Charlesworth & Willis, 2009). Furthermore, the detrimental effects of inbreeding can be exacerbated by environmental stress (Meagher et al., 2000), meaning that inbreeding depression is often stronger in populations experiencing challenging conditions. Consequently, studies of inbreeding are essential for understanding evolutionary mechanisms and the long-term persistence of natural populations inhabiting changing environments.

Traditionally, the study of inbreeding in natural mammalian and bird populations required long-term field observations for the construction of multigenerational pedigrees (Pemberton, 2008). However, pedigrees are exceptionally difficult to build in non-animal systems and this is ultimately impossible for cryptic groups such as fungi. Many studies have therefore used the heterozygosity of small numbers of microsatellites as a proxy for inbreeding (Keller, 2002). However, microsatellite-based measures of inbreeding tend to be highly inaccurate and lack the resolution of genomic approaches (Balloux et al., 2004). Fortunately, the discovery of runs of homozygosity (ROHs) in human genomes (Gibson et al., 2006) and the falling costs of whole genome resequencing have led to a step change in the study of inbreeding in wild populations (Kardos et al., 2016). ROHs occur when a diploid individual inherits two copies of the same IBD haplotype from its parents, resulting in long homozygous tracts in the genome (Gibson et al., 2006). By summing up over these IBD segments, an individual's genomic inbreeding coefficient F_{ROH} can be quantified to base-pair resolution as the proportion of the genome in ROHs (McQuillan et al., 2008). Additionally, ROHs carry information about the antiquity of common ancestry (Kirin et al., 2010). This is because recombination breaks down IBD haplotypes at each successive generation, resulting in smaller ROHs for ancient IBD segments and longer ones for recent IBD segments (Gibson et al., 2006). Hence, ROH length frequency distributions can testify to variation in past effective population sizes.

Since their early discovery, ROHs have been used to uncover global patterns of inbreeding in humans and to elucidate their cultural and demographic determinants (Kirin et al., 2010). Genomic studies have shown that ROHs in contemporary human populations have been shaped by cultural practices that promote consanguineous marriages (Kirin et al., 2010; Pemberton et al., 2012) and carry signatures of historical founder events and population expansions that occurred as mankind spread out of Africa to colonise the world (Pemberton et al., 2012). Furthermore, ROHs have been linked to complex phenotypic traits including height, educational attainment, depression, Alzheimer's disease and cancer (Ceballos et al., 2018). More recently, the increasing availability of genome-wide data has led to a growing number of studies characterising ROHs in both domesticated and wild animals, driving advances in livestock production, evolutionary biology and conservation (Ceballos et al., 2018; Hewett et al., 2023; Kardos et al., 2018, 2023).

Despite the many advantages of ROHs, their potential has not yet been fully realised in plants and fungi. In particular, while a handful of studies have used ROHs to investigate phenotypic traits and inbreeding in plants (Barragan et al., 2024; Kumar et al., 2021; Pavan et al., 2021) we are not aware of any studies of ROHs in fungi. Such studies are urgently needed to improve our understanding of the dynamics of forest ecosystems in an era of environmental change. In particular, ROHs could be used to identify patterns of inbreeding related to past demographic events, which would help to inform contemporary management and conservation. For example, understanding past responses to major climatic events such as the last glacial maximum (LGM) could help us to predict responses to ongoing climate change (Morelli et al., 2016).

The lack of genomic studies of inbreeding in fungi is important because species such as ectomycorrhizal fungi (EMF) play pivotal roles in forest ecosystems. For example, they participate in nutrient cycling and soil carbon sequestration, and they promote tree growth and enhance resilience to environmental stress (Tedersoo et al., 2020). Thus, inbreeding and the loss of genetic diversity due to environmental change could potentially impact forest health not solely through plants, but also through fungi, but this has never been investigated. One reason for the lack of studies of ROHs in fungi could be that inbreeding research in these organisms has typically focused on the special case of selfing, where spores from the same genet cross to produce a viable dikaryon (Billiard et al., 2011). In fungal sexual outcrossing, mating compatibility is determined by alleles carried at one (bipolar mating systems) or two (tetrapolar mating systems) independent loci. For two haploid individuals to successfully mate, they must carry different alleles at these mating loci, subsequently generating diploids (or dikaryons) with heterozygous mating types (Raudaskoski & Kothe, 2010). Hence, mycological theory has long considered tetrapolar species to be less prone to selfing, as only 25% of spores generated by one individual will be cross-compatible (Billiard et al., 2011). However, this may only be true in the absence of strong selection and local adaptation, and when many niches are available (Giraud et al., 2010), which is not always the case in the context of host-dependent organisms like EMF. Furthermore, inbreeding can go beyond selfing, as it also occurs due to the inheritance of IBD haplotypes dating back either a few or many generations. The latter is important because it represents the legacy of major historical events such as the LGM.

Population genetic theory and empirical studies suggest that the LGM had a profound influence on many species of plants and animals inhabiting the northern hemisphere, shaping contemporary distributions as well as levels of genetic diversity and inbreeding (Hewitt, 2000). Around the peak of the LGM, many species experienced strong bottlenecks in small, isolated refugia, leading to a decrease in genetic diversity and an increase in homozygosity. Further diversity was subsequently lost through successive founder events

as the ice retreated and populations recolonised newly available habitats. However, the size and locations of the glacial refugia remain controversial (Tzedakis et al., 2013). For example, Hewitt (1999) argued for a small number of southerly refugia in Europe, whereas recent studies have uncovered evidence for a mosaic of refugia across a diversity of latitudes (Magri, 2008; Magri et al., 2006). While the distribution of fungi during the LGM is poorly characterised, it is likely that fungi associated with plants such as EMF tracked the geographical distributions of their hosts and were thus relegated to refugia during forest range contractions. However, empirical evidence from the fungal perspective is currently lacking, while the majority of studies of fungi to date have used small numbers of genetic markers to quantify genetic diversity, which provide insufficient resolution to resolve ROHs. Hence, population genomic studies of fungi are essential to address a major knowledge gap in the context of current global efforts to understand the resilience and adaptation of forests to large climatic events (Ibáñez et al., 2019).

Boletus edulis Bull. is arguably the most important commercially harvested wild mushroom. It has a broad geographical distribution across Eurasia and North America, where it forms obligate mycorrhizal associations with the dominant northern hemisphere forest tree species (Tremble, Brejon Lamartinière et al., 2023). However, the population dynamics of this species remain poorly understood. Globally, seven distinct and largely non-overlapping lineages have been described, five restricted to North America, one spanning both North America and boreal Asia, and one in Europe (Tremble, Brejon Lamartinière et al., 2023; Tremble, Hoffman & Dentinger, 2023). These lineages split 1.2–2.6 million years ago and show high levels of genomic divergence, despite some of them being sympatric in parts of North America and the presence of occasional gene flow (Tremble, Brejon Lamartinière et al., 2023). On a large geographical scale, within-lineage structure is limited (Tremble, Hoffman & Dentinger, 2023), while on a fine geographical scale, genetic diversity varies in relation to woodland age, with multiple close relatives being found in younger forest patches, indicating restricted dispersal and a high potential for inbreeding (Hoffman et al., 2020).

Here, we used whole genome resequencing data (Tremble, Hoffman & Dentinger, 2023) to characterise ROHs across all seven *B. edulis* lineages. Our dataset covers a broad latitudinal gradient, from the tropics to the Arctic tundra, as well as an elevational range spanning over 2500 m (Figure 1a). This sampling design allowed us to characterise global patterns of inbreeding as well as to investigate the best predictors of inbreeding among a set of geographical and ecological variables. We hypothesised (i) that ROHs would reveal evidence for inbreeding in at least some individuals and lineages and (ii) geographical and ecological proxies of past climatic conditions would explain variation in inbreeding, reflecting previously described patterns in *B. edulis* hosts (Anderson et al., 2006; Magri, 2008). Specifically, we expected inbreeding levels to increase with latitude, elevation and low predicted habitat suitability during the LGM; Finally, we hypothesised that (iii) ROH length distributions would reflect past climatic events, with a large proportion of short ROHs reflecting ancient common ancestry.

2 | MATERIALS AND METHODS

2.1 | Genomic data

We analysed whole genome resequencing data from a total of 225 sporocarps (Tremble, Hoffman & Dentinger, 2023) using the workflow shown in Figure S1. These samples originated from field campaigns covering Alaska, Utah, Germany, the United Kingdom and Guatemala, as well as from museum collections from Colorado, the West Coast and the East Coast of North America, Scandinavia, Iceland, the Mediterranean Coast and Russia. In a previous study, these 225 samples were classified into seven distinct *B. edulis* lineages using a summary coalescent phylogenomic approach (Tremble, Hoffman & Dentinger, 2023). The resulting dataset comprises 29 samples from the Alaska (AK) lineage, 27 from the British Columbia (BC) lineage, 26 from the Colorado (CO) lineage, 52 from the European (EU) lineage, 47 from the West Coast of North America (WC) lineage, 33 from the East Coast of North America (EC) lineage and 11 from the Guatemala (GU) lineage.

2.2 | Genotyping

In order to evaluate broad-scale patterns of population structure and relatedness, we mapped the reads from each sample to the common pseudo-chromosomal reference genome (BD747 from WC lineage) using the mem algorithm of bwa v0.7.13 (Li, 2013) with the default settings. Single nucleotide polymorphisms (SNPs) were then called with GATK v4.4.0.0 (McKenna et al., 2010) using the default settings. The resulting dataset was pruned to retain only SNPs with minor allele frequency (MAF) ≥ 0.1, depth of coverage ≥10, missing data <10% and mapping quality ≥30, and we removed indels using the following command in Vcftools v0.1.17 (Danecek et al., 2011): '--remove-indels --minDP 10 --minQ 30 --max-missing 0.9 --maf 0.1'.

For the analysis of inbreeding, we sought to minimise any potential biases inherent in mapping the reads from different lineages to a common reference. We observed that, across all of the lineages, rates of mapping success were significantly higher and the proportion of uncalled sites was significantly lower when mapping the reads to the own lineage-specific reference in comparison to the common reference (Table S1). This is to be expected given the high genetic divergence of the lineages, but could lead to issues calling ROHs based on the common reference, as missing sites might result in fewer ROHs being detected with our conservative approach (Thorburn et al., 2023). We therefore mapped the reads from each sample to the lineage-specific reference genomes described by Tremble, Brejon Lamartinière et al. (2023): YSU-09856 (N50 = 184 kbp) for the AK lineage, WTU-68809 (N50 = 130 kbp) for the BC lineage, BD-953 (N50 = 329 kbp) for the CO lineage, C-F-109468 (N50 = 170 kbp) for the EU lineage, DUKE-0193972 (N50 = 161 kbp) for the EC lineage, KST39 (N50 = 238 kbp) for the GU lineage, and the pseudo-chromosomal reference BD747 for the WC lineage. We retained all sites with a depth of coverage ≥9, missing data <10% and mapping

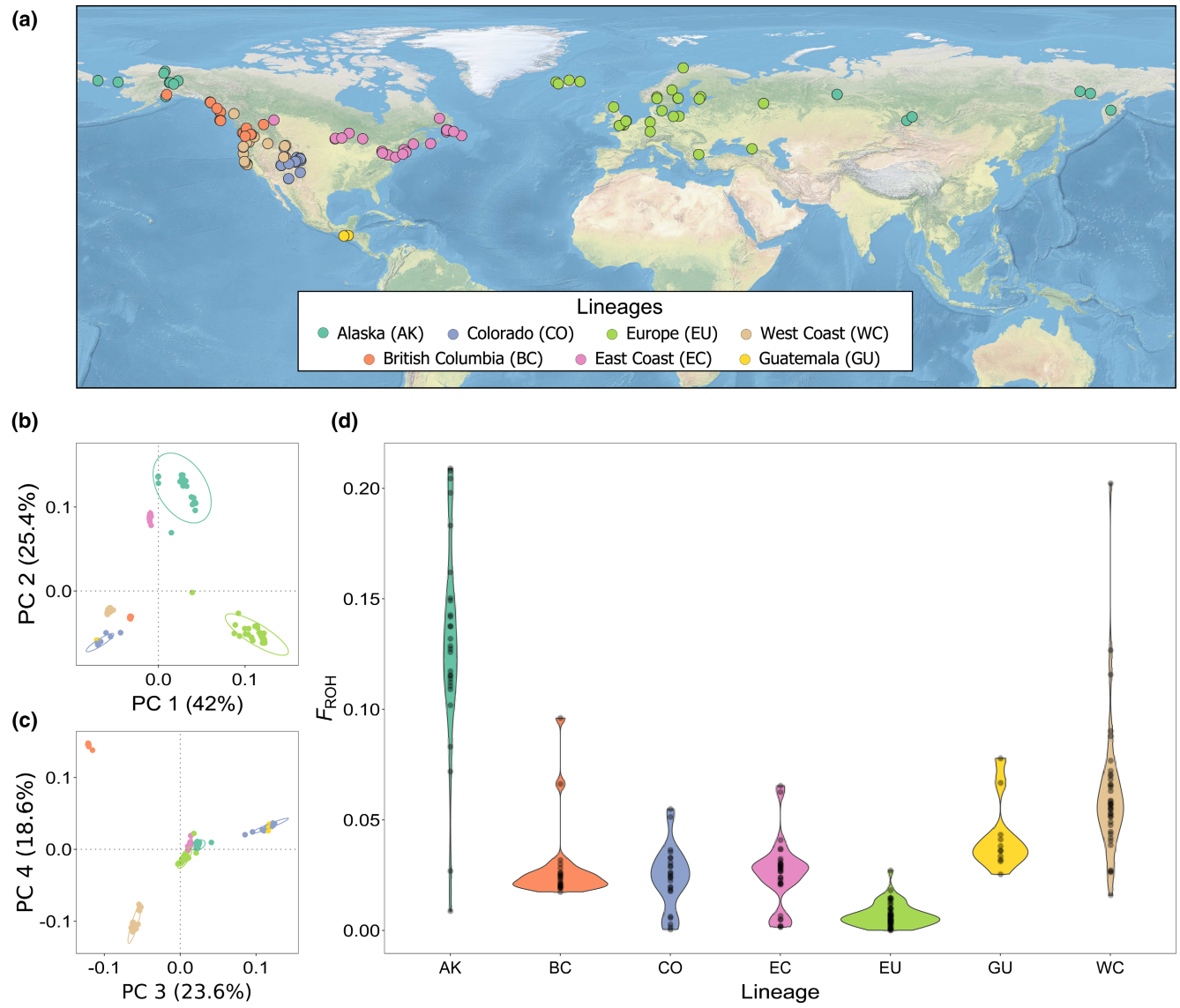

FIGURE 1 Patterns of genomic relatedness and inbreeding among seven globally distributed *Boletus edulis* lineages. (a) Sampling locations of 225 sporocarps across the northern hemisphere. The lineages are colour-coded as shown in the legend. (b, c) Results of the principal component analysis showing variation in the first four principal components. (d) Violin plots of individual inbreeding coefficients (F_{ROH}) for each lineage. Individuals are denoted by points and the shapes represent lineage-specific kernel densities of F_{ROH} values. The map was retrieved from https://www.naturalearthdata.com.

quality ≥30, and we removed indels using the following command in Vcftools: '--remove-indels --minDP 10 --minQ 30 --max-missing 0.9'. No minor allele frequency pruning was performed according to the best practices for ROH calling (Meyermans et al., 2020).

2.3 | Relatedness analysis and clone correction

In order to characterise the relatedness structure of our dataset and to check for the presence of inadvertently resampled clones, we calculated pairwise kinship coefficients separately for each lineage using the KING algorithm (Manichaikul et al., 2010) with the –relatedness2 command from Vcftools. Pairs of samples with kinship coefficients equal to or greater than the conservative default threshold of 0.354 were classified as clones according to the software's best practices. Where clones were present, we randomly selected one sample to represent the individual genet for inclusion in subsequent analyses.

2.4 | Population structure and inbreeding

To contextualise our inbreeding analyses, we first characterised patterns of global population structure. For this, we used the final clone-corrected dataset of 209 individuals mapped to the best common reference. We implemented principal component analysis (PCA) of the genomic dataset, which comprised 152,132 polymorphic, biallelic SNPs, using pcadapt 4.3.3 (Luu et al., 2017). This software takes missing data into account in the computation of the Z-scores, such that

the larger the quantity of missing data, the smaller the Z-score (Luu et al., 2017). We then quantified inbreeding using the lineage-specific vcf files. Specifically, we computed F_{is} using a dataset of 50,000 randomly selected linkage disequilibrium-pruned ($r^2 < .2$) SNPs within Plink v1.90b6.21 (Purcell et al., 2007) and characterised ROHs using the Bcftools 1.11 -roh function (Narasimhan et al., 2016). Only ROHs ≥1kbp were considered. While this threshold is smaller than the minimum length thresholds typically used in mammalian studies, it was adapted to take into consideration the much smaller genome length and the recombination rate estimated from a well-studied agarycomycete (Gao et al., 2018; Larraya et al., 2000). To control for differences in the contiguity of the lineage-specific reference genomes, we standardised the ROH measures according to mapping coverage. Specifically, we used coverage bed files computed by the Bedtools 2.27.1 (Quinlan & Hall, 2010) command 'genomecov' for each individual's bam file as masks in order to restrict ROH calling to only those regions of the genome where the coverage was sufficiently high (≥5) to determine zygosity. To quantify individual inbreeding, we then calculated the genomic inbreeding coefficient F_{ROH} as the proportion of the genome in ROHs. For this, the standardised ROH dataset was used to calculate for each individual the proportion of the mappable genome in ROH as follows:

$$F_{ROH} = \frac{\sum \text{length of standardized ROHs (bp)}}{\sum \text{length of mappable genome (bp)}}$$

To estimate the antiquity of the identified ROHs, we utilised the formula whereby expected ROH lengths follow an exponential distribution according to 1/2g Morgans (Howrigan et al., 2011), with g being the number of generations since the most recent common ancestor. For this, we used a recombination rate of 34 kb/cM estimated from *Pleurotus* spp. (Gao et al., 2018; Larraya et al., 2000).

2.5 | Elevation and LGM habitat suitability

Elevation data were downloaded from the Global Land One-kilometre Base Elevation project website (Hastings et al., 1999). GLOBE DEM files were converted into tif raster files using QGIS 3.28.1 and the elevation value (in metres) for each of our sampling locations was extracted from the tif files using the package raster 3.6.3 (https://CRAN.R-project.org/package=raster). To estimate the habitat suitability of our sampling locations during the LGM, we used MaxEnt v.3.4.1 (Phillips et al., 2006). This software estimates the habitat suitability of a given locality based on available climatic data, predicting past species distributions based on sampling locations as the only input. We used the default settings with the 19 Bioclim2 variables modelled to have occurred during LGM (~22 KYA) (Fick & Hijmans, 2017) at the finest resolution available (30-s, ~1 km^2) and ran the models with 100 replicates. The fit of each resulting model was assessed from area under the receiver-operator curves (AUCs). For each lineage, we used the mean of the 100 replicates to represent the predicted LGM habitat suitability and extracted the values corresponding to each sampling location.

2.6 | Statistical analyses

To identify those forces contributing towards global and local patterns of inbreeding, we performed two sets of analyses. First, we conducted an exploratory analysis of the data in order to identify inter-correlated variables and to determine those factors shaping variation within and among lineages. For this, we computed a PCA of the continuous variables F_{ROH}, elevation, latitude and predicted LGM habitat suitability, while including lineage as a supplementary categorical variable. This analysis was implemented using the r packages factomineR and factoextra (Lê et al., 2008). Then, to formally test for the effects of elevation, latitude and predicted LGM habitat suitability on inbreeding, we implemented post hoc Beta regressions separately for each predictor variable, with and without lineage included as a random effect, using the packages betareg (Cribari-Neto & Zeileis, 2010), lme4 (Bates et al., 2015) and glmmTMB (Brooks et al., 2017).

3 | RESULTS

To investigate inbreeding and its correlates in an ecologically and commercially important wild mushroom, we analysed whole genome resequencing data from 225 *B. edulis* sporocarps belonging to seven globally distributed lineages (Figure 1a; Tremble, Hoffman & Dentinger, 2023). As an initial check, we evaluated the relatedness structure of the dataset and searched for the presence of clones. Levels of relatedness were low for all but one of the lineages, with mean pairwise kinship coefficients being below 0.042 and the majority of samples being unrelated (Table 1). The exception was the GU lineage, for which most pairs of individuals were classified as third-degree relatives and the mean kinship coefficient was 0.059. A total of 18 clonal pairs were identified, primarily from the WC and BC lineages (Table 1). Clone correction left a final dataset of 209 individual genets, comprising 29 from the AK lineage, 23 from the BC lineage, 24 from the CO lineage, 42 from the WC lineage, 31 from the EC lineage, 49 from the EU lineage and 11 from the GU lineage. In line with the results of Tremble, Brejon Lamartinière et al. (2023) and Tremble, Hoffman and Dentinger (2023), the lineages were clearly resolved in the PCA with the exception of CO and GU, which clustered together in the four first dimensions (Figure 1b,c).

3.1 | Global patterns of inbreeding

We found clear evidence for inbreeding, with genomic inbreeding coefficients (F_{ROH}) reaching up to 0.21. By contrast, F_{IS} values were close to or below zero (Figure S2). Considerable variation in F_{ROH} was present both among and within lineages (Figure 1d). AK and WC exhibited both the highest mean F_{ROH} values (0.13 and 0.06 respectively) and the greatest within-lineage variation (SD = 0.05 and 0.03 respectively). The EU lineage was the least inbred (mean F_{ROH} = 0.01)

Relatedness	AK	BC	CO	EC	EU	GU	WC
Clones	0	5	3	2	3	0	5
1st degree	0	0	1	1	0	1	1
2nd degree	8	0	4	1	2	1	3
3rd degree	24	2	24	4	8	45	28
Unrelated	374	399	293	520	1311	8	1043
Mean Phi	−0.145	−0.171	−0.025	−0.075	−0.127	0.059	−0.11

Note: The top row shows the number of clonal pairs in each lineage. Subsequent rows show the numbers of pairs of individuals assigned to various relatedness categories. The bottom row shows average pairwise relatedness for each of the lineages. For the names and sampling localities of the lineages, see Figure 1a.

TABLE 1 Summary of relatedness patterns among 225 *Boletus edulis* whole genomes.

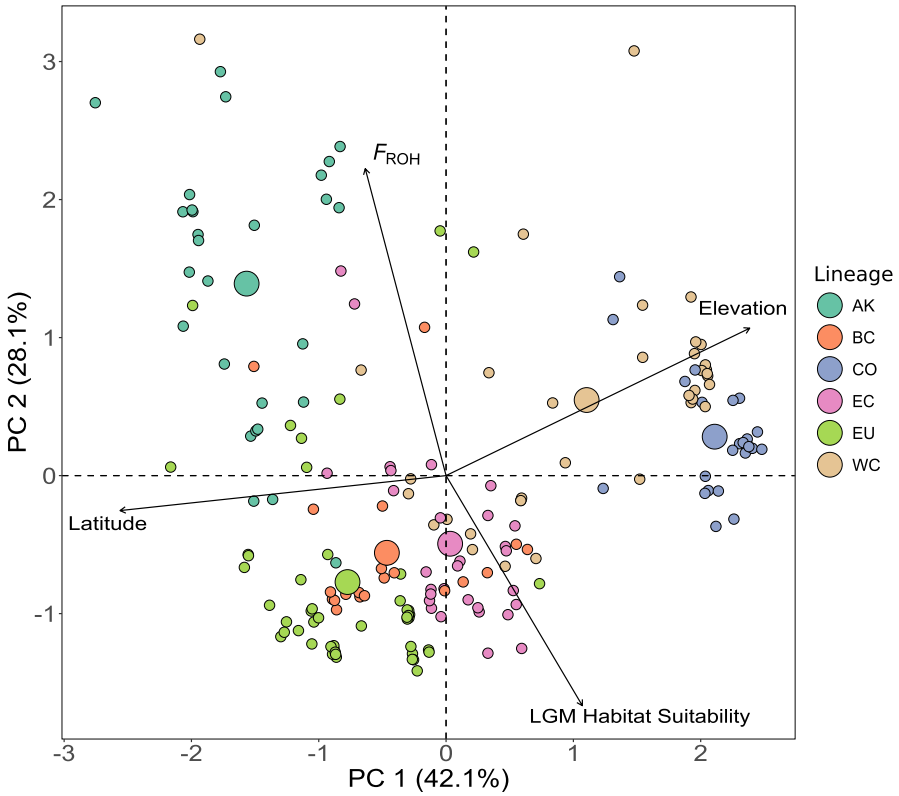

FIGURE 2 Results of the principal component analysis of the dataset based on F_{ROH}, latitude, elevation and predicted last glacial maximum habitat suitability. Correlations between the variables and the principal components are shown as arrows, with arrow length being proportional to the magnitude of the correlation coefficient (shown in Figure S4). Larger points represent the means of each lineage.

and showed the least within-lineage variation (SE = 0.01). The remaining lineages had intermediate levels of inbreeding (mean F_{ROH} = 0.03, 0.02, 0.03 and 0.04 for the BC, CO, EC and GU lineages respectively) and within-lineage variation (SD = 0.02, 0.01, 0.01 and 0.02 for the BC, CO, EC and GU lineages respectively). There was no obvious concordance between inbreeding levels and the underlying population genetic structure. For example, the AK and EC lineages clustered together in the PCA (Figure 1b,c) but differed substantially in their levels of inbreeding (Figure 1d).

3.2 | Correlates of inbreeding

To evaluate general trends and to capture the most important relationships in our dataset, we computed a PCA based on F_{ROH}, elevation, latitude and predicted LGM habitat suitability (Figure 2 and Figure S3). The GU lineage was excluded from this analysis as it did not have enough sampling locations to generate LGM habitat suitability predictions. This approach decomposed the total variation into uncorrelated components, allowing clear visualisation of the major axes of variation (Figure 2), as well as the variables loading upon them (Figure S4). The first two PCs captured over 70% of the total variation. Latitude was strongly negatively correlated with the first PC ($r = -0.90$, $p < .001$) whereas elevation was strongly positively correlated with the first PC ($r = 0.84$, $p < .001$). This opposition highlights the heterogenous nature of our dataset, with samples from the lineages CO and WC being located at the highest elevation and the lowest latitude, while samples from the EU and AK lineages occur at high latitude but low elevation. Inbreeding was strongly positively correlated with the second PC ($r = 0.78$, $p < .001$) while predicted LGM habitat suitability showed a strong negative correlation with the second PC ($r = -0.59$, $p < .001$). This suggests

that the most inbred individuals originate from those locations with the lowest predicted habitat suitability during the LGM. Elevation was also positively correlated with PC2 ($r=0.38$, $p<.001$) suggesting that genets sampled from high elevations tend to be more inbred and are associated with lower predicted LGM habitat suitability values. Furthermore, inbreeding was negatively correlated with PC1 ($r=-0.22$, $p=.002$) suggesting that individuals tend to be more inbred at higher latitudes.

To formally test our hypothesis based on insights from the PCA, we used Beta regressions to evaluate the relationships between inbreeding, elevation, latitude and predicted LGM habitat suitability, both with and without lineage included as a random effect (see 2. Materials and Methods for details). The best predictor of inbreeding was elevation ($z=2.7$, $p=.007$), but only when excluding lineage as a random effect (Table S2), reflecting high levels of among-lineage variation. Post hoc regression analyses revealed an overall tendency for elevation to be positively associated with inbreeding across the majority of lineages (Figure 3a), with the strongest relationship being found for the EU lineage ($p<.003$). The second strongest predictor of inbreeding was latitude ($z=-1.987$, $p<.05$) but only when lineage was included as a random effect (Table S3) reflecting the opposition of the positive global effect and lineage-specific effects. Moreover, contrasting patterns were found across the lineages, with AK and GU showing significant negative relationships ($z=-3.18$, $p<.002$ and $z=-4$, $p<.001$ respectively) while BC and EC showed positive but non-significant trends (Figure 3b). Finally, predicted LGM habitat suitability did not explain a significant proportion of the variation in inbreeding, regardless of whether or not lineage was included as a random effect (Table S4), although many of the lineages showed weak but non-significant negative trends (Figure 3c).

3.3 | Antiquity of ROHs

To estimate the antiquity of genomic inbreeding, we characterised lineage-specific ROH length distributions. Across all seven lineages, the majority (97.5–99.5%) of the ROHs were shorter than 20 kb in length. ROHs larger than 50 kb were ubiquitous but less common, while larger ROHs in the range of 50–175 kb were only occasionally found (Figure S5). This inference appears not to be limited by the contiguity of the lineage-specific references, as 76.6–99.2% of the scaffolds across lineages were sufficiently large to call ROHs of 50 kb or longer (Figure S6). To summarise variation in the antiquity of ROH among lineages, we converted the length of each ROH into the estimated number of generations since the most recent common ancestor (TMRCA) according to Howrigan et al. (2011) using a recombination rate estimate from another agarycomycete species (Gao et al., 2018; Larraya et al., 2000). We found a diversity of ROH lengths spanning from 1 kb (corresponding to approximately 1700 generations ago) to 175 kb (corresponding to approximately 9 generations ago). To investigate patterns of ROH ancestry in our dataset, we grouped the ROHs into categories representing three different time

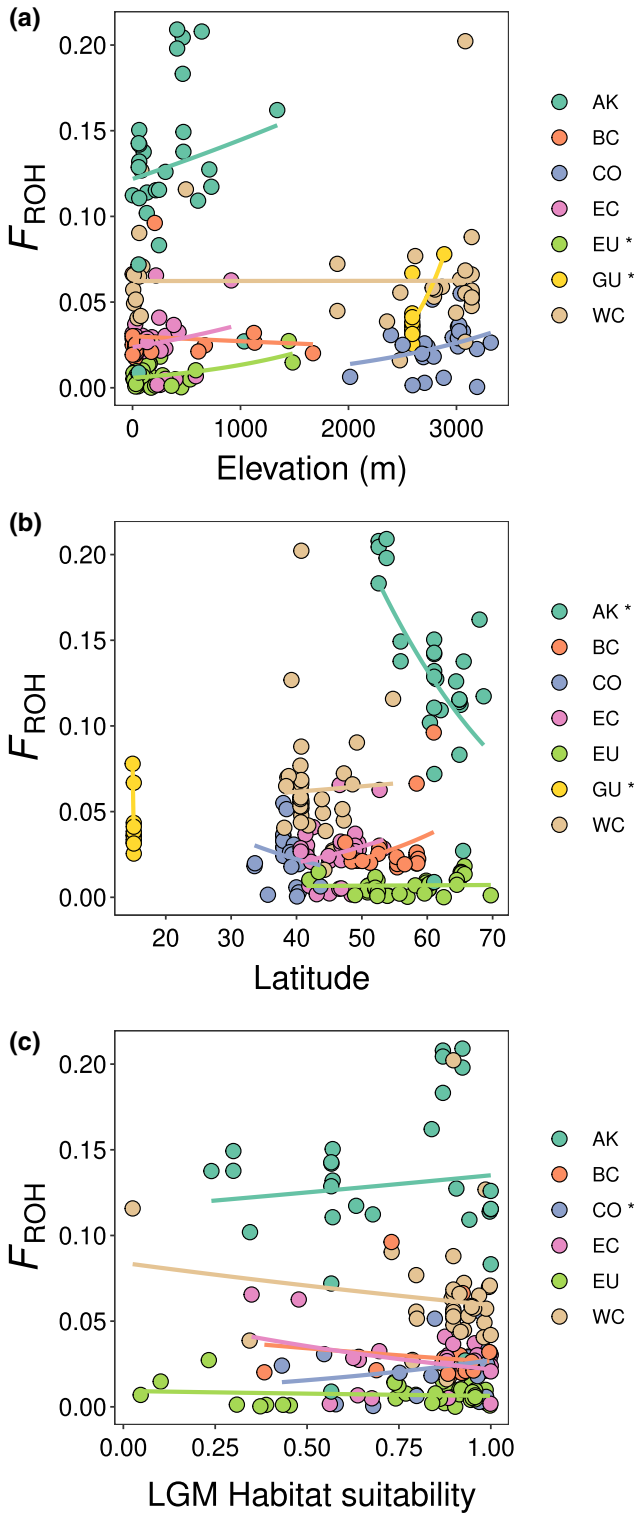

FIGURE 3 Results of post hoc analyses of the effects of (a) elevation, (b) latitude and (c) predicted last glacial maximum habitat suitability on genomic inbreeding levels. Individual sporocarps (closed points) and lineage-specific Beta regressions (solid lines) are colour-coded as shown in the legend. The asterisks indicate statistically significant (alpha = 0.05) Beta regressions.

periods. First, we defined 'recent common ancestry' as ROHs dating back approximately 9–50 generations ago. We used a relatively narrow generational range to ensure that this category captured only

the most recent inbreeding events in our dataset. Next, we defined 'intermediate common ancestry' as ROHs dating back approximately 50–200 generations ago, and 'ancient common ancestry' as ROHs dating back approximately 200–1700 generations ago. Successively broader generational ranges were used for these categories because uncertainty in the relationship between ROH length and TMRCA increases with longer coalescent times due to Mendelian sampling and the stochasticity of recombination (Pemberton et al., 2012). Finally, we computed the percentage of the mappable genome IBD separately for each lineage and category.

Figure 4 shows that ROHs corresponding to 9–50 generations ago are extremely rare, only being found in a handful of individuals from the lineages BC, EC and GU. We did not find any evidence of contemporary inbreeding, such as matings between close kin or even selfing, which should be manifested as longer ROHs dating back fewer generations. Across all of the lineages, the majority of ROHs corresponded to around 200–1700 generations ago, which is indicative of ancient common ancestry. ROHs corresponding to 50–200 generations ago were present in all of the lineages, but were especially abundant in the AK lineage, probably reflecting the high latitude of the respective sampling locations, which would likely have taken thousands of years to recolonise after the LGM.

4 | DISCUSSION

Although genomic approaches have revolutionised the study of inbreeding in animals (Ceballos et al., 2018; Kardos et al., 2018,

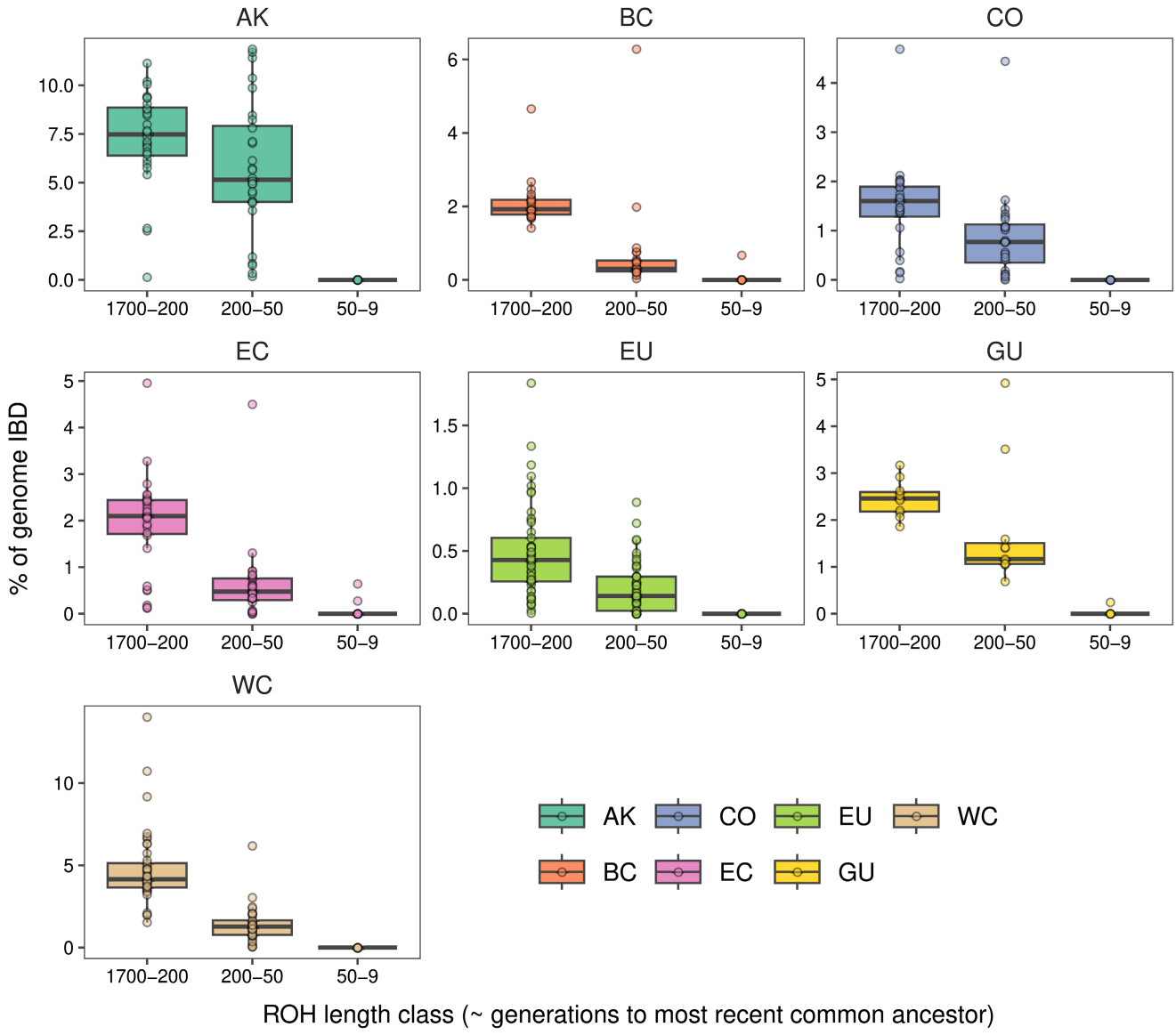

FIGURE 4 The proportion of the genome of each individual assigned to three runs of homozygosity (ROH) length classes. Each point represents the proportion of the genome of an individual within the corresponding class. Box plots indicate the median, first and third quartiles for each class. The ROH length classes are expressed as the approximate number of generations since the most recent common ancestor.

2023), the application of ROHs to plants has been more limited (Barragan et al., 2024; Kumar et al., 2021; Pavan et al., 2021) and we are not aware of any ROH studies in fungi. We therefore conducted the first genomic survey to our knowledge of ROHs in a wild, globally distributed fungus. We uncovered substantial variation in inbreeding both among and within lineages, which appears to mainly reflect ancient rather than recent common ancestry. Furthermore, much of the variation in inbreeding could be explained by geographical and ecological proxies of past climatic conditions, consistent with prevailing theory (Hewitt, 2000) and empirical studies of the dominant forest tree species of the northern hemisphere (Anderson et al., 2006; Magri et al., 2006; Roberts & Hamann, 2015). Our study thereby uncovers the genomic legacy of major climatic events on a forest symbiont.

4.1 | Inbreeding in *Boletus edulis*

One explanation for the lack of studies using ROHs to quantify genomic inbreeding in EMF is that most EMF species lack high-quality reference genomes (Loeffler et al., 2020). A major advantage of using *B. edulis* is that both a single highly contiguous pseudochromosomal reference genome and multiple lineage-specific reference genomes are available. We used the former as a common backbone for broad-scale analyses of population structure and relatedness. However, among-lineage variation in mapping success to the common reference together with a global tendency for mapping success to be higher to the own lineage-specific reference motivated us to use the lineage-specific references for the inference of inbreeding. To control for variation in the total length and contiguity of the lineage-specific references, we standardised the ROH calling according to mapping depth and assembly length. In addition, we used the software recognised as being the least prone to false positive ROH discovery (Narasimhan et al., 2016). Thus, our results are conservative and the variation we observe in inbreeding should be minimally impacted by sequencing or bioinformatic artefacts.

Despite our conservative approach, we found clear evidence for inbreeding in *B. edulis*, with F_{ROH} being as high as 21%. In contrast to previous studies reporting significant F_{IS} values in fungal populations (Abe et al., 2017; Bergemann & Miller, 2002), we found that F_{IS} values were consistently around or below zero in *B. edulis*. This is likely because F_{IS} predominantly captures inbreeding due to contemporary population substructure, whereas ROHs in this species appear to reflect much more ancient common ancestry. Further studies of EMF are needed to generalise our results and to learn more about the magnitude and antiquity of inbreeding and its relevance to fungal population dynamics, including resilience to environmental change and commercial harvesting.

In yeast, regions of homozygosity have also been shown to arise via a mechanism that is independent of inbreeding involving mitotic mutations (Smukowski Heil, 2023). The resulting loss of heterozygosity (LOH) could potentially be conflated with ROHs. However, LOHs are generated through mitotic recombination in diploid (more often in polyploid) nuclei (Dutta et al., 2022), a process that should be less common in Basidiomycete species that predominantly persist as dikaryons such as *B. edulis*, since both haploid nuclei go through mitosis separately (Hiltunen et al., 2022). Therefore, we believe the ROHs we detected in our study are true signals of genomic inbreeding, which is supported by the observation that geographical and ecological variables explain a significant amount of the variation in F_{ROH}.

4.2 | Global patterns of inbreeding

The broad sampling coverage of our study allowed us to uncover appreciable variation in inbreeding both among and within lineages. The best predictor of variation in inbreeding in *B. edulis* was elevation, with positive associations between F_{ROH} and elevation being found in both the PCA and the regression analysis of the global dataset excluding lineage as a random effect. Including the random effect likely removed statistical significance as much of the variation in elevation occurs among lineages and thus, the standard error of the pooled estimate was very high. However, positive associations between inbreeding and elevation were still visible within individual lineages in the post hoc analysis. Furthermore, individually significant associations were observed in the EU and GU lineages, although the latter appears to be driven by a single outlier. As argued by Hewitt (1996) and Willis et al. (2004), a globally positive relationship between elevation and inbreeding likely reflects the impact of past climatic events, primarily the LGM, during which lower elevation sites would have supported larger host tree populations (Gugerli et al., 2001) and hence, historically larger EMF populations.

We also found an effect of latitude on inbreeding, although this relationship appears more complex. Specifically, the PCA revealed a positive relationship between inbreeding and latitude, which was reflected by a positive but non-significant global trend for higher latitude populations to be more inbred in the Beta regression excluding lineage as a random effect. However, latitude became statistically significant when including lineage as a random effect, exposing a clear case of Simpson's paradox (Simpson, 1951). Thus, the global positive relationship appears to be largely driven by the AK lineage, which has the highest level of inbreeding and is located at the highest latitude, while local trends were predominantly neutral or negative. For example, the AK lineage exhibited a strong and highly significant negative association between latitude and inbreeding in the post hoc analysis, while the EU lineage exhibited no relationship. Long-standing theory on the impacts of glacial cycles argues that higher latitudes supported smaller populations during the LGM and/or had to be recolonised from southerly refugia (Hewitt, 1999; Taberlet et al., 1998). In theory, this should be manifest as a positive relationship between inbreeding and latitude (Dussex et al., 2020; Niedziałkowska et al., 2016), which is the opposite to our results for the AK lineage. However, recent discoveries of central refugia in Europe (Magri et al., 2006) and a northern refugium in North America (Brubaker et al., 2005; Shafer

et al., 2010), together with evidence for postglacial recolonisation routes being more complex than previously thought (Magri, 2008; Mee & Moore, 2014) have led to this view being revised. In particular, the presence of a large refugium in the north of Alaska (Beringia) could explain the negative relationship between inbreeding and latitude in the AK lineage, as postglacial recolonisation in this region of North America likely progressed from north to south (Brubaker et al., 2005). Similarly, the lack of a relationship between latitude and inbreeding in the EU lineage is consistent with the presence of multiple central European (micro-) refugia (Magri, 2008) and complex multidirectional postglacial colonisation pathways (Magri et al., 2006). It also refutes the notion of a single large refugia in the European southern peninsula (Hewitt, 1999), which aligns with the results and conclusions of several other recent studies of the main tree hosts of *B. edulis* (Gömöry et al., 2020; Magri, 2010; Willis & Vanandel, 2004).

Predicted LGM habitat suitability was also strongly negatively correlated with the second component of the PCA, while inbreeding was strongly positively correlated. At the same time, predicted LGM habitat suitability was moderately positively correlated with the first component of the PCA, while inbreeding showed a weak negative correlation. We interpret this opposition on both PCs as being suggestive of a negative association between inbreeding and LGM habitat suitability. However, none of the Beta regressions were statistically significant. One explanation for this could be that the PCA was able to capture a weak overall relationship mainly driven by AK, which exhibits by far the lowest LGM suitability values whilst also being the most inbred lineage.

4.3 | Antiquity of inbreeding

Since haplotypes are broken down at each successive generation by recombination, ROHs can be dated back to TMRCA based on their length while making certain assumptions about the recombination rate and generation time. Despite finding genomic evidence for the presence of closely related individuals in the majority of *B. edulis* lineages, ROHs reflecting recent common ancestry (50 or fewer generations ago) were conspicuously absent from all but a handful of individuals from just three lineages. Hence, it appears that inbreeding between close relatives and/or selfing rarely occur in *B. edulis*. This might be expected given the high diversity of mating alleles found in our study populations (Tremble, Hoffman & Dentinger, 2023). Thus, our results suggest that the mating system of *B. edulis* may be efficient at preventing consanguineous matings.

By contrast, short ROHs dating back approximately 200–1700 generations ago were ubiquitous. Precise dating of these IBD segments is not possible because generation time estimates are not currently available for any EMF. However, multiple geographical and ecological proxies of LGM conditions explain a significant amount of the variation in genomic inbreeding in *B. edulis*, suggesting that the ROHs may originate from this period. If indeed some of these ROHs date back to the LGM, then the generation time of *B. edulis* would be somewhere in the order of 10 years. We believe this is plausible for a long-lived mushroom (field observations indicate that *B. edulis* individuals can fruit for at least 15 years, W. Amos, pers. comm.) although extensive monitoring of entire populations over many decades would be required to obtain a more precise generation time estimate.

While short ROHs were abundant and large ROHs were rare across all of the lineages, ROHs of intermediate length (dating back approximately 50–200 generations ago) were present in varying amounts in different lineages. This may testify to variation among the lineages in post-glacial recolonisation patterns. For example, the AK lineage, which carries the greatest proportion of ROHs of intermediate length, is present at high latitude and would therefore have been recolonised potentially hundreds of generations after the more southerly lineages. Thus, these IBD segments could potentially be explained by smaller effective population sizes and successive founder events during the hundreds to thousands of years that followed the LGM. Alternatively, another hypothesis could be that, since the AK lineage experiences the most hybridisation with other lineages (Tremble, Brejon Lamartinière et al., 2023), the divergence between these lineages could lead to underdominance, increasing apparent inbreeding.

By contrast, the EU lineage had the lowest representation of intermediately sized ROHs, probably reflecting more rapid postglacial recolonisation. Interestingly, the EU lineage is also considered to be the least host-specialised of the lineages based on its symbiosis gene content (Tremble, Brejon Lamartinière et al., 2023; Tremble, Hoffman & Dentinger, 2023). Host generalism may have contributed to differences in recolonisation patterns, as the ability to form associations with multiple tree species may increase niche availability, enabling faster recolonisation and larger effective population sizes. This generalist advantage during strong perturbations is a popular concept in the context of both mutualists and parasites (Dennis et al., 2011).

In conclusion, we used a genomic approach based on ROHs to investigate inbreeding in a wild mushroom. We found that inbreeding is widespread among *B. edulis* populations from across the northern hemisphere, with among- and within-lineage variation being explained by elevation, latitude and, to a lesser extent, predicted LGM habitat suitability. These patterns lend support to theoretical and empirical studies of the impact of the LGM on forest ecosystems from the perspective of a key mutualist and forest engineer. Overall, our study emphasises the potential of population genomics to deepen our understanding of the ecology and population biology of fungi.

AUTHOR CONTRIBUTIONS

E.B.L., J.I.H., K.T., K.K.D. and B.T.M.D. conceived the study. K.T. and B.T.M.D. contributed the reference genomes and sequencing data. J.I.H. acquired funding and supervised the PhD student (E.B.L.). E.B.L. and K.T. analysed the data. E.B.L. and J.I.H. drafted the manuscript. All of the authors commented upon and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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DATA AVAILABILITY STATEMENT

The sequences used in this study are publicly available at the BioProject accessions PRJNA1010140 and PRJNA763230. The scripts used to analyse the data are available via https://zenodo.org/records/11122013.

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