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**GENOMIC SEQUENCING REVEALS HISTORICAL, DEMOGRAPHIC, AND SELECTIVE FACTORS ASSOCIATED
WITH THE DIVERSIFICATION OF THE FIRE-ASSOCIATED FUNGUS *NEUROSPORA DISCRETA***

Pierre Gladieux^{1,2*}, Benjamin A Wilson³, Fanny Perraudieu^{1,4}, Liliam A Montoya¹, David Kowbel¹,
Christopher Hann-Soden¹, Monika Fischer¹, Iman Sylvain¹, David J Jacobson¹, John W Taylor¹

¹Department of Plant and Microbial Biology, University of California, Berkeley, USA

²Ecologie Systematique Evolution, Université Paris Sud, Batiment 360, 91405 Orsay, France

³Department of Biology, Stanford University, USA

⁴Ecole Polytechnique, Route de Saclay, 91128 Palaiseau, France

*present address: INRA, UMR BGPI, TA A54/K, F-34398, Montpellier, France; CIRAD, F-34398, Montpellier, France

Correspondence: P Gladieux pierre.gladieux@supagro.inra.fr; UMR BGPI, TA A54/K, F-34398, Montpellier, France;

Phone +33 499 624 863; Fax + 33 499 624 848

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22

23 **ABSTRACT**

24 Delineating microbial populations, discovering ecologically relevant phenotypes, and identifying
25 migrants, hybrids or admixed individuals has long proved notoriously difficult, thereby limiting our
26 understanding the evolutionary forces at play during the diversification of microbial species. However,
27 recent advances in sequencing and computational methods have enabled an unbiased approach
28 whereby incipient species and the genetic correlates of speciation can be identified by examining
29 patterns of genomic variation within and between lineages. We present here a population genomic
30 study of a phylogenetic species in the *Neurospora discreta* species complex, based on the resequencing
31 of full genomes (~37Mb) for 52 fungal isolates from 9 sites in three continents. Population structure
32 analyses revealed two distinct lineages in Southeast Asia, three lineages in North America/Europe with a
33 broad longitudinal and latitudinal range, and limited admixture between lineages. Genome scans for
34 selective sweeps and comparisons of the genomic landscapes of diversity and recombination provided
35 no support for a role of selection at linked sites on genomic heterogeneity in levels of divergence
36 between lineages. However, demographic inference indicated that the observed genomic heterogeneity
37 in divergence was generated by varying rates of gene flow between lineages following a period of
38 isolation. Many putative cases of exchange of genetic material between phylogenetically divergent
39 fungal lineages have been discovered, and our work highlights the quantitative importance of genetic
40 exchanges between more closely related taxa to the evolution of fungal genomes. Our study also
41 supports the role of allopatric isolation as a driver of diversification in saprobic microbes.

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43

44 INTRODUCTION

45 The differentiation of lineages and the evolution of reproductive barriers between them are the
46 processes that create the tree of life. Understanding their underlying mechanisms is a major goal of
47 evolutionary biology. Progress has been made in the past decades on understanding the different
48 factors and traits causing lineage divergence and the evolution of reproductive isolation (Schluter 2000;
49 Coyne and Orr 2004; Nosil 2012; Stukenbrock 2013), or at the opposite, promoting hybridization or
50 genetic exchanges between long divergent lineages (Barton & Hewitt 1981; Arnold 2004; Roper et al.
51 2011; Rius and Darling 2014). In addition, individual genomic features contributing to adaptive evolution
52 and reproductive isolation have been identified (Dettman et al. 2012; Nosil & Schluter 2011; Presgraves
53 2007; Rieseberg & Blackman 2010), and as the phylogenetic breadth and depth of taxa with sequenced
54 genomes has increased, many putative cases of exchange of genetic material between phylogenetically
55 divergent lineages have been discovered (Brand et al. 2013; Gladieux et al. 2014; Novo et al. 2009;
56 Racimo et al. 2015). Whole-genome data sets provide increased resolution that can reveal the sequence
57 and timing of speciation (lineage-splitting) events and the geographic context in which they occurred.
58 They also make it possible to address long-standing questions regarding the extent to which the
59 divergence of populations, or their maintenance, occur in the face of ongoing gene flow: How do
60 genomes diverge during speciation? How do they respond to secondary contact? How permeable are
61 they to post-divergence genetic exchanges?

62 Theory predicts that levels of genetic divergence across the genome will be more heterogeneous
63 in populations diverging in the presence of gene flow (sympatry or parapatry) than in geographically
64 isolated (allopatric) populations. Under the strictly allopatric model, gene flow between species ceases

at every locus in the genome simultaneously so that the genealogical histories of all loci in the genome are compatible with a single time of species divergence. But genomic data reveals that divergence is often heterogeneous, and ancestral relationships among genomic features are not always congruent with known phylogenetic history, suggesting that the timing and genealogical history of speciation might be complex (Garrigan et al. 2012). Hybridization between diverging lineages may therefore create a genomic mosaic of regions where interspecific gene flow has ceased at different times (the genic view of speciation), due to the inability of genomic features directly involved in differential adaptation and/or reproductive isolation to freely permeate species boundaries (Wu 2001). Several population-genetic models that focus on selection at linked sites also predict greater differentiation in regions of low recombination simply as a result of faster sorting of ancestral alleles even in the absence of gene flow (Nachman & Payseur 2012).

Filamentous fungi like the genus *Neurospora* are ideal systems for studying the nature and time course of genetic changes underlying population divergence and species maintenance. Ecological genomics of adaptation and speciation in *Neurospora* fungi is facilitated by their haplontic life-cycle and their relatively small genomes with low proportions of repetitive DNA. Moreover, the genus contains multiple recently diverged species, some of which coexist in sympatry or have large distributions encompassing various environments. Thousands of wild strains representing broad taxon sampling have been collected from all continents and are available from the Fungal Genetic Stock Center (Turner et al. 2001; Jacobson et al. 2004; Jacobson et al. 2006). Reproductive isolation can be measured using well-tested protocols (Dettman et al. 2008; Dettman et al. 2003; Menkis et al. 2009; Turner et al. 2010), and genomic tools and functional annotation resources are available to make connections between genotype, phenotype and fitness (Davis & Perkins 2002; Dunlap et al. 2007; McCluskey et al. 2010; Ellison et al. 2011; Palma-Guerrero et al. 2013; Ellison et al. 2014).

Previous population genomic studies provided insights into the environmental factors that may be driving lineage divergence in *Neurospora*. They also evidenced divergence-with-gene-flow of *Neurospora* populations and an important role of genomic novelties of trans-specific origin as sources of adaptive change. Ellison et al. (2011) discovered two cryptic and recently diverged populations of *N. crassa*, endemic to Louisiana and the Caribbean basin, and genome scans revealed at least two ‘islands’ of extreme divergence between them, with growth-rate assays suggesting that divergence islands may be the result of local adaption to latitudinally distinct environments, an hypothesis validated by gene deletion. Analyses also suggested that the divergent haplotype observed at one of the islands of divergence in the Louisiana population has been introgressed from another, unknown, *Neurospora* species. Phylogenomics of mating type chromosomes also revealed the existence of large introgression tracts in three phylogenetic lineages of the selfing species *Neurospora tetrasperma*, and analyses showed that the introgression likely originated from two closely related outcrossing species (Strandberg et al. 2010; Sun et al. 2012). To extend these findings, and to address their universality, we conducted a population genomic study of another species of *Neurospora*: the Phylogenetic Species 4 of the *N. discreta* species complex (=PS4). This species has the greatest latitudinal distribution of any *Neurospora* species, from central Africa, through Europe and western North America, as far north as Alaska (Dettman et al. 2006). The broad distribution of PS4, and the frequent coexistence of multiple *N. discreta* phylogenetic species in sympatry make them ideal systems to study the general processes involved in the build up or maintenance of population differentiation. We used deep-coverage, and genome-scale DNA sequence data from 52 genomes of PS4 and experimental measures of reproductive isolation to address the following questions: What is the population structure? What is the genealogical history of population subdivision? What are the genome-wide patterns of divergence? What is the degree and genomic distribution of gene flow between related lineages? What is the degree of

reproductive isolation between lineages caused by intrinsic prezygotic barriers and intrinsic early post-zygotic barriers? What is the degree and genomic distribution of introgression from other lineages? What are the genomic features harboring signatures of recent selective sweeps? Our results reveal cryptic population structure within PS4, with two distinct lineages in Southeast Asia, three lineages in North America/Europe with a broad longitudinal and latitudinal range. Demographic and clustering analyses were also consistent with a model of secondary contact following a period of isolation, with varying rates of introgression generating genomic heterogeneity in levels of divergence between lineages.

MATERIALS AND METHODS

Genomic data collection

Isolates from Cote d'Ivoire, Papua New Guinea, Thailand, Switzerland and Spain were provided by the Fungal Genetic Stock Center. Isolates from CA, NM, and WA were obtained from DJ's personal *Neurospora* collection, hosted in JT's lab. Isolates from AK were collected by JT and handled as described in Jacobson et al. (2004).

Isolates were initially germinated on Vogel's minimal medium (Vogel 1956). After growth was observed, cultures were transferred to a Petri dish containing Bird medium (Metzenberg 2004) overlaid by cellophane and left to grow under constant light for 36-48 h before harvesting. Mycelial tissue was placed in the -80 Celsius freezer for 30 minutes, and then incubated at 65°C for 45 minutes, inverting occasionally, adding 0.5 mL of Zirconia/silica 0.5mm beads and 500 µl of lysis buffer with final concentrations of 50 mM Tris-HCl, 50 mM EDTA, and 3% SDS. The mycelium was ground twice for 30s in a bead beater machine, and in between the material was re-suspended by shaking. After a wash with 500 µl chloroform:isoamyl alcohol (24:1), samples were placed in the bead beater machine for another

30s, and then centrifuged ($12,000 \times g$, 5 min) to remove cellular debris. The aqueous phase was collected and genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen Inc., Valencia, CA). Genomic libraries were generated manually or with Apollo 324 NGS Library Prep System (Wafergen Biosystems), and samples were sequenced in Illumina HiSeq2000, 100bp paired-end with a 500bp insert size at the QB3 Vincent J. Coates Genomics Sequencing Laboratory at the University of California, Berkeley. The raw reads were deposited in the Sequence Read Archive and the accession numbers are listed in Table S1 (Supporting Information).

Reads were filtered based on quality and all duplicates and reads with more than 10% Ns, with at least one base with phred score smaller than 20, and with average phred score smaller than 30 were discarded. All good quality paired and orphan reads were then aligned to the reference genome using BOWTIE 2.0 (Langmead & Salzberg 2012). We used --END-TO-END mode, which requires that the entire read align from one end to the other, and preset option --SENSITIVE. We also used option --NO-DISCORDANT, which specifies that pairs of reads that do not align with the expected relative mate orientation and with the expected range of distances between mates are discarded. Alignments were sorted with SAMTOOLS (Li et al. 2009), duplicates were removed with PICARD (<http://picard.sourceforge.net>) and reads with mapping quality below 30 were removed with BAMTOOLS (Barnett et al. 2011). We used REALIGNERTARGETCREATOR and INDELREALIGNER within the GENOME ANALYSES TOOLKIT (McKenna et al. 2010) to define intervals to target for local realignment, and to perform local realignment of reads around indels, respectively, and UNIFIED GENOTYPER to call SNPs. We removed SNPs with quality <30, SNPs with mean coverage below 5 and above 200 and sites with more than 33% missing data using VCFTOOLS (Danecek et al. 2011).

Polymorphism, recombination, divergence

Preliminary phylogenetic analysis based on the full set of SNPs and isolates was carried out using the programs DNADIST and neighbor from the PHYLIP 3.69 package. Polymorphism and divergence statistics were computed using custom-made python scripts calling EGGLIB C++ libraries. d_{xy} was computed following (Nei & Li 1979) as the average number of pairwise differences between sequences from two populations, excluding all comparisons between sequences within populations. F_{ST} was calculated as $(\pi_T - \pi_S)/\pi_T$, where π_T is the nucleotide diversity in the total sample and π_S is the average nucleotide diversity in each population (Nei 1982). Recombination rates were estimated for the 20 largest scaffolds using the INTERVAL program in LDHAT version 2.2 (Auton & McVean 2007). Singletons and sites with missing data were excluded and the reversible-jump Markov-chain Monte Carlo scheme implemented in INTERVAL was run for 5e6 iterations, with a block penalty of 10, samples taken every 5000 iterations and a burnin phase of 5e5. Z , the standardized Fay and Wu's H was calculated using two outgroups (THA and PS4A) and only sites where both outgroups had same alleles were included, to avoid biases caused by homoplasy on the outgroup branch (Fay & Wu 2000; Baudry and Depaulis 2003; Zeng et al. 2006). The coefficient of linkage disequilibrium (r^2) (Hill & Robertson 1968) was computed using VCFtools with missing data excluded. The rate of decay in linkage disequilibrium with physical distance was determined by averaging r^2 for all pairs within a particular distance, with 1kb increments.

Source-sink patterns within lineages

Allelic richness and private allele richness were calculated with ADZE v1.0 (Szpiech et al. 2008) for subpopulations within lineages CA-WA and NM-WA based on haplotypes identified in 10kb windows. Only SNPs without missing data were included. ADZE implements a rarefaction procedure for counting alleles while adjusting for differences in sample size across subpopulations. Calculations were made using a standardized sample size corresponding to the smallest number of observations per population ($n=3$).

178 *Phylogenomics*

179 We used RAxML (Stamatakis 2014) to infer maximum likelihood genealogies based on the full set of
180 SNPs or on 10kb genomic windows. In scans of topological variation along the genome, only branches
181 with bootstrap support above 70% were considered. We used 100 bootstrap replicates.

182 *Admixture*

183 The three-population test is a test for treeness in three population trees including a focal group X and
184 two other groups Y and W. If group X is related to groups Y and W by a simple tree (*i.e.*, without
185 admixture), the f_3 statistic should be positive. If group X results from admixture of groups related to Y
186 and W, f_3 can be negative, and thus a significantly negative value of f_3 provides an unambiguous signal
187 of admixture (Reich et al. 2009). f_3 tests were carried out using TREEMIX (Pickrell & Pritchard 2012) with
188 standard errors computed in blocks of 100 SNPs.

189 *Demographic history inference*

190 We used the PS6 and THA genome sequences as outgroups to infer ancestral alleles and restricted our
191 dataset to include only those SNPs for which both outgroups shared the same allele, in order to limit
192 misidentification of ancestral alleles, which can bias demographic inference. Twenty-three divergence
193 models were considered (Table S2, Supporting Information), and for each model we ran the program
194 multiple times with different starting points to ensure convergence. We used the simplex method
195 implemented in dadi.Inference.optimize_log_fmin for numerical optimization, as we found that the
196 BFGS-based routines often failed to arrive at a good estimate starting from a random point in parameter
197 space. We assessed the model's goodness-of-fit by maximizing the model likelihood and visual
198 inspection of the residuals between the site frequency spectra generated by the inferred model and the

real data. We used 100 nonparametric bootstraps to estimate parameters uncertainties, using 100 pseudo-observed datasets with equal numbers of loci sampled with replacement from the original data set.

Selective sweep analysis

To identify genomic features that have potentially swept through populations we applied the genome scan method implemented in the program H-SCAN, which aims to detect signatures of selective sweeps by measuring the average length of pairwise homozygosity tracts (*H* statistic) in a population sample. The *H* statistic was computed for all variable sites and all lineages.

Mating success

The mating type of each isolate was determined *in silico*, using BLASTN of the *mat A* (Staben & Yanofsky 1990) and *mat a* (Glass et al. 1990) sequences on genomic sequences assembled using SOAPDENOV0. Crosses were made in 13 x 100-mm tubes containing slanted Westergaard synthetic crossing medium. We used filter paper as a carbon source to reduce conidiation (*i.e.*, production of mitotic spores) and because it is an effective substrate for crossing *Neurospora* (Perkins & Turner 1988; Turner et al. 2001). Filter paper was cut into strips and inserted into the tubed medium prior to the autoclaving and slanting processes. Strains were incubated for 4 days at 25°C to allow for the development of receptive protoperithecia (producing specialized hyphae, trichogynes, which are the female gametes), and fertilization was performed by transferring mitotic spores (*i.e.*, male gametes) using a platinum loop. After an additional 14 days of incubation, the reproductive success was scored on a scale with five rating categories under a dissecting microscope (Figure 5). The five categories correspond to different stages in reproductive development, from the development of perithecia, to the formation of black ascospores (Jacobson 1995; Dettman et al. 2003). Unlike other species of *Neurospora*, *N. discreta* ascospores are

not necessarily ejected from perithecia and can simply be oozing out. Hence, we determined the proportion of black ascospores visually after crushing perithecia in water between microscope slide and a cover slip.

RESULTS

Genome alignment and SNP calling

We present a population and comparative genomics analysis of *N. discreta* Phylogenetic Species 4 (PS4) evolution based on the resequencing of 52 genomes (Table 1). We used Illumina short-read technology to generate genome-wide sequences from 43 isolates of the PS4 group B (= PS4B): 8 from Alaska (AK), 15 from California (CA), 8 from New Mexico (NM), 8 from Washington (WA), and 3 non-North American isolates [2 from Europe (EU), one from Thailand (THA)]. We also used 9 isolates of PS4 group A from Papua New-Guinea (=PS4A), and as the outgroup one isolate of *N. discreta* PS6 from Côte d'Ivoire (CIV). Reads were mapped to the 37.3 Mb *N. discreta* PS4 reference genome (hosted on FungiDB <http://fungidb.org>; isolate FGSC 8579, collected in Belen, NM), yielding average alignment depths of 12.4× to 108.6× and covering 77.8% to 98.9% of the reference sequence (Table 1; Table S1, Supporting Information). After a preliminary phylogenetic analysis, the dataset was clone-corrected by keeping a single representative of nearly identical genotypes (*i.e.*, where genetic distance between genotypes is below 0.1; F84 distance, DNADIST program, PHYLIP package; Table S1; Figure S1, Supporting Information). Analyses were based on a final set of 42 genomes representing 1,762,444 high-quality SNPs of which 628,558 had no missing data. We note that the role of conidia for *Neurospora* dispersal in nature has long been suspected (Pandit and Maheshwari 1996; Turner et al. 2001) and interestingly our finding of clonal genotypes provides the first indirect evidence that conidia can start new colonies and contribute to spread the fungus.

Population subdivision

To assess the total evidence for population subdivision and the genealogical relationships among samples, the complete set of SNPs was used to infer a maximum likelihood phylogenetic tree. In the best fitting tree, rooted by the PS6 isolate from Côte d'Ivoire, the PS4B isolate from Thailand branched in between PS4A and other PS4B isolates (Figure 1). A total of 1,189,625 SNPs were specific to the combination of North American/European PS4B and PS4A sequences, of which 15,647 (1.3%) were shared and 355,484 fixed (29.9%) between the two groups. A total of 516,729 SNPs were segregating in the North American/European dataset alone.

Within PS4B sequences from Europe and North America, we observed three distinct lineages, hereafter referred to as the AK-EU, CA-WA and NM-WA lineages (Figure 1). The CA-WA lineage was basal to the group formed by the AK-EU and NM-WA lineages and comparisons between pairs of lineages showed that the vast majority of variable sites corresponded to fixed differences between lineages, or polymorphisms that were exclusive to each lineage (Table 2). Interestingly, isolates from the two lineages found in Washington state (WA) were coexisting in sympatry within the same, small campground at Lake Chelan.

We also analyzed population subdivision and admixture within the North American/European group of samples using the model-based clustering algorithm implemented in FRAPPE. Only runs with $K=2$ and $K=3$ populations converged consistently towards the same likelihood values and towards the same clustering solution, and the greatest increase in likelihood among runs was observed from $K=2$ to $K=3$. Together, these results suggest that the model with $K=3$ populations captures most of the population subdivision signal existing in the North American/European clade (Figure S2, Supporting Information), and the three identified clusters matched the lineages identified using maximum likelihood phylogenetic

inference (Figure 1). Admixture among clusters was only observed in Alaska where one individual of the Alaskan subpopulation had substantial ancestry in the NM-WA lineage (membership coefficient in NM-WA: 12.04%). A genome scan revealed that putatively introgressed segments could be detected and were widespread in the genome of the admixed Alaskan isolate (Figure S3, Supporting Information). This is consistent with relatively recent hybridization and backcrossing between the two lineages, although the isolates from within the NM-WA lineage had not been detected in Alaska.

Turning to the two, sympatric subpopulations coexisting in Washington, using the three-population test, a formal test of admixture based on patterns of allele frequency correlation that can detect gene flow events occurring over hundreds of past generations (Reich et al. 2009; Patterson et al. 2012), we found no support for an admixed ancestry in Washington subpopulations either for the CA-WA lineage (sources: CA,NM; $f_3=0.00091$; $Z\text{-score}=0.76773$) or the NM-WA lineage (sources: CA,NM; $f_3=0.00155$; $Z\text{-score}=3.13093$).

The geographic range of the three lineages could be consistent with recolonization from either southern or northern glacial refugia. To investigate source-sink patterns, and given the few Washington isolates in either the CA-WA or NM-WA lineages ($n=3$ for each Washington component), we used an approach based on the computation of allele richness and private allele richness rather than the more familiar coalescent-based approach. The allele richness approach rests on the assumption that recently founded populations retain only a subset of the variability existing in parental populations. We estimated allele and private allele richness in 10kb windows (*i.e.*, 10kb haplotypes defined the different 'alleles'), using a rarefaction procedure designed to avoid biases due to differences in sample size between populations from each pair (Szpiech et al. 2008). We found higher levels of allele richness (AR) and private allele richness (PAR) in the WA subpopulations for both lineages, suggestive of histories of independent southward migrations from WA to CA and from WA to NM (Table 3).

Summary of genetic variation

Summaries of variation are listed in Table 4. Variability, estimated by the nucleotide diversity (π), the proportion of polymorphic sites per base (ϑ_w), number of haplotypes (K) and haplotype diversity (He) averaged across 10kb windows, was higher in PS4A ($\pi=0.0032/\text{bp}$; $\vartheta_w=0.0036/\text{bp}$; $He=0.923$; $K=7.993$), intermediate in CA-WA ($\pi=0.0021/\text{bp}$; $\vartheta_w=0.0020/\text{bp}$; $He=0.880$; $K=7.910$), and lower in AK-EU ($\pi=0.0009/\text{bp}$; $\vartheta_w=0.0009/\text{bp}$; $He=0.773$; $K=5.788$) and NM-WA ($\pi=0.0007/\text{bp}$; $\vartheta_w=0.0007/\text{bp}$; $He=0.823$; $K=6.094$). The recombination rate was on the order of 10 Morgans/kb for all lineages, compared to 1 to 3 Morgans/kb for populations of *Saccharomyces cerevisiae* (Tsai et al. 2008) (Table 4). Linkage disequilibrium decayed to half its maximum value in less than 1kb to 3kb (Table 4), compared to 0.78kb in *N. crassa* (Ellison et al. 2011) and ~3kb in *S. cerevisiae* (Liti et al. 2009). Computations based on local subpopulations (*i.e.*, the AK, CA, NM and two WA samples) instead of lineages that had been identified using clustering algorithms gave the same pattern (data not shown).

We observed a large number of high-frequency derived mutations in all lineages, leading to U-shaped (*i.e.*, non-monotonic) derived Site-Frequency Spectra (SFS) (Figure 2). Model fitting using the PYTHON package DADI (Gutenkunst et al. 2009) indicated that observed SFS were significantly different from those expected under the standard neutral model ($\chi^2>300$; $P<0.001$). We observed a systematic excess of high-frequency derived variants, and nearly all lineages also displayed an excess of low frequency variants compared to neutral expectations (Figure 2). The observed excess of high-frequency derived mutations was not due to misidentification of ancestral states, as we restricted the dataset to include only those SNPs for which the two outgroups had same alleles to avoid biases caused by homoplasy on the outgroup branches. The fact that the non-synonymous SFS was more skewed toward low-frequency variants than the synonymous SFS in all lineages but CA-WA suggests that negative

selection, which maintains deleterious mutations at low frequencies, contributes to the excess of rare polymorphisms, possibly in combination with recent population expansion. Moreover, the fact that the synonymous SFS was more skewed toward high-frequency variants than the non-synonymous SFS suggests little role for genetic draft (Gillespie 2001; Maynard-Smith et al. 1974) in the large proportions of high frequency derived polymorphisms. The fitting of multiple-populations models indicated that this latter category of variants may be explained by migration among populations (see below).

Genealogical discordance across the genome

Unlike the simple allopatric model which predicts a single split time for all genomic regions, most non-allopatric speciation models predict discordant genealogical histories among loci (Garrigan et al. 2012; Martin et al. 2013). To characterize the genome-wide distribution of genealogical histories resulting from divergence within PS4B, we inferred maximum likelihood genealogies for 10kb windows using PS6 as an outgroup. Of 3797 windows, 41.8% presented ‘resolved’ genealogies, that is, with the outgroup specifying one of the North American/European lineages as basal and the other two lineages forming a monophyletic, sister group. CA-WA was identified as the basal group in 17.5% of genomic windows while discordant topologies with either the NM-WA or the AK-EU lineages as basal groups were found in trees for 12.7% and 11.7% of genomic windows, respectively. The most supported phylogeny of North American/European lineages based on the full set of SNPs ((AK-EU,NM-WA),CA-WA) was retrieved in 12.7% of 10kb windows, and it was a subset of the 17.5% genealogies with an internal branch between CA-WA and the remaining AK-EU and NM-WA isolates, regardless of the separation between these latter two lineages.

Inter-lineage gene flow

331 The finding of admixture between AK-EU and NM-WA suggested that the different lineages may also
332 have been connected by gene flow in the past. To test whether the observed widespread phylogenomic
333 discordance across PS4B genomes was due to post-divergence gene flow, we inferred a complete
334 demographic model using the DADI package (Gutenkunst et al. 2009), which infers demographic
335 parameters based on a diffusion approximation to the site frequency spectrum. We used a sequential
336 approach, as the method cannot handle more than three population samples. The compared models
337 included scenarios of strict isolation, continuous post-divergence gene flow, ancient migration and
338 secondary contact, assuming or not heterogeneous migration rates across the genome. Given a model,
339 we used DADI to generate an expected site frequency spectrum, to maximize the similarity between the
340 expected and observed site frequency spectra over the parameter values space, and to evaluate fit by
341 computing a composite-likelihood. We first inferred the demographic history of lineages PS4A and PS4B-
342 US-EU, the latter corresponding to the pooling of the three North-American/European lineages. We
343 started from a simple bifurcating model with constant population size and no migration, and we
344 progressively increased model complexity by adding parameters. The most supported model included a
345 size change in the ancestral population, simultaneous size changes in descendant lineages, and relatively
346 recent gene flow following secondary contact between them (Figure 3; Supplementary Table 2).
347 Examination of residuals indicated that the resulting model reproduced the observed spectra well for
348 intermediate-frequency variants, but we also found an excess of high frequency alleles and a deficit of
349 low frequency alleles in our data compared with what is predicted by the model (Figure 2; Figure S4,
350 Supporting Information). In order to convert parameter estimates to physical units, we estimated the
351 ancestral population size (N_{AB1}). We used the population mutation rate $\vartheta=4N_{AB1}\mu L$, where μ was
352 assumed to be approximately $1e - 9$ per generation (Lynch 2010), and L is the genome size (ca. 37Mb).
353 This ancestral population size was then used to transform time estimates from DADI (in units of $2N_{AB1}$)

into actual generations. The estimated divergence time was 1.8e6 generations ago (*i.e.*, $2.9e5 + 3.6 + 1.5e6$; Figure 3; Table 5). Parameter estimates indicated relatively recent and severe population shrinkage in PS4B-US-EU (from $8.3e9$ to $9.2e4$ individuals) and ancient, strong population increase in PS4A (from $2.1e5$ to $1.6e8$ individuals), with both size changes estimated to have occurred 2.9e5 generations ago. We also inferred an ancient population expansion in the ancestral population (ca. 1000 fold increase, 1.5e6 generations prior to lineage splitting). Secondary contact and gene flow was estimated to have started at approximately the same time as population size changes. The migration rate was substantial into PS4A ($6.4e-8$ genetic replacement per generation) and nearly zero into PS4B-US-EU ($2.1e-42$ genetic replacement per generation); the corresponding effective number of migrants per generation was on the order of $1e-1$ and $1e-36$, respectively.

We then inferred the demographic history of lineages within PS4B-US-EU. We started from the inferred total evidence maximum-likelihood tree as the true population phylogeny, and we progressively increased model complexity by adding parameters. The best-supported model included a strong demographic bottleneck in the ancestral population prior to divergence and relatively recent heterogeneous migration among lineages following secondary contact (Figure 3; Table S2, Supporting Information). Overall fit was also good, with the resulting model reproducing the observed spectra well, as seen in residuals (Figure 2; Figure S4, Supporting Information). Parameter estimates indicated a ~1000 fold decline in the global joint ancestral population, a small effective size and short lifespan of the population ancestral to lineages AK-EU and NM-WA, and effective population sizes on the order of $1e4$ to $1e5$ for derived lineages (Table 5; Figure 3). The three lineages diverged ca. $4.4e5$ generations ago, and gene flow following secondary contact began $7.4e4$ generations ago. Most of the genome (85.1%) experienced migration rates on the order of $1e-7$ genetic replacement per generation (corresponding effective number of migrants per generation on the order of $1e-3$ to $1e-2$), except for migration into

NM-WA from AK-EU, which was zero. A smaller fraction of the genome (14.9%) experienced migration rates that were ~100 times higher from NM-WA into CA-WA, ~10 times higher from NM-WA into AK-EU, similar from CA-WA into AK-EU, ~10 times smaller from CA-WA into NM-WA and from AK-EU into CA-WA, and on the order of $1e-8$ genetic replacement per generation from AK-EU into NM-WA (dramatically higher migration than the $1e-13$ estimated for the other 85% of the genome). Gene flow along the internode carrying the AK-EU and NM-WA lineages could not be estimated (data not shown) and the corresponding parameters were therefore not included in the models compared.

Genome-wide patterns of divergence

To measure the extent of isolation between lineages and subpopulations at different levels of geographic separation, while accounting for genomic heterogeneity in levels of divergence, we characterized patterns of genetic divergence across the genome using measures of relative divergence (F_{st} , also known as a measure of 'differentiation') and absolute divergence (d_{xy}) (Figure 4). The divergence metrics were computed at the scale of non-overlapping 10kb windows in order to increase precision by minimizing noise from individual site-based estimates. Overall, levels of divergence were consistent with the phylogenetic relationships among taxa: average divergence was greater between PS4A and PS4B than between lineages within PS4B, and greater between PS4B lineages than between subpopulations within them (Table 6).

Theory predicts that variation in genome-wide levels of divergence as a result of the mode (with or without gene flow) and stage of separation between populations. Thus, levels of divergence are expected to be low and homogenous during early stages of speciation when gene flow is high, and rise in level and heterogeneity (due to unevenness in susceptibility to introgression) as the speciation process proceeds, and reach a high and more homogenous level after the speciation process is

completed (Barton & Bengtsson 1986; Via 2009; Feder et al. 2012; Roux et al. 2013). In our comparisons we found that the coefficient of variation followed this trend, with lowest levels of heterogeneity in divergence in comparisons between PS4A and PS4B, and highest levels in comparisons between subpopulations from within the same lineage. Complicating the picture is the hypothesis that heterogeneity in levels of divergence is affected by the existence and strength of linked selection and by genomic variation in rates of recombination such that positive or negative selection are expected to increase levels of relative divergence in regions of low recombination (Nachman & Payseur 2012). We found no significant, positive correlation between local recombination rate and nucleotide diversity (not shown), suggesting no strong impact of background selection and/or hitchhiking (Hill-Robertson effects) on patterns of diversity, and thus no strong contribution of selection at linked sites to heterogeneity in levels of relative differentiation.

Theory also predicts that the variation in levels of divergence across the genome is greater in populations diverging in the presence of gene flow (sympatry or parapatry) than in geographically isolated populations. Levels of divergence between sympatric populations of the CA-WA and NM-WA lineages were broadly similar in mean and variation to those between their allopatric counterparts (Table 6). This result suggests that gene flow was not higher between sympatric populations relative to allopatric ones, consistent with the lack of evidence for recent hybridization between these sympatric subpopulations in analyses of admixture (Figure 1).

Genome scan for selective sweeps

In a selective sweep, rare adaptive variants and linked variation are driven to high population frequencies by positive selection, while non-adaptive alleles are removed from the population. Selective sweeps are classified as ‘hard’ sweeps, where only a single adaptive allele sweeps through the

population, and ‘soft’ sweeps, where multiple adaptive alleles sweep at the same time through the same population or through different populations of a genetically structured species (Messer & Petrov 2013). Hard and soft sweeps are both expected to increase linkage disequilibrium over neutral expectations and generate unusually long tracts of homozygosity around the adaptive site.

To identify genomic features that have potentially swept through populations we applied the genome scan method implemented in the program H-SCAN, which aims to detect signatures of selective sweeps by measuring the average length of pairwise homozygosity tracts (H statistic) in a population sample. We measured the average pairwise homozygosity tract length (in bp) extending in both directions from each SNP. We weighed the tract lengths by the number of SNPs in each pairwise homozygous region to account for variable SNP density. The H statistic was computed for all variable sites and all lineages (Figure S5, Supporting Information). However, the highest H values all corresponded to relatively small regions of elevated pairwise homozygosity (8400bp on average), indicating a lack of recent detectable selective sweeps in PS4 lineages (Figure S5; Table S3, Supporting Information).

Experimental measures of reproductive success

The lack of admixture between subpopulations coexisting in sympatry at the Washington site suggested the existence of strong reproductive isolation barriers between the CA-WA and NM-WA lineages. To test this hypothesis and more generally to gain insight into the strength and nature of reproductive isolation among subpopulations and lineages, we experimentally measured several components of mating success of crosses between 29 of the 31 North American and European isolates of PS4B. We also crossed isolates to the original testers strain of *N. discreta sensu stricto* (Mat A P581 and Mat a 8127), in an effort to assign them to a biological species. Mating types were determined computationally and we

measured mating success for $16 \times 14 = 240$ crosses. Each isolate was used as both a female or a male, so that each cross consisted in two matings, and reproductive success was reported as the most successful of the two matings (Figure 5).

The vast majority of all attempted crosses were compatible (23 category 5, 208 category 6). Five out of eight incompatible interactions were observed between isolates from the same lineage, two in crosses with one of the tester strains, and only one unsuccessful cross involved isolates from different lineages (Figure 5). Several isolates tended to be sterile when acting as the perithecial parent, but were competent when functioning as the fertilizing parents, confirming previous observations that reproductive success is influenced by the interaction of both parental genotypes (Dettman et al. 2003). In general, mat A isolates showed a female sterility pattern, but not in all intra- or inter-group matings, as illustrated by the success of crosses between CA-WA isolates or crosses between AK-EU mat A and CA-WA mat a, suggesting that interactions between parental genotypes are modulated by the geographic origin of the parents.

DISCUSSION

To investigate the evolutionary factors responsible for the diversification of fungal populations and species in the presence of gene flow, we used population genomics of 52 resequenced isolates from 9 sites in 3 continents combined with experimental measures of reproductive isolation to study population structure in a fungus with an exceptionally broad latitudinal and longitudinal range, the fire-associated fungus *Neurospora discreta*, a relative of *N. crassa*. Clustering analyses identified several long-diverged lineages within the phylogenetic species PS4, and analyses of the genomic landscape of divergence revealed extensive heterogeneity in levels of genomic divergence between lineages. Haplotype-based statistics and analyses of genomic patterns of variation revealed no signature of

selective sweeps and linked selection, indicating that genomic heterogeneity in divergence is not simply an incidental consequence of selection at linked sites reducing within lineage variation relative to divergence between populations. Modeling a number of demographic histories revealed that genomic heterogeneity was most likely caused by differential gene flow following secondary contact between allopatric lineages.

Biogeography and Neurospora's life cycle

Previous studies revealed that PS4 is the most broadly distributed species of *Neurospora* (Dettman et al. 2003; Jacobson et al. 2004; Jacobson et al. 2006). Our study demonstrates clear and deep population structure within PS4, revealing the power of genomic sequencing to reveal cryptic diversity that may be missed by more traditional methods of species recognition, such as phylogenetic investigations of diversity (Taylor et al. 2000; Dettman et al. 2003). Model-based inferences of past demographic history indicated that PNG and North American/European lineages diverged 3.6e6 generations ago, and lineages within North America/Europe about 4.4e5 generations ago. Relating these estimates with past geologic or climatic events requires an estimate of generation time, which is a perennial question in fungi. Extensive examinations of *Neurospora* colonies at burn sites in North America led to only a single observation of fruiting bodies beneath the bark of woody species (Jacobson et al. 2004). This suggests that sexual reproduction in *Neurospora* may be induced by wildfires, and thus that the generation time of *Neurospora* may correspond to the fire returns intervals. Previous work estimated a frequency of wildfires ranging from 20-25 years for shrubs and grasslands to 300 years for pine forest in the Yellowstone National Park (Houston 1973; Romme and Despain 1989), and ca. 100 years for the boreal forest of Quebec (Lesieur et al. 2002). These estimates, however, are based on tree fire-scars chronologies, which do not go further than one millenium, and it is not certain that fire returns intervals

remained constant through time. Assuming 100 years as a gross estimate of the generation time of *Neurospora*, the North-American/European and Papua-New-Guinean lineages of PS4 would have diverged about 400 million years ago, which corresponds to the age of Ascomycota/Basidiomycota divergence (Berbee & Taylor 2006), and is much older than the divergence between *N. discreta sensu lato* and other *Neurospora* heterothallic species estimated from phylogenies (ca. 7 My; Corcoran et al. 2014). This conflict suggests that fires were more frequent than estimated and/or that sexual cycles in *Neurospora* are not necessarily synchronized with wildfires. Assuming, very conservatively, that the divergence between PS4A and PS4B is the age of *N. discreta sensu lato* (7 My), our per-generation estimate of 3.6e6 generations would actually imply a generation time of ca. 2 years, which is much shorter than the estimated frequency of wildfires in North America.

Field observations (Jacobson et al. 2004) and experimental inoculations of pine seedlings (Kuo et al. 2014) suggest that species of *Neurospora* may be living as an endophyte. An endophytic lifestyle implies strong dependence of the migration pathway of *Neurospora* species on their hosts, although the lack of host specificity make them less dependent on changes in the geographic range of a particular species than on movements of the whole plant community. The oldest split detected within our dataset, between North-American and Asian lineages of PS4, may be related to the dispersal and subsequent isolation of plant species between landmasses across what is now the Bering land bridge, which existed from the Miocene (23-5 Mya) toward the end of the Pliocene (3.5 Mya) (Wen 1999).

The geographical extent of our sampling effort limits detailed inferences on the phylogeography of North-American lineages, but a number of hypotheses can nonetheless be proposed. Divergence within North American PS4B may have been driven by climate oscillations of the Pleistocene (2.6Mya-11kya), followed by secondary contact and limited hybridization along the Pacific Northwest, which is an important suture zone between multiple Northward and Southward postglacial expansion routes

(Swenson & Howard 2005). The presence of the same lineage in Alaska and Europe may result from long-distance dispersal, possibly via the transportation of logs or other woody material carrying the fungus. This hypothesis is consistent with the lack of divergence between Alaskan and European samples in the phylogeny inferred from the full set of SNPs. Such anomalies in the distribution of *Neurospora* lineages have already been reported in *N. crassa* (Radic et al. 2012) and *N. tetrasperma* (Corcoran et al. 2014), and molecular data provided evidence that even fungal species without long-distant colonization abilities can successfully establish themselves on new continents (Fontaine et al. 2013; Gladieux et al. 2015).

Reproductive barriers between sympatric lineages

Biological species in *Neurospora* have historically been delineated by measuring the proportion of black ascospores in laboratory crosses (Perkins et al. 1976; Jacobson et al. 2004). When using this broad criterion, we find that all investigated strains belong to a single biological species, in accordance with their membership in the same phylogenetic species *N. discreta* PS4. However, despite compatibility *in vitro* and the relatively recent divergence among North-America/European lineages, our resequencing data provided no evidence for admixture between lineages CA-WA and NM-WA coexisting at Washington sampling site. This lack of evidence suggests reproductive isolation between these lineages, although the non-zero estimates of gene flow between them indicates that reproductive isolation became effectively complete only recently, or is not complete between all populations of the two lineages. As is always the case, it is not known if increased sampling might also reveal admixed individuals that were not included in our study.

The widespread compatibility of crosses attempted in our *in vitro* study is not contradictory with the fact that lineages mostly maintain cohesion, in particular in sympatry (Gladieux et al. 2011; Taylor et

al. 2015; Taylor et al. 2000). The observed interfertility simply indicates that the relative reproductive isolation between lineages is not caused by the intrinsic reproductive barriers which have been evaluated in vitro, such as intrinsic premating barriers (*i.e.*, assortative mating by mate choice), intrinsic postmating prezygotic barriers (*i.e.*, gametic incompatibility) or a form of intrinsic early postzygotic isolation (*i.e.*, zygotic mortality). Instead, the relative reproductive isolation between lineages appears to be caused by one or several of the following extrinsic barriers (Table 1 in Nosil et al. 2005): temporal isolation (*i.e.*, different mating times), habitat isolation (*i.e.*, different mating sites), ecological barriers (*i.e.*, immigrant inviability, selection against hybrids), or intrinsic late postzygotic barriers (*i.e.*, hybrid sterility), or another form of intrinsic early postzygotic isolation (*i.e.*, hybrid inviability).

Among possible barriers to gene flow, the contribution of intrinsic barriers could be measured experimentally, by germinating ascospores to estimate hybrid viability, and by using the resulting mycelia to assess the self-fertility of hybrids. In *N. tetrasperma*, incorporating characters such as viability and fertility of offspring resulted in a better congruence between biological species delineation and actual patterns of historical gene flow (*i.e.*, phylogenetic species recognition; Menkis et al. 2009). Quantifying the role of extrinsic barriers to gene flow is more challenging in organisms that are difficult to study in nature like *Neurospora*, but ecological barriers can nonetheless be investigated, indirectly, using a reverse ecology approach (Ellison et al. 2011; Branco et al. 2015). In the case of North-American/European lineages of PS4, overall levels of genomic divergence were too high, despite substantial levels of gene flow, to allow the identification of candidate genomic features involved in the local adaptation using genome scans. However, the finding of a late generation hybrid between NM-WA and AK-EU lineages in Alaska suggest that reproductive isolation is not complete among all lineages, and there might be opportunities to identify hybrid swarms that would allow fine mapping of genomic

regions resisting to gene flow because of endogenous (*i.e.*, incompatibilities) or exogenous (*i.e.*, divergent ecological selection) reproductive barriers.

A comprehensive survey of the literature revealed a general lack of intrinsic premating isolation between sympatric species of pathogenic Ascomycetes (Le Gac & Giraud 2008) which is reminiscent of the case of sympatric lineages of *Neurospora* (Dettman et al. 2003; Menkis et al. 2009; this study). The authors proposed that the lack of intrinsic premating barriers between pathogenic Ascomycetes results from a peculiarity of their life cycle, with mating in these fungi taking place within the host, so that mating only occur between individuals adapted to the same host and hence host specificity pleiotropically controls assortative mating (Le Gac & Giraud 2008; Giraud et al. 2010; Gladieux et al. 2011). However, *Neurospora* species are not pathogens, but more likely endophytes, and albeit probably mating within their hosts, they lack host specificity so it is unlikely that this form of habitat specialization contributes to reproductive isolation. Another hypothesis is that the general lack of intrinsic premating barriers between *Neurospora* species results from other factors, such as the existence of constraints on the evolution of such barriers (Turner et al. 2010; Turner et al. 2011).

Ongoing admixture and post-divergence gene flow

We inferred relatively high migration rates following secondary contact between North-American/European lineages (median of effective migration rates on the order of $1e^{-1}$), and between PS4B-US-EU and PS4A (on the order of $1e^{-1}$, unidirectional into PS4A), providing evidence that lineages were connected by gene flow in the recent past, and therefore that their distributions have been overlapping to some extent. The geographic distance between Papua New Guinea and North America undoubtedly surpasses intrinsic dispersal abilities of *Neurospora* ($>10,000$ km) and our time estimate for secondary contact (ca. $3e5$ ybp) greatly exceeds the first long-distance human travels across the Pacific

Ocean (Jobling et al. 2004). One possibility is that detected gene flow might in fact be caused by introgression by an unknown species of intermediate divergence between the two lineages, giving the false impression that genetic exchanges occurred between the two allopatric lineages. Endemicity of this putative species in Papua New Guinea may account for the observed asymmetry in migration patterns (unidirectional migration into PS4A).

In North-America/Europe, the different lineages may have diverged following repeated periods of isolation in separate glacial refugia, interspersed with periods of secondary contact potentially permitting gene flow that only began relatively recently, as suggested by the finding of a late generation hybrid in Alaska indicative of ongoing admixture. Patterns of allele richness and private allele richness in populations from CA, WA and NM were consistent with histories of southward migrations, but a larger sampling is required to conclude about the phylogeography of PS4 in North America. Patterns of genetic variation consistent with allopatric isolation and subsequent secondary contact were also detected North American lineages within the *N. tetrasperma* species complex by Corcoran et al. (2014).

Using a secondary contact demographic model with heterogeneous gene flow across the genome, we were able to show that a small fraction of the genome experienced higher migration rates between certain pairs of lineages. The indirect effects of selection presumably explain this heterogeneity in introgression rates among genomic regions, with limited gene flow in the neighborhood of barrier loci (genetic incompatibilities or genomic features under divergent ecological selection), maximal gene flow at adaptively introgressed regions and basal gene flow in regions not affected by selection (Roux et al. 2013). It is important to note that the failure to account for genomic heterogeneity in introgression rates would have provided support to an inappropriate demographic model of divergence, biasing conclusions about the effects of natural selection on effective migration rates (Roux et al. 2014).

Concluding remarks

Population genomics offers great potential for enhanced understanding of the population biology and evolution of fungi. Here, we reported on the population structure and demographic history of Asian, North American and European isolates of the phylogenetic species PS4 of *N. discreta*. We found evidence for cryptic population subdivision, underlying a history of strict isolation followed by introgression through secondary contact, with genomic heterogeneity in rates of post-divergence gene flow. Despite the inferred history of long-term isolation, experimental measures of mating success revealed a lack of early intrinsic reproductive barriers between lineages (*e.g.*, mate choice or zygotic mortality), suggesting that the barriers that limit gene flow following secondary contact are mostly extrinsic barriers (*e.g.*, immigrant inviability) or late intrinsic barriers (*e.g.*, hybrid inviability or sterility). In terms of *Neurospora* biogeography, our study revealed histories of southward migration in North-American lineages, which reinforces previous findings that established *Neurospora* species as common inhabitants of northern forests, and not only primarily tropical or subtropical fungi as was long believed (Jacobson et al. 2004; Dettman et al. 2006; Perkins & Turner 1988; Turner et al. 2001a). Further sampling in the northern latitudes should increase our understanding of the diversity and distribution of temperate *Neurospora* spp. High resolution data sets focusing on currently admixing lineages should also help determining the extent and cause of introgression and in particular whether it is driven by natural selection, either directional or stabilizing. Similar investigations on other models will allow to quantify how common are the factors uncovered in our study in the diversification of fungal lineages.

DATA ACCESSIBILITY

The raw reads were deposited in the Sequence Read Archive and under accession numbers SRS1087846 to SRS1087920. Polymorphism data (VCF files), all input data as well as resulting tree files underlying NJ and maximum likelihood phylogenies, mating types and crossing results were deposited in Dryad (doi:10.5061/dryad.1538f).

622

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630 **AUTHOR CONTRIBUTIONS**

631 PG and JT designed research. PG and JT wrote the paper, with contribution from BW and DJ. All authors
632 read and approved the manuscript. DJ and JT collected samples. PG analyzed data, following preliminary
633 analyses by FP, and with contribution from BW for the selective sweep analysis. CHS and PG designed
634 the pipeline for read processing and SNP calling. PG and DK prepared sequencing libraries. MF and IS
635 determined mating types of all isolates, and carried out DNA extraction with PG. LM, DJ and PG
636 conducted the mating compatibility experiment.

TABLES

Table 1. Isolates and genome sequencing information

Taxon/Origin	<i>n</i>	Proportion of ref. genome covered (%)	Alignment depth
<i>N. discreta</i> PS4A			
Papua New Guinea	9 (9)	80.1	22.2
<i>N. discreta</i> PS4B			
Alaska	8 (8)	95.9	42.5
California	15 (8)	92.4	49.6
New-Mexico	8 (7)	98.7	68.8
Washington	8 (6)	96.7	75.5
Switzerland	1 (1)	96.9	95.1
Spain	1 (1)	96.7	38.9
Thailand	1 (1)	86.8	25.8
<i>N. discreta</i> PS6			
Ivory Coast	1 (1)	58.2	17.5

n is sample size and between parentheses is the number of genomic sequences included in the study after clone-correction (*i.e.*, after keeping only one representative of nearly identical genomic sequences).

Table 2. Fixed differences, shared and exclusive polymorphisms.

Category	AK-EU vs CA-WA	AK-EU vs NM-WA	CA-WA vs NM-WA	US-EU vs PS4A
Total number of biallelic sites	366958	272408	354410	1172183
Fixed differences	34.9%	43.7%	38.6%	30.3%
Shared polymorphisms	5.0%	2.4%	3.0%	1.3%
Exclusive polymorphisms lineage 1	20.3%	32.2%	43.0%	31.2%
Exclusive polymorphisms lineage 2	39.2%	21.6%	15.0%	28.2%

US-EU is the pooling of all three North American/European lineages (*i.e.*, AK-EU $\cup$ CA-WA $\cup$ NM-WA)

Table 3. Mean allelic richness (AR) and Mean private allele richness (PAR) per 10kb-window after correction for sample size by rarefaction using ADZE.

Lineage	Subpopulation	mean AR (standard error)	mean PAR (standard error)
CA-WA			
	CA	1.2237 (0.0182)	2.2151 (0.0087)
	WA	1.6714 (0.0168)	2.6628 (0.0084)
NM-WA			
	NM	2.0907 (0.0079)	0.6413 (0.0132)
	WA	2.4233 (0.0089)	0.974 (0.0125)

Sample size was standardized to n=3 (size of WA subpopulations).

Table 4. Summary of population genomic variation

Population ID	n	π (%)	ϑ_w (%)	Tajima's D	He	K	ρ (M/kb)	LD 50% decay
AK-EU	10	0.098 (0.151)	0.093 (0.128)	-0.213 (0.946)	0.773 (0.203)	5.788 (2.083)	47.1	3kb
CA-WA	11	0.208 (0.216)	0.201 (0.193)	-0.035 (1.131)	0.88 (0.161)	7.91 (2.249)	24	<1kb
NM-WA	10	0.07 (0.106)	0.073 (0.1)	-0.31 (0.873)	0.823 (0.166)	6.094 (1.92)	15.1	2kb
PNG	9	0.318 (0.162)	0.36 (0.154)	-0.621 (0.957)	0.923 (0.227)	7.993 (1.955)	56.4	<1kb

n is sample size; ϑ_w is Watterson's ϑ ; π is nucleotide diversity; He is haplotype diversity; K is the number of haplotypes; ρ is the recombination rate, averaged across the 20 largest scaffolds; ϑ_w , π , He , K are average values across 10kb windows with standard deviation between parentheses; LD 50% decay is the average physical distance over which the coefficient of linkage disequilibrium (r^2) decays to half its maximum value.

Table 5. Likelihood and parameter estimates of an isolation-with-migration model fitted to the data using the python package DADI

Dataset/Parameter	Point estimate	Average	SD
<i>PS4A and PS4B-US-EU</i>			
<i>Log-likelihood</i>	-8.13E+04	-7.96E+04	1.13E+04
N_{AB1}	1.41E+06	1.39E+06	1.98E+05
$m_{PS4A \leftarrow PS4BUSEU}$	6.40E-08	3.12E-06	2.14E-05
$m_{PS4BUSEU \leftarrow PS4A}$	2.11E-42	1.43E-12	8.17E-12
N_{PS4A1}	2.12E+05	4.16E+05	5.95E+04
N_{PS4A2}	1.58E+08	3.07E+08	4.42E+07
$N_{PS4BUSEU1}$	8.34E+09	2.30E+10	1.15E+10
$N_{PS4BUSEU2}$	9.20E+04	1.80E+05	2.57E+04
T_{AB1}	1.50E+06	1.47E+06	2.11E+05
T_{AB2}	1.48E+06	1.45E+06	2.07E+05
T_{AB3}	3.61E+00	1.70E+00	2.12E+00
T_{AB4}	2.94E+05	2.87E+05	4.10E+04
<i>AK-EU, CA-WA and NM-WA lineages within PS4B-US-EU</i>			
<i>Log-likelihood</i>	-7.66E+03	-7.77E+03	1.04E+02
N_B	1.50E+06	1.47E+06	3.52E+04
$m_{AKEU \leftarrow CAWA}$	3.38E-07	3.43E-07	1.51E-08
$m_{AKEU \leftarrow NMWA}$	3.23E-06	3.18E-06	8.32E-08
$m_{CAWA \leftarrow AKEU}$	4.55E-08	4.15E-08	8.23E-09
$m_{CAWA \leftarrow NMWA}$	2.79E-05	2.79E-05	1.28E-06
$m_{NMWA \leftarrow AKEU}$	9.30E-08	1.02E-07	1.05E-08
$m_{NMWA \leftarrow CAWA}$	1.04E-08	1.07E-08	4.88E-09
$m_{AKEU \leftarrow CAWA}^{'}$	1.15E-07	1.12E-07	4.55E-09
$m_{AKEU \leftarrow NMWA}^{'}$	2.21E-07	2.25E-07	6.29E-09
$m_{CAWA \leftarrow AKEU}^{'}$	1.87E-07	1.86E-07	5.71E-09
$m_{CAWA \leftarrow NMWA}^{'}$	2.41E-07	2.42E-07	7.35E-09
$m_{NMWA \leftarrow AKEU}^{'}$	3.88E-13	6.51E-12	3.98E-11
$m_{NMWA \leftarrow CAWA}^{'}$	1.07E-07	1.06E-07	3.35E-09
N_{AKEU}	5.40E+04	1.08E+05	1.04E+03
N_{B1}	9.14E+03	1.75E+04	1.73E+03
N_{CAWA}	1.27E+05	2.53E+05	1.51E+03
N_{B2}	8.73E-02	1.66E-01	1.70E-02

N_{NMWA}	7.12E+04	1.43E+05	8.21E+02
T_{B1}	1.30E+04	1.21E+04	1.39E+03
T_{B2}	8.05E-02	7.67E-02	7.74E-03
T_{B3}	3.74E+05	3.74E+05	2.45E+03
T_{B4}	7.40E+04	7.42E+04	3.35E+03
ρ	1.49E-01	1.49E-01	2.66E-03

Parameter are presented in [Figure 3](#)

Table 6. Summary of genomic divergence between lineages and subpopulation

Pair	average $F_{ST} \pm$ S.D.	coefficient of variation (F_{ST})	average $d_{xy} \pm$ S.D.	coefficient of variation (d_{xy})	Pearson's r (P -value)
PS4B-US-EU vs PS4A	0.650 ± 0.096	0.147	0.01486 ± 0.00311	0.209	0.344 (<1e-92)
AK-EU vs CA-WA	0.513 ± 0.232	0.453	0.00358 ± 0.00341	0.951	0.371 (<1e-107)
AK-EU vs NM-WA	0.613 ± 0.193	0.314	0.00253 ± 0.00274	1.083	0.217 (<1e-35)
CA-WA vs NM-WA	0.584 ± 0.206	0.353	0.00470 ± 0.00374	0.796	0.227 (<1e-39)
CA vs $WA_{(CA-WA)}$	0.081 ± 0.128	1.592	0.00207 ± 0.00220	1.063	-0.018 (0.297)
NM vs $WA_{(NM-WA)}$	0.089 ± 0.120	1.349	0.00069 ± 0.00102	1.488	-0.047 (0.007)
AK vs EU	0.116 ± 0.159	1.366	0.00049 ± 0.00089	1.806	-0.006 (0.728)
CA vs NM	0.613 ± 0.206	0.336	0.00472 ± 0.00382	0.004	0.217 (<1e-35)
$WA_{(CA-WA)}$ vs $WA_{(NM-WA)}$	0.603 ± 0.240	0.399	0.00547 ± 0.00435	0.004	0.178 (<1e-24)

S.D. is the standard deviation. The coefficient of variation is a normalized measure of dispersion calculated as the S.D. to the mean. Pearson's r was used to measure and test the correlation between F_{ST} and relative node depth (Nachman and Payseur 2002). Relative node depth is a measure of divergence that corrects for differences in mutation rate among loci, it is calculated as d_{xy} divided by divergence to an outgroup (here THA).

FIGURES

Figure 1. Rooted maximum likelihood genealogy inferred from full genomic sequences (A), Map of sampling sites (B), Cluster-based analysis using FRAPPE (C). In panel A, asterisks denote nodes that received >99% bootstrap support, and thin vertical lines denote the different fungal lineages identified. In panel C, each individual is represented by a thick vertical line divided into K colored segments that represent the individual's estimated membership proportions in the K clusters assumed (vertical scale at the left-hand side of the bar plot). In panels A and C, individuals IDs are colored according to collection locality (panel B), and individuals ID include ISO country codes and ANSI codes for US states: AK: Alaska, CA: California, WA: Washington, ESP: Spain, CIV: Côte d'Ivoire, CHE: Switzerland, THA: Thailand, PNG: Papua New Guinea.

(A) AK-EU

(B) CA-WA

(C) NM-WA

(D) PS4A

Figure 2. Observed site-frequency-spectra at all sites, synonymous sites or non-synonymous sites, and site-frequency-spectrum fitted using the python package DADI under the standard neutral model or the most supported isolation-with-migration models. The higher fraction of SNPs observed in the data ('Observed') compared to neutral expectations ('Standard Neutral Model') on the left-hand side of the graphs indicates an excess of low frequency derived variants. The lower fraction of SNPs observed in the data ('Observed') compared to neutral expectations ('Standard Neutral Model') on the right-hand side of the graphs indicates an excess of high frequency derived variants.

(A) PS4BUSEU - PS4A

(B) AKEU - CAWA

(C) CAWA - NMWA

(D) AKEU - NMWA

(E) CA - WA

(F) NM - WA

(G) AK - EU

10kb Genomic Windows

Figure 4. Genome-wide distribution of divergence at 10kb windows between pairs of lineages or pairs of subpopulations within lineages. Divergence was estimated in terms of absolute divergence (dxy) and relative divergence (Fst). Only the 20 largest scaffolds are shown. Smooth fitted lines were obtained using a Gaussian kernel method with a bandwidth of 10.

(A) Lineages →

↓ a↓A→	AK-EU						CA-WA						NM-WA			Tester
	AKFA2	AKFA24	AKFA31	AKFA6	ES9991	CH9992	WACL1050	WACL1070	WACL1073	CAMH1105	CAWE973	CAWE978	WACL1036	WACL1040	NMBE791	P581
AK-EU	AKFA10 1/1	5/5	6/6	6/6	6/5	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/1	6/5
	AKFA12 6/1	5/0	6/0	6/0	5/0	6/0	6/0	6/0	6/6	6/1	6/6	6/0	1/1	6/0		1/0
	AKFA20 0/5	0/6	0/6	1/5	0/6	0/6	1/6	0/6	0/6	0/6	6/6	6/0	0/6	0/6	0/6	2/2
CA-WA	CAMH1108	5/6	6/6	6/6	6/6	6/6	6/6	6/6	6/2	6/6	1/1	6/6	0/6	6/6	1/6	6/1
	CAMH1110	6/6	6/6	6/6	6/6	5/5	6/6	6/6	6/6	6/6	6/6	6/6	5/6	6/5	6/6	6/5
	CAWE990	5/5	6/6	6/6	6/6	6/5	6/6	6/6	6/6	6/6	6/6	6/6	1/6	6/6	1/6	6/5
	CAWE992	5/6	5/6	6/6	6/6	6/6	5/6	6/4	6/6	6/4	6/6	6/6	1/6	5/6	1/6	5/5
	WACL1039	6/5	6/5	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	0/6	5/1
NM-WA	NMBE781	5/0	6/6	5/0	6/1	5/0	6/0	6/0	5/5	6/0	6/0	6/0	5/1	6/0	6/0	0/0
	NMBE786	6/1	6/6	6/1	6/5	6/6	6/6	6/6	6/5	6/6	6/6	6/6	1/1	6/6	1/1	6/2
	NMBE792	6/0	6/1	6/0	6/1	6/5	6/0	6/1	6/5	6/0	6/1	6/6	1/1	6/0	0/1	5/1
	NMBE793	6/2	6/5	6/5	6/0	6/2	6/6	6/1	6/1	6/4	6/6	6/6	6/6	6/1	5/5	1/6
	NMBE794	5/0	6/6	6/0	6/0	2/5	6/0	6/0	6/0	6/0	6/5	6/0	6/1	6/0	5/0	6/0
	NMBE796	6/2	6/0	6/0	6/6	6/6	6/0	6/0	0/6	0/5	6/6	6/6	6/6	0/5	0/5	6/1
Tester	TX8127	6/1	6/6	5/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	5/1	6/6	1/6	6/1

(B)

Category of Reproductive Success			
6	> 50% black ascospores	2	perithecia developed ostioles, no spores
5	15-50% black ascospores	0 & 1	sterile, no perithecia produced; & barren perithecia, no ostiole developed
3 & 4	<1% black ascospores ; & 1-15% black ascospores		

Figure 5. (A) Reproductive success of 239 crosses (478 matings). Columns represent the 16 *mat A* strains, and rows represent the 15 *mat a* strains, with isolates numbers along the row and column headings of the matrix. Numbers within matrix cells indicate the reproductive success ratings (see B) of the two reciprocal matings of the cross between the corresponding isolates (*mat a* isolate as the perithecial parent/*mat A* isolate as the perithecial parent). The matrix cells have been shaded in proportion to the reproductive success of the best mating (see B). Additional row and column headings indicate the lineage designation (see Figure 1). Isolates P581 and TX8127 are tester strains of *N. discreta sensu stricto*. (B) Categories of reproductive success, corresponding to different stages in reproductive development, from the development of perithecia, to the formation of black ascospores.

Supplementary Table S1. Sample and sequencing information

Taxon/Isolate	Origin	% reference sequence covered	sequencing depth	standard deviation of sequencing depth	included in clone corrected dataset	SRA accession number
<i>N. discreta</i> PS4B						
AK-10	Fairbanks, Alaska	93.2	24.1	43	yes	SRS1087866
AK-12	Fairbanks, Alaska	97.5	37.8	16.9	yes	SRS1087856
AK-16	Fairbanks, Alaska	96.2	42.6	28.3	yes	SRS1087865
AK-2	Fairbanks, Alaska	96.5	61.6	27.6	yes	SRS1087873
AK-20	Fairbanks, Alaska	96.2	40.9	19.3	yes	SRS1087864
AK-24	Fairbanks, Alaska	95.1	25.2	55.3	yes	SRS1087855
AK-31	Fairbanks, Alaska	96.3	46.2	23.2	yes	SRS1087863
AK-6	Fairbanks, Alaska	96.4	61.4	25.6	yes	SRS1087854
CA-1099	Morgan Hill, California	89.9	23.3	58.4	no	SRS1087872
CA-1100	Morgan Hill, California	94	40.3	20.1	no	SRS1087857
CA-1101	Morgan Hill, California	94.1	39.9	22.3	no	SRS1087862
CA-1102	Morgan Hill, California	94.4	49.4	23.6	yes	SRS1087852
CA-1105	Morgan Hill, California	94.1	50.3	21.6	yes	SRS1087851
CA-1106	Morgan Hill, California	94.1	43.9	23.6	no	SRS1087861
CA-1108	Morgan Hill, California	94.4	35.4	20.4	yes	SRS1087860
CA-1110	Morgan Hill, California	94.7	62.5	28.2	yes	SRS1087850
CA-1001	Weaverville, California	81.9	14.3	42.2	no	SRS1087867
CA-967	Weaverville, California	94.5	74.8	29.3	yes	SRS1087920
CA-973	Weaverville, California	96	89.1	38.9	yes	SRS1087910
CA-978	Weaverville, California	94.6	73.8	30.1	yes	SRS1087913
CA-990	Weaverville, California	94.7	108.6	41.2	yes	SRS1087915
CA-992	Weaverville, California	92.5	25.6	44.9	yes	SRS1087847
CA-995	Weaverville, California	82.7	12.7	37.3	no	SRS1087858
NM-778	Belen, New Mexico	98.7	83.7	26.6	no	SRS1087871
NM-781	Belen, New Mexico	98.8	81.1	26.4	yes	SRS1087870
NM-786	Belen, New Mexico	98.8	71.6	24.2	yes	SRS1087849
NM-791	Belen, New Mexico	98.7	54.6	16.6	yes	SRS1087859
NM-792	Belen, New Mexico	98.7	52.6	19.2	yes	SRS1087846
NM-793	Belen, New Mexico	98.7	57.8	18.5	yes	SRS1087868
NM-794	Belen, New Mexico	98.7	62.7	17.8	yes	SRS1087848
NM-796	Belen, New Mexico	98.6	86.5	27.7	yes	SRS1087869
ESP-9991	Macanet de la Selva, Spain	96.7	104.1	38.9	yes	SRS1087914
THA-9956	Khao Yai, Thailand	86.8	25.8	16.8	yes	SRS1087909
WA-1036	Lake Chelan, Washington	98.7	90	25.7	yes	SRS1087916
WA-1039	Lake Chelan, Washington	98.8	99.5	28.6	yes	SRS1087919
WA-1040	Lake Chelan, Washington	98.9	76.2	43.6	yes	SRS1087900
WA-1050	Lake Chelan, Washington	94.8	46.9	47.3	yes	SRS1087899
WA-1056	Lake Chelan, Washington	94.3	84	32.9	no	SRS1087917
WA-1068	Lake Chelan, Washington	98.8	84.3	47.2	no	SRS1087911
WA-1070	Lake Chelan, Washington	94.9	78.9	49	yes	SRS1087875
WA-1073	Lake Chelan, Washington	94.5	44	27.8	yes	SRS1087853
CHE-9992	Leuk, Switzerland	96.9	95.1	32.6	yes	SRS1087912

N. discreta PS4A

PNG-8765	Tiaba, Papua New Guinea	79	13.9	11.2	yes	SRS1087908
PNG-9940	Tiaba, Papua New Guinea	80.5	21.7	16.5	yes	SRS1087907
PNG-9945	Wau, Papua New Guinea	79.9	17.5	14	yes	SRS1087906
PNG-9946	Marinville, Papua New Guinea	78.7	15.6	12.4	yes	SRS1087905
PNG-9947	Sogeri Road, Papua New Guinea	77.8	12.4	9.9	yes	SRS1087904
PNG-9948	Sogeri Road, Papua New Guinea	80.1	22	16.8	yes	SRS1087903
PNG-9949	Rouna, Papua New Guinea	80	20.2	15.4	yes	SRS1087902
PNG-9950	Rouna, Papua New Guinea	80.3	19.3	14.9	yes	SRS1087901
PNG-9951	Hiri, Papua New Guinea	84.3	56.9	40.4	yes	SRS1087918
<i>N. discreta</i> PS6						
CIV-9959	Gahelile, Côte d'Ivoire	58.2	17.5	25.2	yes	SRS1087874

Table S2. Comparison of demographic models

Dataset/Model	Free parameters	Ln L	AIC
<i>PS4A and PS4B-US-EU*</i>			
No migration, constant population sizes	4	-225655	451318
Migration, constant population sizes	6	-165782	331576
Migration, size change in ancestral population	7	-162765	325544
Ancient migration, size change in ancestral population	8	-163345	326706
Secondary contact, size change in ancestral population	8	-153321	306658
Secondary contact, heterogeneous migration, size change in ancestral population***	11	-153321	306664
Secondary contact, size change in ancestral population and simultaneously in both lineages	11	-81314	162650
Migration, size change in ancestral population and sequentially in lineages PS4A and PS4B-US-EU****	12	-81314	162652
Migration, size change in ancestral population and sequentially in lineages PS4B-US-EU and PS4A****	12	-81314	162652
<i>AK-EU, CA-WA and NM-WA lineages within PS4B-US-EU**</i>			
No migration, constant population sizes	6	-21891	43794
Continuous migration, constant population sizes	12	-15475	30974
Continuous migration, size change in ancestral population	14	-12044	24116
Continuous migration, size change in ancestral population and along interior branch	16	-12045	24122
Ancient migration, size change in ancestral population	15	-12099	24228
Secondary contact, size change in ancestral population	15	-11105	22240
Secondary contact, heterogeneous migration, size change in ancestral population	22	-7658	15360
Secondary contact, heterogeneous migration, size change in ancestral population and simultaneously in all three lineages	26	-9000	18052
Secondary contact, heterogeneous migration, size change in ancestral population and sequentially in lineages AK-EU, CA-WA and NM-WA	28	-9000	18056
Secondary contact, heterogeneous migration, size change in ancestral population and sequentially in lineages AK-EU, NM-WA and CA-WA	28	-9000	18056
Secondary contact, heterogeneous migration, size change in ancestral population and sequentially in lineages CA-WA, AK-EU and NM-WA	28	-9000	18056
Secondary contact, heterogeneous migration, size change in ancestral population and sequentially in lineages CA-WA, NM-WA and AK-EU	28	-9000	18056
Secondary contact, heterogeneous migration, size change in ancestral population and sequentially in lineages NM-WA, AK-EU and CA-WA	28	-9000	18056
Secondary contact, heterogeneous migration, size change in ancestral population and sequentially in lineages NM-WA, CA-WA and AK-EU	28	-9000	18056

*PS4B-US-EU corresponds to the pooling of lineages AK-EU, CA-WA and NM-WA

**All models assumed the topology ((AK-EU,NM-WA),CA-WA) as the genealogical relationship among lineages. The 'interior branch' is the branch holding lineages AK-EU and NM-WA.

***identical to model without heterogeneous migration, as the estimate was zero for the p parameter modelling the relative proportion of two classes of migration rates

****identical to model with simultaneous size change, as the estimate was zero for the interval between the two size changes

Table S3. Genomic coordinates of peaks of highest average pairwise homozygosity H as measured by the product of average pairwise homozygosity tract length extending in both directions from each SNP and the number of SNPs in each pairwise homozygous region to account for variable SNP density (bp x S). The corresponding lengths of the region of high average pairwise homozygosity are

Lineage	Scaffold	Coordinate	H (bp)	H (bp x s)
AKEU	2	2589111	6109.27	6.81E+07
AKEU	7	156165	12629.2	7.37E+07
AKEU	8	1250434	10103.2	7.61E+07
CAWA	3	2066258	8291.8	1.27E+08
CAWA	7	354872	11756.2	8.16E+07
CAWA	4	1340904	19909.4	5.85E+07
NMWA	1	838281	8826.13	1.55E+09
NMWA	4	731500	2342.6	1.85E+09
NMWA	7	187	6384.73	1.65E+09
PS4A	1	1409210	1300.67	5.48E+06
PS4A	3	2180404	5729.94	4.81E+06
PS4A	5	2528828	7681.25	3.64E+06
PS4B-US-EU	1	843407	2626.09	1.58E+09
PS4B-US-EU	7	231261	8213.07	6.08E+08
PS4B-US-EU	2	455323	2506.37	5.80E+08

Supplementary Figure S1. Neighbor-joining based on Felsenstein's 1984 genetic distance, calculated on the full set of SNPs. Asterisks indicate nearly identical genotypes that were subjected to clone-correction.

Figure S2. Numbers of modes (*i.e.*, clustering solutions) and average likelihoods over 10 Frappe replicated runs plotted as a function of the putative number of clusters (K).

Number of candidate introgressed
variants per 10kb windows

10kb Genomic windows

Figure S3. Genome-wide distribution of the number of candidate introgressed variants per 10kb windows in isolates of the AK-EU lineage. For a focal isolate, candidate introgressed variants are SNPs which are fixed for alternate alleles between the NM-WA isolates and the focal isolate on the one hand, and the rest of AK-EU isolates on the other hand. Only the 20 largest scaffolds are presented. On average, the number of candidate introgressed variants was 4.95 per 10kb window for isolate AKFA12 and less than 0.47 for other isolates.

Figure S4. Comparison of the observed joint allele frequency spectrum with that expected under the demographic model fitted using *dadi*, and corresponding residuals. The 3-dimensional Joint frequency spectrum is represented as three 2-dimensional marginal spectra plotted using heat-maps: (A) AK-EU vs CA-WA, (B) CA-WA vs NM-WA, (C) AK-EU vs NM-WA and (D) PS4B-US-EU vs PS4A, with PS4B-US-EU corresponding to the pooling of AK-EU, CA-WA and NM-WA. The color of a cell at position (x,y) in the marginal spectrum corresponds to the number of derived alleles that are at frequency x in one lineage and frequency y in the other. The residuals represent the difference in the number of alleles predicted by the model compared with that found in the data for each bin in the spectrum (red, model predicts too many; blue, model predicts too few).

Figure S5. H-scan for recent selective sweeps along scaffolds of four lineages of *N. discreta* PS4. Only scaffolds with more than 100 variable sites were considered (*i.e.*, the 18 to 20 longest scaffolds, representing 88% to 90% of the full genome size). Each dot represents the H statistic value calculated at site that is variable within a given lineage.

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