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Keaton Tremble (✉ [keaton.tremble@utah.edu](mailto:keaton.tremble@utah.edu))

University of Utah <https://orcid.org/0000-0002-0788-2830>

Joseph Hoffman

University of Bielefeld

Bryn Dentinger

Natural History Museum of Utah/University of Utah

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# Contrasting continental patterns of adaptive population divergence in a holarctic fungus

Keaton Tremble<sup>1,2\*</sup>, Hoffman, J.I.<sup>3</sup> and Bryn T.M. Dentinger<sup>1,2</sup>

<sup>1</sup>Univeristy of Utah, School of Biological Sciences, Salt Lake City, Utah, 84112.

<sup>2</sup>Natural History Museum of Utah, Salt Lake City, Utah, 84108.

<sup>3</sup>Bielefeld University, Department of Animal Behaviour, Bielefeld, Germany.

\*Corresponding author(s). E-mail(s): [keaton.tremble@utah.edu](mailto:keaton.tremble@utah.edu);

## Abstract

Understanding the population processes and genetic mechanisms that give rise to new species remains one of the most elusive goals of modern evolutionary biology. In the hyperdiverse and ecologically important Fungi, the process of speciation is virtually unknown, including for the more than 20,000 species of obligate ectomycorrhizal mutualists that play essential roles in ecosystem function. We investigated patterns of genome-wide differentiation in the ectomycorrhizal porcini mushroom *Boletus edulis*, a globally distributed species complex with broad ecological amplitude. By whole genome sequencing 160 individuals from across the Northern Hemisphere, we identified 792,923 SNPs and used these to elucidate the demographic and adaptive processes shaping global population differentiation. We show that *B. edulis* exhibits deeply contrasting patterns of genomic divergence between continents, with multiple lineages being present across North America, while a single lineage dominates Europe over a vast geographic scale. These geographical lineages are inferred to have diverged between 2.66 and 1.62 million years ago, corresponding to a period of climatic upheaval and the onset of glaciation during the Pliocene-Pleistocene boundary. High levels of genomic differentiation were observed among lineages despite evidence of substantial and ongoing introgression. Furthermore, genome scans, demographic inference and ecological niche models all suggest that genomic

33 differentiation is maintained by environmental adaptation and not phys-  
34 ical isolation. Our study uncovers striking differences on a truly global  
35 scale and emphasizes the importance of local adaptation and ecologically  
36 mediated divergence, rather than prezygotic barriers such as allopa-  
37 try or genomic incompatibility in population differentiation in Fungi.

38 **Keywords:** population genomics, speciation, ectomycorrhizae, fungi, local  
39 adaptation

## 40 1 Introduction

41 Understanding the complex evolutionary pathways by which populations  
42 diverge and new species are formed has long been a goal of evolutionary biol-  
43 ogy [1, 2]. Beyond gaining fundamental knowledge about the mechanisms that  
44 generate and maintain diversity, understanding the evolutionary history of  
45 genotypes and phenotypes allows us to predict how these characteristics will  
46 be impacted by changing climate. However, this can be challenging as the evo-  
47 lutionary processes that drive divergence such as demography, introgression,  
48 and adaptation rarely act in isolation [3]. Moreover, the dynamic interaction  
49 of these processes can produce differing signatures of divergence depending  
50 on the geographic and temporal scale of the study [4–6]. To overcome the  
51 limitations of scale, and disentangle the complex evolutionary web weaved  
52 by the speciation process, research must be conducted within a truly global  
53 perspective.



**Fig. 1** Examples of phenotypic variation across the global distribution of *B. edulis*. Numerous attempts have been made to segregate groups of *B. edulis* into new species based on this phenotypic variation, yet these attempts have not been corroborated by genetic evidence.

54 In order to study the processes that give rise to divergence in wild organ-  
55 isms, it is important to identify tractable systems that exhibit dynamic  
56 properties for which hypotheses can be generated or tested. Species that  
57 are common, have broad geographic or ecological distributions, and exhibit  
58 morphological variation are ideal targets for determining the genetic basis

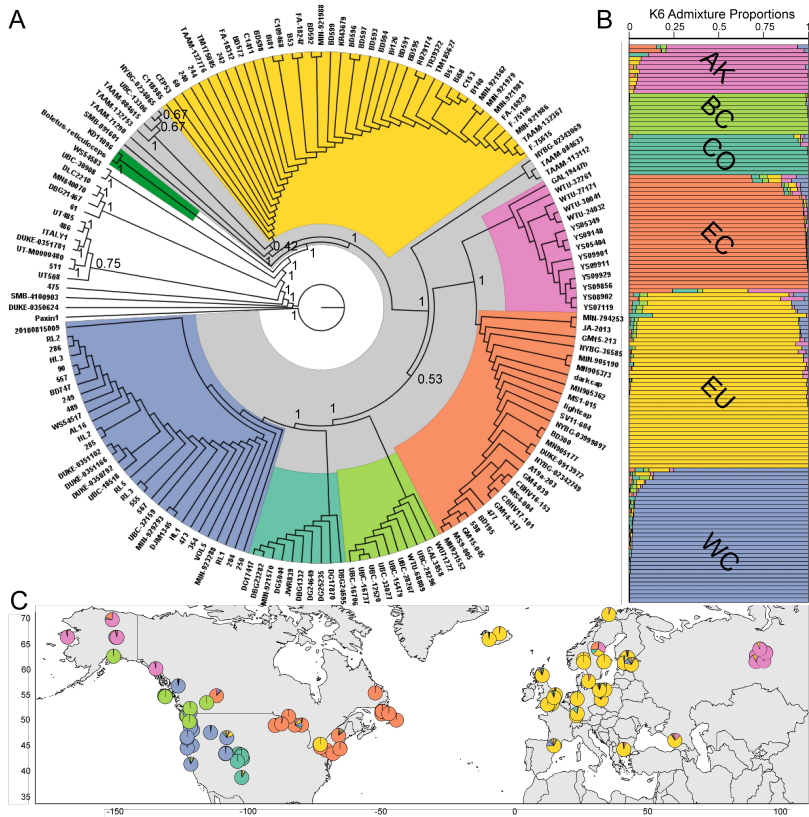
of divergence [7]. For example, systems such as the monkey flowers (*Mimulus sp.*) [8–10], black cottonwood (*Populus trichocarpa*) [11–13], and three-spine stickleback (*Gasterosteus aculeatus*) [14–16] have been used to understand how the complex interaction of demography, introgression, and local adaptation give rise to variation and population differentiation, ultimately resulting in the formation of new species. However, none of these systems exhibit a truly global distribution or continuous ecological breadth. This limits our ability to investigate the interaction of evolutionary processes at scale, unconstrained by geography or ecology. Furthermore, there is an over-representation of animal and plant taxa in these systems, limiting their application to under-studied groups such as the Fungi [3, 17, 18] despite their ecological importance, enormous diversity, and far more tractable (cost-effective) genomes. The Fungi as a group represent an enormous gap in our fundamental understanding of the process of population divergence [3, 17, 18]. For example, the geographic scale at which populations form and the degree to which distant groups maintain gene flow is entirely unknown in all but a few select taxa within the *Basidiomycota* [19], a phylum containing over 30,000 species [20]. To study the processes that give rise to divergence at global scales, and provide a novel insight into fungal population dynamics we here highlight the globally distributed prized edible “porcini” mushroom, *Boletus edulis*.

*Boletus edulis* Bull. (“porcini”) is a well known ectomycorrhizal species complex that is found in nearly all temperate ecosystems across the northern hemisphere [21]. Due to extensive phenotypic variation (Fig. 1), the taxonomy of *B. edulis* is a matter of some controversy [22]. This complex has been variously treated as a single taxon with as many as 22 forms, subspecies, and varieties, or segregated into multiple species based primarily on morphological features [23, 24]. However, supporting molecular evidence has not led to conclusive segregations, indicating that this complex group is somewhere in the evolutionary process of speciation [3, 22, 25, 26]. Taken together, the taxonomic uncertainty, both from continuous morphological variation and ambiguous molecular evidence, enormous ecological amplitude, continuous Holarctic distribution, and small genome (ca. 50 Mbp) makes *B. edulis* an ideal non-model system to investigate, on a global scale, the roles of demography, introgression, adaptation, and isolation in the speciation process in wild organisms. Here, we utilize global-scale population genomics, ecological niche modeling, and demographic modeling within a novel system, *B. edulis*, to identify the primary forces driving lineage divergence and loci under putative selection, within a global and historical context.

## 2 Results and Discussion

### 2.1 Striking population structure found in the continuously distributed *B. edulis*

To investigate the demographic history of a globally distributed taxon and to elucidate the process of population differentiation at the most inclusive



**Fig. 2** Population structure analysis of *B. edulis*. A) Coalescent phylogenomic reconstruction of the group from 702 single copy gene gene-trees. *B. edulis* (highlighted grey) is defined here as all individuals sister to *B. reticuloceps* (highlighted dark green). Colors indicate geographically structured clades, *B. edulis* individuals not in groups are ambiguously placed predominately due to poor genome assembly and low recovery of signal copy genes. B) LEA admixture analysis assuming 6 ancestral populations from 52,112 high-confidence, LD-pruned variants, and C) distribution of admixture across the range of *B. edulis*

102 scale, we constructed a dataset of whole genomes from 161 specimens of *B.*  
 103 *edulis* and three outgroup species. We first generated a high quality reference  
 104 genome from a North American *B. edulis* specimen (N50 = 149KB; 16573  
 105 protein-coding regions; 97.3% BUSCO completeness) and re-sequenced 160 *B.*  
 106 *edulis* specimens from across its global distribution together with three addi-  
 107 tional members of the "porcini" *sensu stricta* [22]. Coalescent phylogenomic  
 108 reconstruction of 702 single copy genes revealed a monophyletic *B. edulis* com-  
 109 posed of six clades that share a most recent common ancestor with the Asian  
 110 endemic, *B. reticuloceps* [27] (Fig. 2 A). Five of the six nodes had 100% sup-  
 111 port, and the 6th received full support in a separate concatenated supermatrix  
 112 phylogenomic reconstruction that recovered the same *B. edulis* topology (Fig  
 113 S2). Thirteen specimens were redetermined as other species, illustrating the

114 degree to which taxonomic uncertainty of *B. edulis* has compromised reliable  
115 identification. To prevent the inclusion of misidentified specimens in subse-  
116 quent analyses we retained only individuals that were clustered within these  
117 six, *B. edulis* lineages (147 of 160 initial specimens). These groups are strongly  
118 geographically structured and are hereafter referred to as the Europe (EU),  
119 Russia/Alaska (AK), eastcoast North America (EC), British Columbia (BC),  
120 Colorado (CO), and westcoast North America (WC) lineages.

121 To determine the magnitude and spatial distribution of admixture between  
122 these lineages we identified 792,923 high confidence genome-wide variants from  
123 137 individuals that remained after quality filtering. The six lineages were  
124 recovered in both admixture (K=6) (Fig. 2 B,C) and PCA analysis (Fig.  
125 S3), further indicating strong phylogeographic structure. The extent of admix-  
126 ture varied widely between lineages (mean admixture AK=8.7%, BC=0.6%,  
127 CO=2.0%, EC=5.9%, EU=6.5%, WC=3.4%). However, four out of the six lin-  
128 eages (AK, EC, EU, WC) contained at least one individual exhibiting >30%  
129 mixed ancestry, suggesting a recent history of migration. Surprisingly, we  
130 identified contrasting patterns of admixture between geographically adjacent  
131 lineages. Asian individuals within the AK lineage exhibit a moderate signa-  
132 ture of shared ancestry with EU, yet North American individuals within the  
133 same lineage exhibit admixture predominately with the EC group. In addition,  
134 lower values of K (putative ancestral populations) collapse the AK lineage into  
135 hybrids of EC and EU, further indicating a long history of migration between  
136 AK and the EC and EU lineages (Fig. S4). In contrast, the WC lineage has  
137 more admixed ancestry with the EU lineage than either BC or CO despite  
138 geographic and phylogenetic proximity. This suggests that admixture is con-  
139 text specific, and not strictly limited to geographic proximity. Interestingly,  
140 one individual collected in North America carries a EU genotype, with little  
141 admixture (Fig. 2 C). This specimen was collected in a monodominant stand of  
142 "Norway Spruce" *Picea abies*, a tree species native to Europe that is commonly  
143 introduced in North America [28]. This strongly suggests that this individual  
144 represents a recent anthropogenic introduction, so was removed from further  
145 analyses. Altogether, admixture analysis of *B. edulis* indicates that admixture  
146 is lineage and context specific; geographic proximity appears to predict admix-  
147 ture for some lineages, while other groups experience minimal admixture with  
148 close neighbors.

## 149 2.2 Contrasting geographic structure at the continental 150 scale

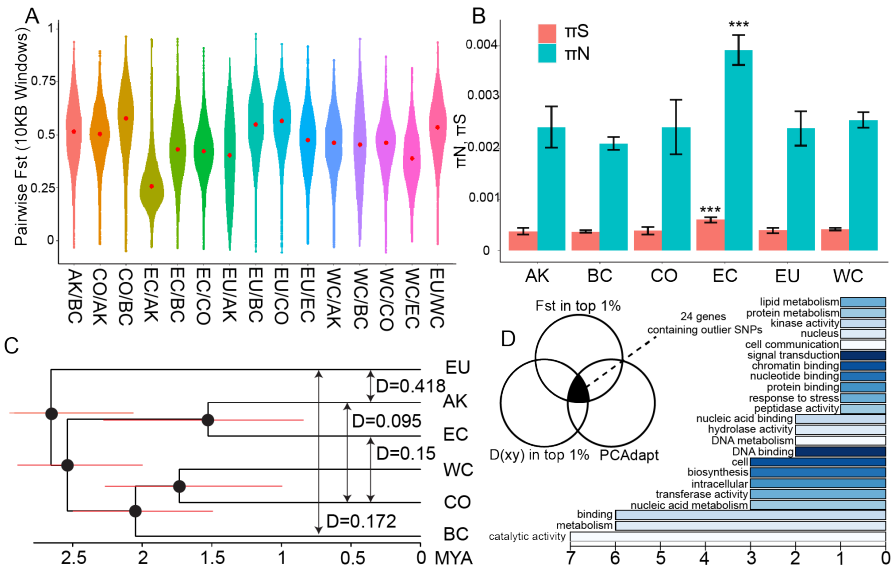
151 Within lineage PCA (Fig. S5 left) and neighbor-joining trees (Fig. S5 right)  
152 show no clear patterns of within-lineage geographic clustering for all five of the  
153 lineages except AK. The AK lineage has clear geographic structure; comprised  
154 of three regions: Eastern Russia, Western Russia, and Alaska. To corroborate  
155 these findings, we utilized multiple-matrix-regression [29] to empirically test  
156 for isolation-by-distance (IBD) and isolation-by-environment (IBE) (accord-  
157 ing to euclidean distance between samples using PCA of 19 bioclim variables)

158 and found that IBD was not significant ( $P > 0.05$ ) for all lineages except AK  
159 ( $P < 0.05$ ), and IBE was only significant for the WC population. Given the  
160 enormous spatial distribution of the AK lineage and patchy sampling included  
161 in this dataset, this signature of IBD is unsurprising. However, it is striking  
162 that the EU lineage, which persists across the entirety of Europe, including  
163 west to Iceland and east to Georgia, does not show any signatures of geo-  
164 graphic sub-structure. Most, if not all systems previously utilized for large  
165 scale population genomic analysis exhibit some degree of IBD, particularly at  
166 the regional to continental scale of the EU lineage. For example, studies in  
167 both *Populus trichocarpa* and *Mimulus guttatus* found limited IBD at local  
168 scales, but strong IBD at regional scales suggesting dispersal limitation across  
169 landscapes [11, 30]. It is possible that the lack of IBD in the EU lineage is a  
170 product of cryptic environmental adaptation of dispersed environments [31].  
171 However, the multiple-matrix-regression method used here empirically tests for  
172 the association of genetic distance and the interaction of geographic distance  
173 and environmental distance, controlling for IBE in the assessment of IBD.

174 On the continental scale, we found contrasting signatures of population  
175 structure between North America and Europe. In Europe, there is a single  
176 widely distributed lineage, yet in North America there is strong geographic  
177 separation of five lineages. Differences in phylogeographic or population struc-  
178 ture between closely related groups have been identified in other systems [32]  
179 and is often associated with differences in dispersal capacity. However, to the  
180 best of our knowledge, no other system exhibits such contrasting signals of geo-  
181 graphic structure between continents. Differences in dispersal capacity could  
182 potentially produce similar patterns of contrasting geographic structure [33],  
183 yet only the widely distributed and sparsely sampled AK lineage exhibited any  
184 degree of IBD or sub-population structure. This would suggest that the dis-  
185 persal capacity of these populations may exceed far beyond their current, in  
186 some cases narrow (BC, CO), range distributions, which may indicate a degree  
187 of local environmental adaptation.

### 188 2.3 Rampant introgression despite high levels of 189 differentiation and ancient divergence

190 To understand the evolutionary history of divergence in *B. edulis*, we per-  
191 formed phylogenetic divergence dating using STARBEAST2 and the multiple-  
192 species coalescent using a 100 MYA MRCA [34] of the *Boletaceae*, and found a  
193 surprisingly ancient split between extant lineages. The MRCA of *B. edulis* was  
194 dated to 2.66 MYA (2.14-3.17 95% HPD), and the most recent split, between  
195 AK and EC lineages, to 1.52 MYA (0.72-2.35 95% HPD) (Fig. 3 C). A root age  
196 of 2.66 MYA corresponds with a period of well-documented climatic upheaval  
197 and onset of glaciation during the Pliocene-Pleistocene boundary [35–37]. The  
198 subsequent rapid three-way separation of Europe from the two North Ameri-  
199 can/Asian groups is suggestive of a single widely-distributed ancestral lineage  
200 that split geographically, which is a similar pattern to that found in the *Picea*  
201 *likiangensi* species complex at the Pliocene-Pleistocene boundary [38].



**Fig. 3** Population genomic analysis of *B. edulis* population differentiation, divergence, and introgression. A) Pairwise  $F_{st}$  values calculated across 10 KB windows. Red points indicate median value, B) Per site nucleotide diversity  $\pi$  calculated at nonsynonymous and synonymous sites for all genic regions on the 1.6 MBP scaffold 1, C) Divergence time estimation of *B. edulis* populations utilizing starbeast2, and all significant results of ABBA/BABBA introgression analysis. Horizontal red bars represent 95% confidence range of node divergence date, and vertical black arrows with corresponding D statistic between specific population pairs. D) Outlier analysis of potentially adaptive loci. Outlier genes were identified as loci in the 99% quantile of mean pairwise  $F_{st}$ , and  $D_{xy}$  that contained a significant ( $P < 0.05$ ) putative locally adapted loci identified by PCAdapt (left). All potential GO terms for the 24 outlier genes were placed into categories by SlimGO (right). The number of genes with each category is indicated by values along X axis.

202 Pairwise population differentiation  $F_{st}$ , and divergence  $D_{xy}$  revealed moderate  
 203 to strong genome-wide differentiation (Fig. 3 A, Fig. S7 Bottom) in  
 204 *B. edulis*. Most lineage pairs possessed  $F_{st}$  and  $D_{xy}$  values (total  $F_{st}$  mean  
 205 across 10kb windows = 0.471,  $D_{xy}$  = 0.1282) as large or larger than values  
 206 commonly found in groups of well differentiated taxa [6, 39–41]. Furthermore,  
 207 there was no significant difference between  $F_{st}$  and  $D_{xy}$  values calculated  
 208 across 10kb windows and genes, indicating that divergence is genome-wide and  
 209 not limited to regions with low selective constraint. In contrast, one lineage  
 210 pair, AK/EC, exhibited significantly lower  $F_{st}$  (mean=0.2739,  $p < 0.0001$ ),  
 211 well within the range of populations experiencing ongoing gene-flow [42–44],  
 212 and exhibited a skewed distribution of  $F_{st}$  values with an excess of highly  
 213 diverged loci (Fig. S8). Visualizing the distribution of  $F_{st}$  values across the  
 214 genome can provide insights into the degree of connectivity between popula-  
 215 tions [45]. Allopatric populations generally exhibit values of divergence that  
 216 are normally-distributed around a global mean with a greater-than-expected  
 217 abundance of conserved house-keeping loci. In contrast, parapatric populations



218 have a skewed distribution, with most of the genome exhibiting low divergence,  
219 homogenized by gene flow, with an excess of highly-diverged regions around  
220 barrier loci [6, 46]. QQ plots show that only the AK/EC exhibits a skewed  $F_{st}$   
221 distribution, indicating that most lineages of *B. edulis* are highly diverged and  
222 exhibit limited signals of ongoing geneflow.

223 The EC lineage exhibited significantly higher nucleotide diversity ( $\pi$ ) across  
224 both nonsynonymous and synonymous positions, and there was no significant  
225 difference between any of the other lineages. The substantially increased diver-  
226 sity within the EC lineage in combination with a skewed distribution of  $F_{st}$   
227 values (Fig. S8), provides strong evidence for ongoing gene flow, predominately  
228 from AK into EC. The similarity in diversity found between all other lineages  
229 is surprising given the difference in range distribution between these groups.  
230 For example, the CO lineage is distributed across a subset of the American  
231 southwest, a fragmented landscape of habitat islands surrounded by desert  
232 basins, and exhibited similar nucleotide diversity ( $\pi$ ) to the EU lineage with a  
233 continent-wide distribution. Landscape fragmentation is often found to be neg-  
234 atively associated with nucleotide diversity [47, 48], in contrast to our results.  
235 This may be explained by a recent rapid expansion in the EU lineage, sug-  
236 gested by a highly negative genome-wide Tajima's  $D$  (mean = -1.149, Fig. S7  
237 Top). In addition, this could be explained by high dispersal capacity regardless  
238 of range size or landscape fragmentation.

239 Ancient divergence, high degree of genomic differentiation, and low levels  
240 of admixture strongly suggest that lineages of *B. edulis* are well segregated.  
241 To empirically test for signatures of introgression and gene-flow we calculated  
242 the four population ABBA-BABA (Patterson's  $D$ ) [49] statistic utilizing our  
243 data set of 792,923 high-quality SNPs. The ABBA-BABA four-population test  
244 has been widely utilized across a diverse array of taxa [38, 50–52] to identify  
245 a significant excess of shared alleles between two groups over what would be  
246 expected from incomplete lineage-sorting, alone. We found highly significant  
247 positive  $D$  values (indicating signatures of gene flow) between all lineage quar-  
248 tets that are compatible with the species tree (Fig. 3 C). Significant signatures  
249 of introgression were identified between even the two most highly differenti-  
250 ated lineages, BC and CO. However, it is not possible to determine the amount  
251 of introgression between taxon pairs from the ABBA-BABA test alone, as  $D$   
252 is not an unbiased estimator [53]. Still, the presence of highly significant  $D$   
253 values across all lineages, regardless of geographic or phylogenetic distance,  
254 implies that introgression events are not rare and isolated occurrences within  
255 *B. edulis*. Nonetheless, the role these events are playing in the divergence of *B.*  
256 *edulis* lineages is unclear. Introgression and hybridization have received exten-  
257 sive recent attention as potential drivers of novel phenotypes and genotypes  
258 in hybrid populations [51, 54, 55]. However, the role that introgression plays  
259 in the breakdown of genomic differentiation between disparate groups is less  
260 well known [56]. We found that high levels of genomic differentiation are being  
261 maintained within *B. edulis* despite substantial introgression. Theoretical work  
262 has also indicated that differentiation can remain after introgression, although

263 only under low migration demographic conditions [57]. In contrast, we identi-  
264 fied a lack of IBD or sub-population structure within widely dispersed lineages,  
265 suggesting high dispersal capacity and potentially high migration rates. This  
266 paradox indicates that forces such as local adaptation may be maintaining  
267 reproductive isolation between *B. edulis* lineages in the face of high dispersal  
268 capacity.

## 269 2.4 Diverse selective pressures acting on *B. edulis*

270 Little is known about the selective pressures that contribute to divergence in  
271 ectomycorrhizal fungi. However, genome-wide outlier analyses can identify loci  
272 carrying signatures of local adaptation, utilizing no *a priori* information on  
273 the ecology of the organism. To identify putative adaptive loci contributing  
274 to lineage divergence, we performed a genome-wide scan to identify highly  
275 divergent genes (Mean pairwise *Fst* and *Dxy* in top 1% of each metric) that  
276 also contained putative locally-adaptive SNPs (identified by PCAdapt) (Fig.  
277 3 D). We identified 20 putative outlier genes, of which eight, five, three, three,  
278 and one gene(s) were most divergent in the EU, BC, WC, CO, and EC lineages,  
279 respectively. Of these 20 genes, 12 had known predicted functions (Fig. 3 D).

280 Two of the three most significant outliers have been the subject of previ-  
281 ous work in other fungal taxa, and have functions that align with mechanisms  
282 previously proposed to contribute to divergence in ectomycorrhizal fungi. The  
283 first, EDU-000167, divergent in the CO lineage, is predicted to produce a Pyri-  
284 doxal phosphate-dependent transferase (IPR015421). A recent study found  
285 that the Pyridoxal-5-phosphate-dependent transferase is involved in the pro-  
286 duction of indolalkaloids in *Curvularia* [58]. Ectomycorrhizal indol alkaloids,  
287 specifically Hypaphorine, were the first fungal molecules identified that regu-  
288 late host symbiosis-related genes during the colonization process and may be  
289 involved in host specificity [59–61]. Moreover, this function was identified in  
290 *Pisolithus tinctorius*, a taxon within the order *Boletales*, the same order as *B.*  
291 *edulis*. Shifts in host specificity have been widely thought to be a primary driver  
292 of divergence in ectomycorrhizal fungi [62]. In addition, they have been shown  
293 to evolve rapidly leading to numerous patterns of compatible and incompatible  
294 host associations within a single ectomycorrhizal genus [63–66] and can lead  
295 to dynamic changes in genome evolution [67]. Regardless, divergence within  
296 loci involved in the complex chemical handshake between ectomycorrhizae and  
297 host can likely lead to reproductive barriers, facilitating lineage divergence. *B.*  
298 *edulis* is well-known to colonize a diverse array of hosts in Europe [22, 26, 68].  
299 However, it is thought to be restricted to gymnosperm hosts in North Amer-  
300 ica [21], though this has not been empirically assessed. If divergence in the  
301 gene EDU-000167 is driving, or indicative of, shifts in host preference in the  
302 CO lineage, this would provide one explanation for the limited admixture and  
303 high degree of divergence found in CO, despite geographic and phylogenetic  
304 proximity with the WC lineage.

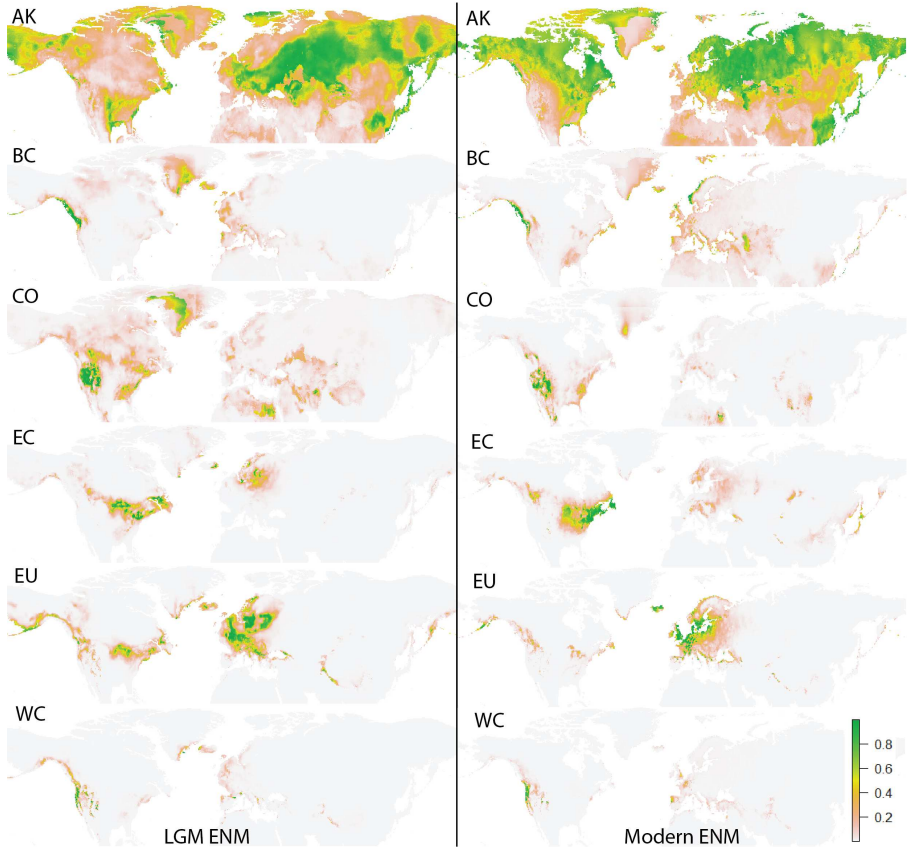
305 The second putative outlier locus, EDU-010436, divergent in BC, was  
306 predicted to have aspartic protease activity (GO:0004190, IPR013320) and

307 contained a predicted secretion signal suggesting that it has extracellular func-  
308 tion. Aspartic proteases have been shown to be the primary component of  
309 extracellular protease activity in numerous ectomycorrhizal taxa [69], and are  
310 well known to be intricately involved in litter degradation [70]. In addition,  
311 EDU-010436 exhibited the lowest nucleotide diversity ( $\pi$ ) of the three outliers  
312 ( $\pi = 0.00605$ ), well within the lowest 3% of all genes, providing strong evi-  
313 dence that it is currently under selection in the BC lineage. While originally  
314 thought to have little saprotrophic activity, recent work indicates that most  
315 ectomycorrhizal fungi retain some capacity to decompose organic material and  
316 actively acquire carbon and nitrogen from decomposition [71–76]. Moreover,  
317 saprotrophic capacity and the conditions under which saprotrophic activity is  
318 conducted, differ between closely related taxa, suggesting a potential difference  
319 in selective pressures along the mutualist to facultative-saprotroph spectrum  
320 [77]. If selection in EDU-010436 indicates some shift in saprotrophicity in the  
321 BC lineage, this could have created an ecological reproductive barrier where  
322 hybrids experience reduced fitness as they exhibit an intermediate saprotrophic  
323 phenotype. In addition, the coast of British Columbia, where the BC lineage  
324 is predominately distributed, is well known for high levels of precipitation  
325 and moderate seasonal temperatures, which may allow for high decomposition  
326 rates and large pools of readily accessible C and N [78], hypothetically relax-  
327 ing the purifying selection that would maintain saprotrophic capacity in the  
328 BC lineage. However, even among this well-characterized outlier, it is unknown  
329 if it serves the same purpose in *B. edulis* or has been co-opted for novel  
330 function, necessitating further research. Regardless, genomic outlier analysis  
331 indicates a degree of divergence in loci putatively involved in adaptation to  
332 local environmental conditions, lending evidence to the role of local adaptation  
333 in maintaining lineage differentiation.

## 334 2.5 Non-overlapping ecological niche distributions 335 suggest local environmental adaptation

336 Due to limited knowledge about the forces that structure ectomycorrhizal  
337 niches, it is unknown if the strong geographic structure seen in *B. edulis* could  
338 be due to strong selection for adaptation to local environmental conditions.  
339 Moreover, there is little *a priori* knowledge of the capacity for lineages of  
340 *B. edulis* to occupy unique climatic or ecological niches, capacities that may  
341 help to explain the observed pattern of strong genomic divergence despite  
342 introgression. To overcome this, we utilized ecological niche modeling [79] to  
343 create models of geographic distribution likelihoods (strictly likelihoods of per-  
344 sistence) based on historic 19 bioclim variables (1950-2000), calculated niche  
345 overlap, and assessed whether populations occupy discrete climatic niches.

346 As expected given the strong geographic structure of *B. edulis* lineages,  
347 ecological niche models identified low to moderate levels of niche overlap  
348 between lineages, (Table SX), and relatively limited distribution overlap (Fig. 4  
349 right). Even within North America, where there is currently geographic overlap  
350 between several lineages, niche models suggest that the samples we analyzed



**Fig. 4** Suitability distribution of *B. edulis* populations determined by ecological niche modeling using the 19 Bioclim variables modeled during the last glacial maximum 22 KYA (LEFT) and empirical values measured from 1950-200 (RIGHT). Regions in green indicate areas with high likelihood of persistence.

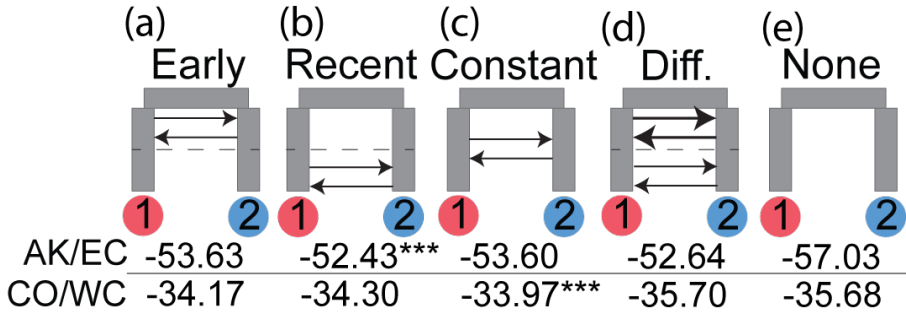
351 may lie at the edge of their potential distributions. However, the ecological  
 352 niche model for the AK lineage indicates an expansive range of environmental  
 353 suitability, stretching from eastern Europe to the east coast of North America,  
 354 and greatly overlapping in distribution with the EU and EC lineages. The AK  
 355 lineage also has the highest level of admixture, predominately with the EU  
 356 and EC lineages, suggesting that niche overlap may result in increased gene  
 357 flow. Across all models, bioclim 8 (Mean Temperature of Wettest Quarter)  
 358 explained the most variation, followed by bio 3 (Isothermality) and 6 (Min  
 359 Temperature of Coldest Month), respectively (Fig S9). Overall, niche model-  
 360 ing suggests that all lineages other than AK have limited geographic and niche  
 361 overlap, potentially indicating the occupation of unique climatic niche space  
 362 and further reinforcing the potential role of local adaptation in maintaining  
 363 lineage fidelity in the face of high dispersal capacity.

## 2.6 Isolation during glacial maxima does not greatly contribute to genomic differentiation in *B. edulis*

Separation during glacial maxima is well-understood to contribute to the differentiation of populations through the reduction of homogenizing gene flow between groups, allowing genomic incompatibilities to accumulate and prezygotic reproductive barriers to form [80–82]. While we hypothesize that local adaptation is the primary driving force behind the pattern of divergence found in *B. edulis*, periods of allopatric isolation due to glaciation cycles can create similar patterns of geographic structure with limited local adaptation. To determine the relative strength of local adaptation versus allopatric isolation in population divergence, we again used ecological niche modeling to identify the potential geographic distributions of lineages during the last glacial maximum (LGM 22KYA). We then calculated niche overlap [83] to identify lineages that potentially shared refugia, and calculated the change in niche overlap between lineage pairs from the LGM to current distributions to identify whether these lineages are presently experiencing more or less potential contact than during the LGM.

Ecological niche modeling of *B. edulis* lineages during the LGM found that, on average, niche overlap was greater (mean = 0.4206) during the LGM than the amount of contemporary overlap (mean = 0.3971). However, the impact of the LGM climate on niche overlap varied widely between lineage pairs (Table SX). For example, niche overlap between the AK/EC lineages increased from the LGM to modern day and a clear break in population connectivity throughout Canada can be seen in the AK lineage. This pattern is consistent with the presence of large glacial ice sheets known to have persisted across this region during the LGM [84]. In contrast, niche overlap between the CO and WC lineages decreased slightly from the LGM to modern day, presumably due to an increase in low-elevation habitat availability due to lower LGM temperatures in North America [84], a pattern also found in numerous tree taxa, including putative ectomycorrhizal hosts [81]. Overall, there appear to be contrasting signatures of isolation during the LGM, where most lineage pairs experienced no change to moderate gain of potential population connectivity (CO/WC), while a few lineages exhibited a dramatic reduction in connectivity (AK/EC).

However, ecological niche modeling does not empirically test whether populations with niche overlap are experiencing migration during periods of contact or isolation. To identify if lineage pairs are indeed experiencing periods of isolation with limited gene flow, we used coalescent simulations to model five demographic scenarios between two pairs of sister lineages, AK/EC and WC/CO, with contrasting glaciation dynamics (Fig. 5). Coalescence demographic modeling found best support for a secondary-contact model (Fig. 5 b) between the AK/EC lineages where AK/EC most likely experienced a period of post-divergence isolation and is now experiencing gene flow following their reintroduction from post-glacial migration. In contrast, the demographic model with the highest likelihood for WC/CO was a constant gene-flow model with no



**Fig. 5** Representations of the five demographic models tested using two pairs of sister lineages (AK/EC, CO/WC). From left to right, (a) gene flow after lineage divergence with subsequent loss of connectivity, (b) secondary contact with initial period of isolation, (c) constant gene flow since split, (d) differential gene flow with high initial gene flow then accumulation of reproductive barriers, (e) and no gene flow after divergence. Values are mean likelihood values across 100 replicates. "\*\*\*" indicates significance for the highest likelihood values ( $p < 0.00001$ ). The recent gene flow, and constant gene flow models had the highest likelihood for the AK/EC and CO/WC lineages respectively.

408 period of isolation (Fig. 5 C). These results corroborate ecological niche modeling, which suggested that connectivity between AK/EC was highly reduced  
 409 or cut off during the LGM, while WC/CO experienced little to no change in  
 410 connectivity between the LGM and modern day. Despite the strong evidence  
 411 for periods of isolation during glacial maxima, the AK/EC pair exhibit the  
 412 least population differentiation, genic divergence, and have the strongest signatures  
 413 of contemporary gene-flow of all lineages in *B. edulis*. This is in stark  
 414 contrast to the WC/CO lineages which have no evidence for true isolation yet  
 415 maintain strong genome-wide differentiation. Moreover, at the lowest end of  
 416 the proposed range of divergence times between AK/EC (720 KYA), these  
 417 lineages will have experienced eight major glaciation cycles [85], indicating  
 418 gene-flow can be resumed after numerous recurrent periods of isolation acting  
 419 over almost a million years. This suggests that allopatric isolation is not  
 420 a necessary step in the process of divergence and that differentiation can be  
 421 initiated despite connectivity between groups.  
 422

## 423 Conclusions

424 The contrasting roles of demography, introgression, and adaptation on the  
 425 origin and shaping of lineage divergence is poorly understood in wild populations  
 426 of non-model organisms. Here, we found that lineages with high potential  
 427 niche overlap maintain gene flow and have low levels of genomic differentiation,  
 428 despite numerous cycles of true isolation that should allow for genomic  
 429 incompatibilities to form. Moreover, lineages that potentially occupy distinct  
 430 niches, yet do not experience cyclic isolation, possess far greater levels of  
 431 genomic differentiation. In *Fungi*, it has been suggested that true prezygotic  
 432 reproductive isolation due to genomic incompatibilities rarely develops [18].  
 433 For example, introgression has been identified between highly diverged groups

434 across a diverse array of fungal taxa [86–89], indicating that long-separated  
435 groups can hybridize. As a consequence, this places a greater emphasis on the  
436 role of other mechanisms that can generate reproductive barriers, such as local  
437 adaptation, in the speciation process. Our study provides further evidence for  
438 the importance of local adaptation and divergent selection acting on ecological  
439 conditions, rather than prezygotic barriers such as allopatry or genomic  
440 incompatibility, in the role of population divergence in the *Fungi*.

## 441 3 Methods

### 442 3.1 Sample collection, Sequencing, and Population 443 structure analysis

444 160 putative *B. edulis* specimens were gathered from both targeted field collec-  
445 tions and institutional loans from varied fungaria in North America, Europe,  
446 and Asia (Table S1). gDNA from specimens was resequenced on Illumina  
447 MiSeq, HiSeq 2500, and Novaseq (Table S1), and assembled for phylogenomic  
448 analysis. A coalescent tree [90] was produced from maximum-likelihood [91] trees  
449 of 702 single-copy genes extracted from assemblies. Divergence dates were  
450 estimated using StarBEAST2 [92] with a 100 MYA estimate for root of the  
451 *Boletaceae* [34]. For variant calling, we produced a highly-contiguous, anno-  
452 tated, hybrid *de novo* assembly (scaffolds = 613, N50 = 153Kbp, size = 47.5  
453 Mbp, BUSCO=97.3%, 16,573 protein coding regions) (see supplemental meth-  
454 ods for further details). All reads were aligned to this assembly and variants  
455 were called with GATK (v4.0) [93] according to best practices, and quality  
456 filtered. For full details of filtering thresholds, and which datasets were used  
457 specific downstream practices see SUP info. Admixture was calculated with  
458 K=4:10 using the `snmf` function of the LEA R package (v3.2.0) [94], and  
459 PCA and NJ trees were produced with Adegenet R package [?] to visualize  
460 population sub-structure. IBD and IBE analyses were calculated using Mul-  
461 tiple Matrix Regression [29] with 999 permutations, with collection location  
462 coordinates and environmental conditions (19 Bioclim). Population differentia-  
463 tion ( $F_{st}$ ) and divergence ( $D_{xy}$ ) were calculated with PIXY (v1.2.2) [95].  
464 Nucleotide diversity ( $\pi_N$ ,  $\pi_S$ ) was calculated across the first scaffold (1.6Mbp)  
465 using SNPgenie [96].

### 466 3.2 Introgression, and adaptation

467 To identify signatures of introgression between clades of *B. edulis* we calculated  
468 ABBA-BABA statistics [52] (Pattersons D) using the `Dquartets` function and  
469 of DSUITE [49] across all population quartets that are compatible with the  
470 species tree. To identified genomic regions under selection we identified locally  
471 adaptive loci using a three-part genome-wide scan for highly diverged genes  
472 that contained significant putative adaptive snps. Mean  $F_{st}$  and  $D_{xy}$  vs.  
473 all was calculated by finding the mean population pairwise  $F_{st}$  and  $D_{xy}$ )  
474 of each gene for each population. Highly divergent genes, defined as genes in

475 the top 1% of each statistic, were retained for further analysis. To account for  
476 demography and identify genes containing fixed snps, we used PCAdapt [97],  
477 a program that utilizes PCA to account for evolutionary relationships in the  
478 identification of locally adapted SNPs. Highly divergent genes were further  
479 thinned to only those that included a putative locally adapted SNP identified  
480 by PCAdapt (K=3) after bonferoni correction (alpha = 0.01, p < 0.05).

### 481 3.3 Ecological Niche Modeling and Population 482 Demographic Modeling

483 To identify whether clades of *B. edulis* occupy distinct climatic niches, both  
484 today and during the last glacial maximum (22Kya) we used Maxent v3.4.1  
485 [98] (default settings, 100 replicates) to model the distribution likelihoods using  
486 collection location information, and the 19 bioclim variables for modern day  
487 and the LGM [99, 100]. Niche overlap was calculated using the ENMtools R  
488 package (v1.0) [101] across all pairwise combinations. To identify if population  
489 pairs have experienced periods of isolation, we utilized fastsimcoal2 v2.6.0.3  
490 [102] to model five demographic scenarios (Fig. 5) of gene-flow post-divergence  
491 between two pairs of sister-clades, CO/WC and AK/EC. These pairs were  
492 chosen because they have the most recent divergence and have contrasting  
493 patterns of range growth and contraction during glaciation cycles. Models were  
494 replicated 100 times and the run with the highest AIC score was selected for  
495 each model. To identify the model with the highest support, we replicated  
496 the best run for each model using identified parameter values 100 times and  
497 identified the model that significantly had the highest likelihood score.

498 **Data Availability.** All short-read genome sequences, and the newly created  
499 *B. edulis* reference genome are publicly available on the Short Read Archive  
500 and Genbank under the ascension number JAIQWV000000000.

501 **Supplementary information.** Supplementary information includes an  
502 expanded methods section (supplementary methods 1), supplementary figures  
503 S1-S16, and supplementary tables 1-4 which include all specimen collection  
504 information, results of pairwise population analyses, 19 bioclim values for all  
505 specimens included in study, and results of outlier loci analysis.

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512 wrote the manuscript. Hoffman, J.I. provided technical expertise and collabor-  
513 orated on the manuscript, Bryn T.M. Dentinger worked on study design,  
514 specimen and funding acquisition, and collaborated on the manuscript

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