Long-lasting coexistence of multiple asexual lineages alongside their sexual counterparts in a fungal plant pathogen

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Abstract

Sexual-to-asexual transitions within species are crucial for understanding reproductive evolution, yet the coexistence of both modes of fungal species in the same environment is poorly documented. Here, we report this transition in a plant pathogen species that coexists within the same geographical environment. Our biological model is the poplar rust fungus *Melampsora larici-populina,* which displays a complex life cycle typical of rust fungi (Pucciniales). It alternates between two unrelated hosts to complete an obligate sexual life cycle once a year. We conducted a comprehensive population genetic analysis, using 21 microsatellite markers and data from 2,122 individuals gathered over 30 years from various locations in France. Our results demonstrate the existence of many distinct lineages that reproduce asexually through the years, skipping the sexual phase. Clustering analysis identified a group of multilocus lineages that displayed all hallmarks of the genetic consequences of asexual reproduction, including highly negative and large variance among loci of the inbreeding coefficient (F_{IS}). This indirect evidence for asexual reproduction was confirmed by the direct observation of these asexual lineages being repeatedly sampled across multiple years. This result demonstrates the coexistence of these lineages with their sexual counterparts in the same ecological niche, challenging conventional assumptions about geographical sorting of reproductive modes. These considerations are of paramount importance for understanding the contemporary evolution of major pathogen species. This switch from sexual to asexual reproduction has contributed to devastating epidemics worldwide.

Keywords: sexual-asexual transition | evolution of reproductive modes | population genetics | rust fungi | time samples.

1 Introduction

The evolution of sex remains a fundamental question and an intriguing topic in evolutionary biology (Culotta & Hines, 1998; Otto, 2009; Hartfield & Keightley, 2012). While sexual reproduction may increase genetic diversity, purging deleterious mutations, and enhancing adaptation to changing environments (Schurko et al., 2009; Pierre et al., 2022). It incurs costs such as time and energy to find mates, combined with the biological cost of meiosis (Roughgarden, 1991; Aanen & Hoekstra, 2007; Hörandl, 2013). Asexual reproduction, on the other hand, produces offspring without a mate, allowing for efficient reproduction, maintenance of lineages adapted to local stable conditions, and quick multiplication (Shick et al., 1979; Lodé, 2013). Due to a lack of recombination, asexual lineages are more vulnerable to the accumulation of deleterious mutations (Lynch et al., 1993; Peck, 1994; Keightley & Eyre-Walker, 2000), and more prone to losing allele diversity by genetic drift (Rouger et al., 2016). The study of species displaying both sexual and asexual reproduction enables pinpointing the pros and cons of each mode of reproduction (Butlin, 2002; Simon et al., 2010; Billiard et al., 2012). In particular, the study of cases where there is a dynamic transition from sexual to asexual reproductive modes is likely to highlight scenarios in which each reproductive mode could be favored.

The success of asexual reproduction frequently relies on specific environmental conditions. Invasive species often shift towards asexual reproduction in their exotic range, while reproducing sexually in their native environment (Song et al., 2011; Platt & Jeschke, 2014; Gladieux et al., 2015; Tilquin & Kokko, 2016). Ant species can exhibit such diverse reproductive strategies, with asexual reproduction frequently being dominant in invasive populations, as observed in *Wasmannia auropunctata* and *Cerapachys biroi* (Foucaud et al., 2006; Kronauer et al., 2012). Plant pathogenic fungi also adopt diverse reproductive strategies, with asexual reproduction being responsible for the most damaging epidemics (Ashu & Xu, 2015; Gladieux et al., 2015; Drenth et al., 2019). Potato late blight is a devastating disease caused by the oomycete pathogen *Phytophthora infestans.* Both sexual and asexual reproduction take place in the native range, but it is asexual reproduction only that led to the devastating Irish Potato Famine in the 1840s (Gavino et al., 2000; Ristaino, 2002; Fry, 2020). The wheat yellow rust fungus *Puccinia striiformis* f. sp. *tritici* displays a similar pattern, with different reproductive modes depending on the region. The fungus reproduces sexually in China and Pakistan, which is highlighted by its high genotypic

diversity, whereas it reproduces asexually in the rest of the world (Bahri et al., 2009; Mboup et al., 2009; Ali et al., 2014). The rice blast fungus *Magnaporthe oryzae* (Syn: *Pyricularia oryzae*) reproduces sexually in a limited area of Southeast Asia, its native range, whereas epidemics globally are driven by asexual lineages (Saleh et al., 2012).

The coexistence of both reproductive modes in the same geographical environment is still poorly documented, despite offering a unique opportunity to decipher the evolutionary and ecological dynamics of sexually or asexually derived lineages. While rare in fungi, such studies are common in other taxa, such as in aphids (Halkett et al., 2006; Simon et al., 2010), daphnia (Lehto & Haag, 2010), and a cyprinid fish *Carassius gibelio* (Barbuti et al., 2012). The phytopathogenic fungus *Melampsora larici-populina*, causing poplar rust, belongs to the taxonomic order of Pucciniales (Steenackers et al., 1996). This species follows a complex life cycle with an obligate sexual reproduction that alternates with rounds of clonal multiplication (Figure 1). Two unrelated host plants are required to complete the life cycle (Hacquard et al., 2011). Clonal multiplication occurs on poplar trees (*Populus* spp.), while sexual reproduction takes place on larch trees (*Larix* spp.) (Lorrain et al., 2019). In the following, lineages that complete the entire life cycle, including the obligate sexual reproduction phase, are referred to as sexual lineages. The sexual reproduction on larch ensures genetic recombination and obliterates all traces of the previous rounds of clonal multiplication (Xhaard et al., 2011; Rouger et al., 2016).

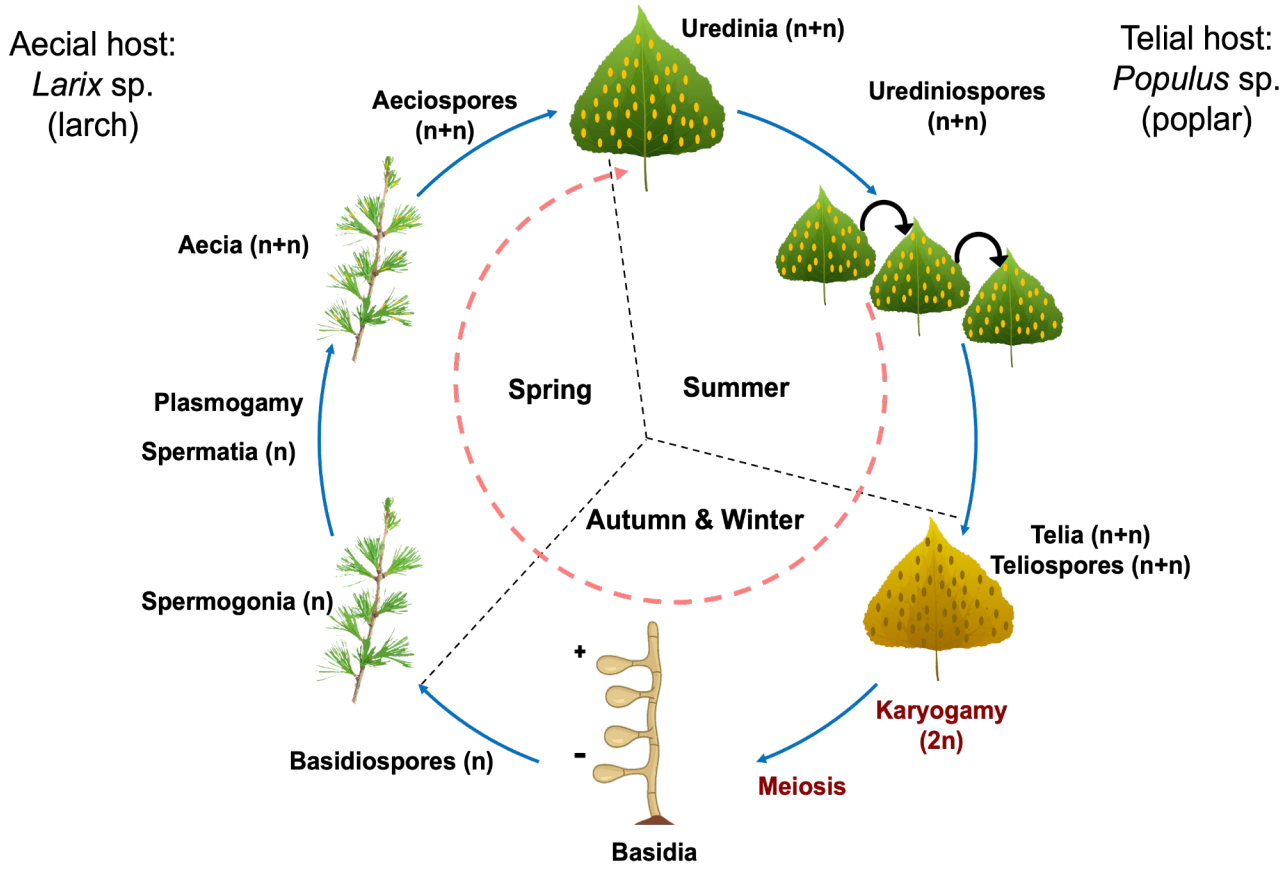


Figure 1

The *Melampsora larici-populina* life cycle. The blue arrows indicate the heteroecious life cycle, the clonal multiplication phase on poplar tree leaves during summer, and the sexual phase on the larch tree. The dashed red arrow indicates pure asexual multiplication on poplar, skipping the sexual phase.

A previous population genetic study (Xhaard et al., 2011) has provided evidence for some variability in reproductive mode, with an obligate sexual genetic group coexisting (i.e., the same geographic region) with the genetically distant group that displays all hallmarks of long-lasting asexual reproduction. This suggests that some lineages of the poplar rust fungus could survive alone through clonal multiplication on poplar. However, this preliminary study did not investigate the ecological and evolutionary dynamics of the asexual group, as it relied on a single sampling year. The coexistence of sexual and asexual reproduction within the same geographic region makes

90 *M. larici-populina* an ideal evolutionary model for elucidating the transition from one reproductive
91 mode to another, as both modes can be found in the same environment.

92 In this study, we provide an in-depth characterization of the reproductive strategies of *M. larici-*
93 *populina*, with an emphasis on the (spatio-temporal) persistence of asexual lineages and their
94 genetic characteristics. Our study also examines the ecological and spatial factors influencing the
95 distribution of sexual and asexual populations, thereby offering insights into the evolution of
96 reproductive modes in rust fungi. This study encompasses the analysis of 2122 individuals sampled
97 across France over 30 years of population surveys that have been genotyped using 21 microsatellite
98 markers.

99 We used a two-fold approach to assess the reproductive mode variation among poplar rust
100 individuals. First, we employed an indirect population genetic approach based on the clustering
101 among lineages and subsequent evaluation of the variation in population genetic indices known to
102 be signatures of asexual reproductive mode, namely: repeated genotypes, linkage disequilibrium,
103 and overall negative F_{IS} value and large variance over loci (Balloux et al., 2003; Halkett et al.,
104 2005; Stoeckel & Masson, 2014). Second, we benefited from time-samples to investigate the
105 resampling through years of the same multilocus lineage (MLL), which is direct evidence for their
106 asexual survival. Furthermore, the genetic relatedness of all individuals was analyzed to
107 investigate evolutionary relationships among asexual lineages and with their sexual counterparts,
108 which points to multiple events of the emergence of asexual lineages. Finally, examining the
109 coexistence of sexual and asexual lineages, their long-term persistence, and their spatial
110 distribution suggested that environmental and ecological factors potentially play a role in shaping
111 the survival and maintenance of asexual lineages in this species.

112

2 Material and Methods

2.1. Isolate collection and sampling strategy

To characterize the long-term genetic structure of *Melampsora larici-populina* in France, we analyzed a total of 2,122 isolates collected from 61 administrative departments over 30 years (1992-2024) (Figure S1). The dataset (Table S1) combines previously published genotypes with new collections, structured into three distinct sampling categories: (i) National surveys (broad spatial scale): We integrated data from two major countrywide sampling campaigns. The first corresponds to the 2009 survey (23 locations, n=428) previously described by Xhaard et al. (2011). The second is a new dataset from a 2011 survey, extending coverage to 60 locations (n=1,374). All survey samples were collected in late summer (August-September), coinciding with the peak of clonal multiplication but prior to the sexual cycle (Figure 1); (ii) Targeted monitoring and historical collections (temporal scale): We included 246 isolates from a long-term epidemiological monitoring project in the Durance River valley (2004, 2008, 2020) (Xhaard et al., 2012; Becheler et al., 2016). These were supplemented by 34 historical isolates from our laboratory collection, spanning seven years between 1992 and 2014. (iii) Overwintering assessment: To investigate potential overwintering mechanisms, we performed a specific sampling campaign in February 2024 (n=40) at a unique stand in Cavalaire-sur-Mer (southern France). Unlike typical poplar stands, this site retains green foliage through winter, offering a rare opportunity to sample active rust populations outside the standard growing season.

2.2 Genotyping

The DNA extraction process was guided by a protocol that employs the BioSprint 96 DNA Plant Kit and the BioSprint 96 automated workstation (Qiagen). Samples were genotyped using 21 microsatellite loci [Mlp_91, Mlp_83, Mlp_50, Mlp_71, Mlp_96, Mlp_95, Mlp_55, Mlp_66, Mlp_12, Mlp_68b Mlp_54, Mlp_57, Mlp_58, Mlp_49, Mlp_93, Mlp_82b Mlp_56, Mlp_87, Mlp_97, Mlp_77, Mlp_94 (Xhaard et al., 2009)]. These loci have been extensively utilized in several population genetic studies of this species (Xhaard et al., 2011; Becheler et al., 2017; Persoons et al., 2017; Louet et al., 2023; Saubin et al., 2024). The amplification through PCR with the Multiplex PCR Kit (Qiagen) was performed as described by Xhaard et al. (2011). It was conducted across three multiplex reactions containing eight, five, and eight loci, ranked by

142 amplification size, respectively. The PCR products were then pooled and analyzed using an ABI
143 3730 Genetic Analyzers (Applied Biosystems) by the company GenoScreen. Fragment sizing
144 utilized a LIZ-1200 size standard, and allele scoring was performed with GENEMAPPER V4.0.
145 Samples that failed to amplify at more than six loci were excluded from further analysis.

146 **2.3 Population genetic and statistical analyses**

147 First, to handle genetic data of microsatellite markers in the R programming language v.4.4.1, we
148 converted the data format from a data frame to a genuine object using the `df2genind` function
149 from the **adegenet** package v.2.1.10. This function optimizes multilocus genotype data and
150 accommodates missing data if it is found in some individuals or loci. The automated workflow
151 code provides full details on subsequent analyses (https://doi.org/10.5281/zenodo.15100450)
152 (Abdalrahem et al., 2026) .Moreover, specific analyses were executed using the GenAPoPop 1.0
153 software due to specific methodological requirements.

154 *Identification of repeated genotypes:* The prerequisite to an accurate assessment of reproductive
155 mode is the proper delineation of repeated genotypes derived from clonal multiplication (Halkett
156 et al., 2005; Arnaud-Haond et al., 2007). Individuals with the same combination of alleles (the so-
157 called multilocus genotypes, MLG) were consistently identified through both an ad hoc procedure
158 in Excel during the manual curation of chromatographs and using `mlg.filter` from the **poppr**
159 package v.2.9.3 (Kamvar et al., 2014). Genotypes differing by a few alleles were double checked
160 by returning to the original chromatographs. All remaining differences between genotypes are
161 therefore confirmed and reliable. We computed (*Pidu*) and (*N_Pidu)* to test for the discrimination
162 power of our marker set, and to rule out the probability of finding repeated MLGs by chance (that
163 is deriving from sexual reproduction) using GenAPoPop 1.0 (Evett & Weir, 1998; Waits et al.,
164 2001; Stoeckel et al., 2024).

165 *Delineation of multilocus lineages:* As the accumulation of somatic mutations over time can lead
166 to slight differences among multilocus genotypes within a given asexual lineage (Arnaud-Haond
167 et al., 2007), we subsequently grouped MLGs into multilocus lineages (MLL). So as not to apply
168 an arbitrary threshold for MLL delineation, we first computed the distribution of pairwise shared
169 allele distance as advised by Arnaud-Haond et al. (2007), and Bailleul et al. (2016). As intra lineage
170 differentiation can vary across MLLs, we chose to use the UPGMA clustering method, which

171 aggregates MLGs one by one by examining the nearest neighbors until the MLL boundaries are
172 found (Kamvar et al., 2014, 2015). The threshold on genetic distance between neighbor genotypes
173 was set using the function `cutoff_predictor` from **poppr**.

174 *Clustering procedure to indirectly identify asexual lineages:* As the asexual reproductive mode
175 freezes genotypes, it retains ancestral allelic states. Asexual lineages can thus rapidly diverge from
176 their sexual counterparts, the latter being still influenced by genetic drift (Rouger et al., 2016). In
177 line with this statement, we previously found large genetic differences between sexual and asexual
178 groups of *M. larici-populina* (Xhaard et al., 2011). Assigning poplar rust individuals would thus
179 be an effective approach to identify asexual lineages without any *a priori*. The main pitfall of this
180 method being that the clustering algorithm should not rely on gathering individuals according to
181 Hardy-Weinberg expectations, which is the common assumption of any Structure-like approach
182 (Pritchard et al., 2000). We thus carry out our clustering analysis using a PCA and discriminant
183 analysis approach based on allelic distribution (Jombart et al., 2010). We used the `find.clusters`
184 function from the **adegenet** package to reduce the dimensionality of the dataset. Discriminant
185 analysis of principal components (DAPC) was then used to refine the identification of clusters by
186 maximizing the variance between two clusters, while minimizing within cluster variance (Jombart
187 et al., 2010). Cross-validation (`xvalDapc`; 100 replicates, 90% training set) identified 10 principal
188 components (PCs) as optimal for DAPC, with a mean group assignment success of 99% and 46%
189 of total genetic variance retained. The optimal number of clusters was determined to be K=2, a
190 choice supported by two complementary lines of evidence. First, the Silhouette score K=2
191 (Shahapure & Nicholas, 2020), as a statistical support (Figure S2). Second, this clustering aligns
192 with our biological question to disentangle the two major reproductive modes (sexual vs. asexual
193 lineages); individuals were grouped into two clusters using the `dpca` function with K=2. To ensure
194 the reliability of the assignment, only individuals with a high membership coefficient (≥0.8) were
195 retained for further analyses of within-cluster genetic characteristics.

196 *Resampling multilocus lineages (MLLs) as direct evidence for asexual reproduction.* Observing
197 the same genotype over several years is an indication that this lineage survived asexually - unless
198 sexual reproduction could have resulted in the same combination of alleles by chance, which can
199 be ruled out by a *Pidu* analysis (Evett & Weir, 1998; Stoeckel et al., 2024). To track the persistence
200 of MLLs over time, we recorded the years each MLL was detected and calculated its frequency

across different years. A lineage that persisted for at least two years was considered asexual. We tested the overlap between the direct and indirect approaches for assessing asexual reproductive mode by performing Fisher's exact test (Clarkson et al., 1993) on the contingency table of MLL sorting according to the direct or indirect approach by using the `fisher.test` function from the **stats** package v.4.4.1. In the following, a given MLL is considered asexual if it meets at least one of the two criteria: the assignment to an asexual cluster or the inter-annual resampling over a minimum of two years.

Computation of classical population genetic indices: As asexual reproductive mode distorts allele repartition within and among individuals, we computed several classical population genetic indices used to characterize within cluster and within MLL genetic signatures. Observed heterozygosity (H_O), unbiased expected heterozygosity (H_E) (Nei, 1978), genotype richness (R), and multilocus linkage disequilibrium corrected for the number of loci $\bar{r}_d$ were calculated using the **poppr** package (basic function). Allelic richness (A_R), and F-statistics, including the inbreeding coefficient (F_{IS}) (Weir & Cockerham, 1984) were computed using the **hierfstat** package v.0.5-11. The clonal evenness was measured by calculating the *Pareto* β index, which summarizes the distribution in size of MLLs using GenAPoPop 1.0 (Arnaud-Haond et al., 2007; Stoeckel et al., 2024).

Spatial analysis of the repartition of sexual and asexual lineages: To illustrate the geographical distribution of sexual and asexual individuals across France, the **maps** package v3.4 was utilized. This enabled us to obtain information not only on spatial distribution, but also on the potential coexistence of the two reproduction modes within the same geographical area. Using the `glmer` function from the **lme4** v.1.1-35.5 package, a generalized linear mixed model (GLMM) with a binomial distribution and a logit as a link function was employed to assess the regression coefficients (Bolker et al., 2009). To further test the relationship between the reproductive mode variation and the geographic locations, we performed a regression analysis on the count of sexual and asexual lineages per site. The independent variables (fixed effect) were the geographic location represented by latitude and longitude. The effect of the year of sampling was added as a random effect. This analysis was performed on the 2009 and 2011 data originating from the two countrywide surveys, so as not to overrepresent certain locations. To further assess the distribution and relative abundance of asexual MLLs across time and space, we performed Fisher's exact test

231 for fluctuation in MLL abundance across years and locations. Given the large size of the data sets,
232 we employed a Monte Carlo simulation approach with 100,000 replicates to estimate p-values.

233 *Genetic relationships between and among sexual and asexual lineages:* To further elucidate the
234 evolutionary trajectories of sexual and asexual individuals, and to address the question of the
235 dynamics of transition from sexual to asexual reproduction and vice versa, a discrete matrix based
236 on the relative dissimilarity between individuals was calculated by `diss.dist` with the **poppr**
237 package to create a neighbor-joining tree (Saitou & Nei, 1987) using `nj` from the **ape** package
238 v5.8.1.

239 *Inference of the rate of clonality:* Finally, to estimate the rate of clonality in the *M. larici-populina*
240 populations, we employed the ClonEstiMate Bayesian method. This method analyzes the change
241 of genotype frequencies from the same population over generations (Becheler et al., 2017). We
242 calculated based on two scenarios: i) merging all individuals within a mixed population (that is
243 any individuals can survive asexually by chance), or ii) considering two distinct sexual and asexual
244 populations, based on the lineage sorting detailed above. Since the ClonEstiMate method should
245 be applied on temporal samplings of the same kind of populations, we used the data collected
246 during the countrywide surveys carried out in the years 2009 and 2011.

3 Results

3.1 Delineation of multilocus lineages

We identified 1470 multilocus genotypes (MLGs) over the 2121 genotyped individuals. The 21 markers we used provided enough statistical power to ensure unambiguous discrimination between genotypes. The overall probability of obtaining the same combination of alleles by chance under panmictic sexual reproduction (*Pidu*) is approximately 10^{-16}. This implies that 118 million individuals would have to be genotyped before observing two sexually produced descendants with identical genotypes (*N_Pidu*). We thus confidently considered that all the repeated genotypes we found were clones. We then grouped closely related MLGs into MLLs to account for the few somatic mutations that could occur during clonal multiplication. The delineation of MLLs was undertaken using a genetic distance threshold of 4.6, as determined by the UPGMA clustering algorithm. This grouping led to the delineation of 1381 MLLs, so slightly fewer than the number of MLGs, showing that the vast majority of MLGs have no closely related genotype.

3.2 Reproductive mode assessment

The clustering approach indicated that almost all individuals exhibited a high probability of membership in one of two genotypic clusters: 450 individuals were unambiguously assigned to cluster one, while 1665 individuals were assigned to cluster two (Figure S3). Only one isolate had membership coefficients below the threshold and was excluded from further analyses.

A comprehensive population genetic analysis of each cluster (Table 1) was conducted to assess the value of key population genetic indices, thereby revealing a contrast between the two clusters. Cluster 1 exhibits a highly negative F_{IS} value, low genotypic diversity, and high linkage disequilibrium (LD), all of which are indicative of a strong rate of clonal reproduction (Balloux et al., 2003; Halkett et al., 2005; Arnaud-Haond et al., 2007). The high variance of F_{IS} across loci further points to rare events of recombination within a population reproducing essentially asexually over the long term. In contrast, cluster 2 exhibited slightly positive F_{IS} values and higher genetic diversity, suggesting a higher degree of allelic recombination, a feature of sexual reproduction. The reduced linkage disequilibrium in this cluster further supported this classification. Consequently, cluster 1 was labeled as asexual, and cluster 2 as sexual. It is

275 noteworthy that similar levels of observed heterozygosity were found in both clusters, but a much
276 reduced allelic richness and gene diversity in the asexual cluster 1. Further, the *Pareto β* index was
277 low (~ 0.2), indicating a small number of dominant MLGs, which was consistent with high
278 clonality in cluster 1. In the sexual cluster 2, the *Pareto β* was greater than 1, reflecting a more
279 even distribution of genotypes due to genetic recombination.

Table 1. Characteristics of the two genetic clusters identified in *M. larici-populina* individuals.

	N	MLG	MLL	*R*	*Pareto β*	A_R	H_O	H_E	F_{IS}	Var (F_{IS})	$\bar{r}_d$
Clustering approach											
Cluster 1	450	67	15	0.147	0.226	4.754	0.523	0.361	-0.448	0.093	0.345
Cluster 2	1665	1403	1367	0.843	1.078	9.566	0.506	0.544	0.069	0.004	0.016
Resampling approach											
Multi-years	485	87	18	0.178	0.248	5.364	0.517	0.390	-0.324	0.130	0.339
Single-year	1630	1383	1363	0.848	1.057	9.695	0.508	0.545	0.064	0.004	0.012
Combination of approaches											
Asex	499	96	27	0.191	0.252	5.554	0.517	0.394	-0.314	0.119	0.327
Sex	1616	1374	1354	0.850	1.051	9.760	0.508	0.544	0.067	0.004	0.012

MLG*:* number of multilocus genotypes, MLL*:* number of multilocus lineages, *N*: number of individuals, *R*: genotypic diversity, *Pareto β*: evenness among repeated genotypes; A_R: allelic richness, H_O*:* observed heterozygosity, H_E*:* expected heterozygosity, F_{IS}: inbreeding coefficient, Var (F_{IS}): variance of inbreeding coefficient, and $\bar{r}_d$: multilocus linkage disequilibrium.

The resampling approach revealed two groups: 488 individuals with lineages spanning more than one year due to asexual reproduction and 1,633 sexual individuals without resampled lineages exceeding two years. The contingency table (Table 2), which compares the clustering and resembling approaches, shows a discrepancy. Among the 54 individuals identified as sexual through cluster analysis, subsequent resampling evidence validated their clonality. In contrast, 11 individuals were clustered as asexual; however not resampled.

Table 2. Contingency table showing the relationship between clustering and resampling approaches for reproductive mode definition in the dataset. Values represent the number of individuals assigned to each category.

	Asex (Resampling approach)	Sex (Resampling approach)
Asex (Clustering approach)	436	14
Sex (Clustering approach)	49	1616

A Fisher's exact test was performed to evaluate the correlation between the assignment of reproductive modes from clustering and resampling analyses. The results were highly significant (p-value $< 2.2 \times 10^{-16}$), thereby rejecting the null hypothesis of no association, with a 95% confidence interval. This indicated a robust relationship between the two classification methods. Therefore, a combination of clustering and resampling approaches was used to determine the reproductive mode of each individual. The subsequent analysis involved the calculation of the population genetic indices for both groups. The inference of reproductive mode using two approaches as well as a combination of both, yielded consistent results over all population genetic indices, confirming the robustness of the classification (Table 1). However, there are slight differences in the values of population genetic indices among classification methods, with the

300 asexual group determined by the clustering approach revealing a stronger signature of asexual
301 reproduction.

3.3 Spatial distribution of reproductive modes

The spatial distribution of both sexual and asexual lineages of *M. larici-populina* individuals in France revealed distinct patterns (Figure 2). Sexual lineages were widespread across most regions, predominating in the northern and central parts of the country. In contrast, asexual lineages were more concentrated in the southern regions.

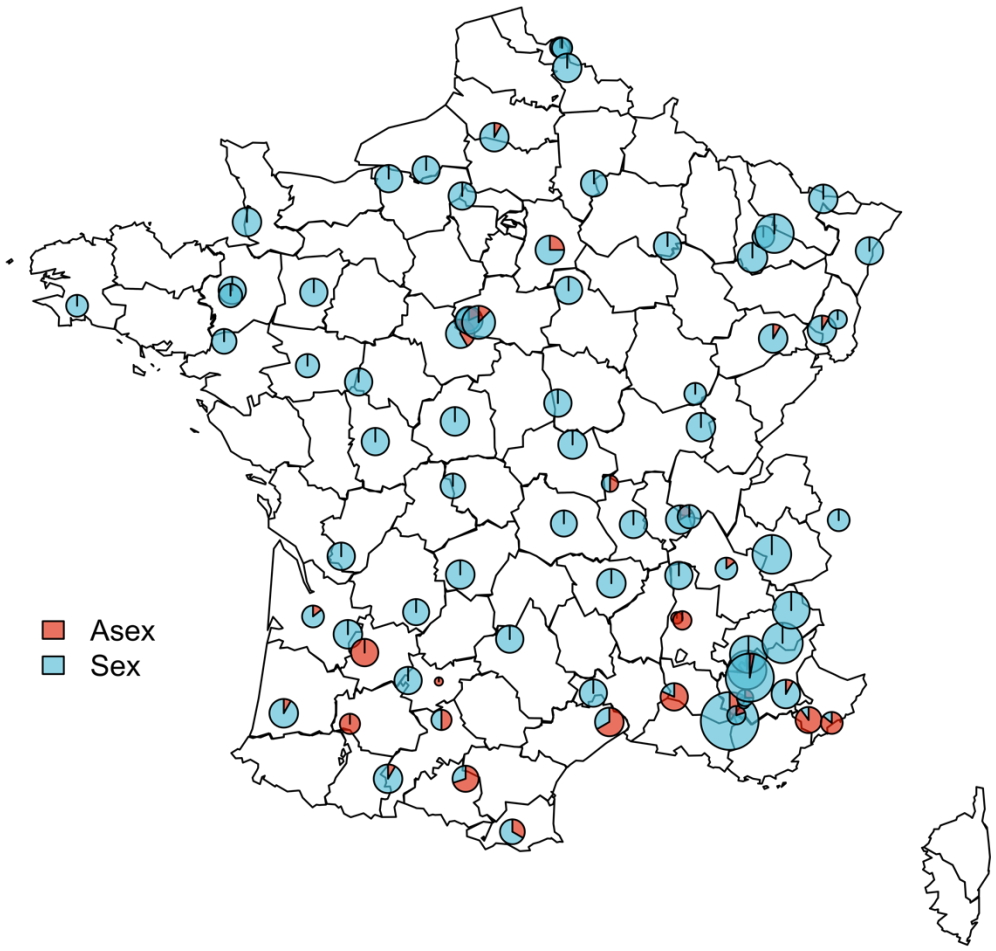


Figure 2

Geographic distribution of sexual and asexual lineages of *Melampsora larici-populina* individuals collected in 2009 and 2011 across France. The pie charts show the proportions of asexual (red) and sexual (blue) lineages at each sampling location. The size of each pie chart corresponds to the total number of individuals collected from that site.

To further investigate the factors that influence the prevalence of asexual lineages, a GLMM binomial regression analysis was conducted. The analysis (Table 3) revealed a significant effect of both latitude and longitude (Figure S4) on the proportion of sexual and asexual lineages, with positive coefficients indicating that the relative abundance of asexual individuals increases with lower latitude (the closer we get to the south of the country). Additionally, the random effect of years (variance = 13.44, sd = 3.666) indicated that the temporal distribution of individuals was not associated with a specific reproductive mode.

Table 3. Results of the binomial regression model predicting the effect of spatial distribution on the reproductive mode of *M. larici-populina*

Predictor	Estimate	Std. Error	z-value	p-value	Significance
(Intercept)	-22.998	2.476	-9.126	< 2e-16	***
Lat	0.464	0.047	9.798	< 2e-16	***
Long	0.093	0.029	3.199	0.001	***

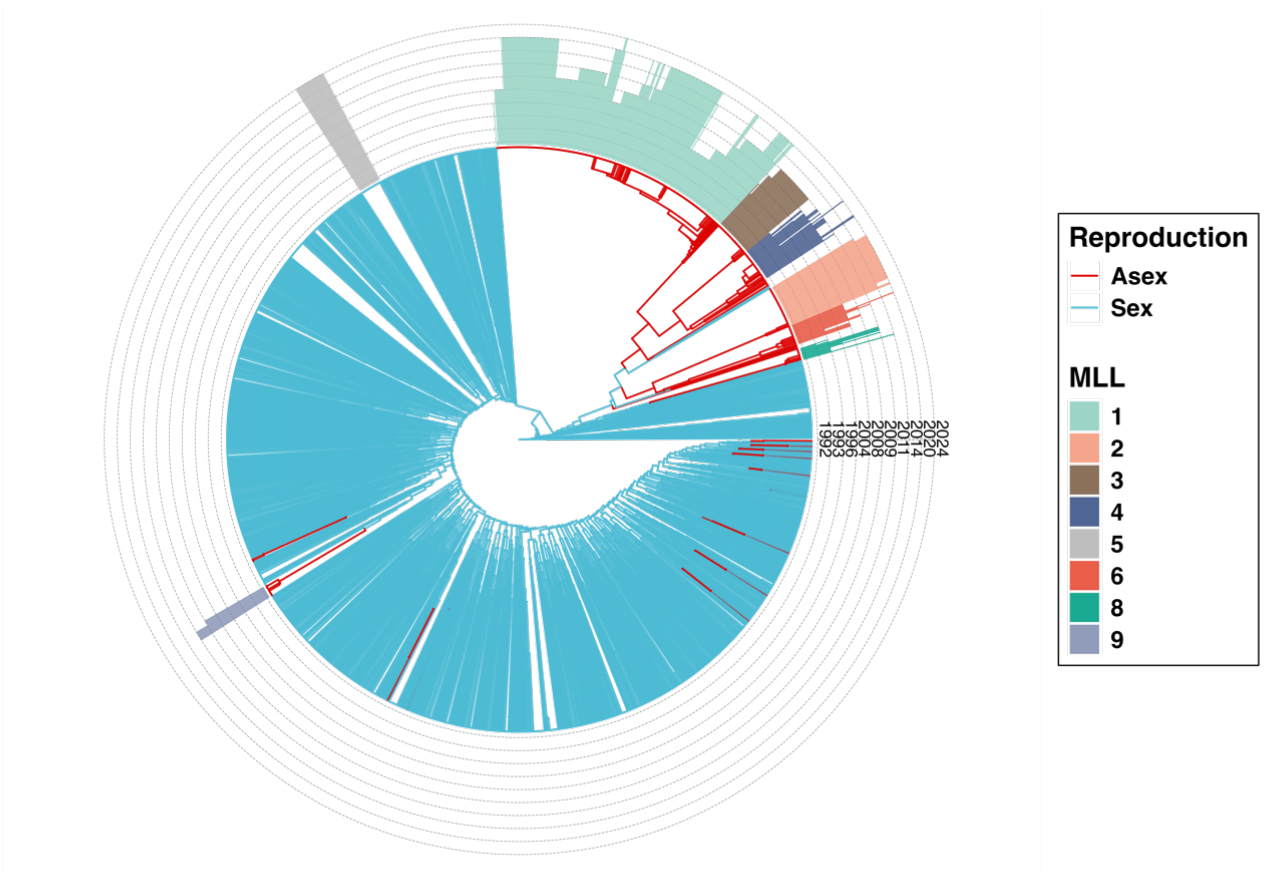
Estimate (regression coefficient), Std. Error (standard error of the estimation), z-value (z-statistic), p-value (statistical significance), and Significance power indicated by asterisks (0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1).

The ClonEstiMate Bayesian analysis yielded insights into the rate of asexual reproduction in *M. larici-populina*. The 99% credible interval from the posterior probability obtained for each group's clonal rates. In the context of a mixed group, where sexual and asexual lineages are merged into one population, the estimated clonal rate was between 0.22 to 0.31, indicating that about 27% of the population reproduces asexually. When the analysis was performed under the assumption of

330 two distinct populations, the clonal rate was between 0.93 to 0.99 for the asexual group and
331 between 0 to 0.21 for the sexual group.

3.4 Genetic relationships between sexual and asexual lineages

The dendrogram tree shows different clades for sexual and asexual lineages (Figure 3), highlighting the seven main asexual lineages. However, some asexual lineages also appeared as closely related sister branches from the sexual clade. Barring MLL9, one of the most abundant asexual lineages, all of these asexual MLLs, closely related to the sexual clade are of small size. These small asexual lineages were thus sporadic, unlike the seven most abundant asexual MLLs that show persistence through time (Figure 3, Table 4). These main asexual MLLs also displayed some reticulation, indicating they consist of different MLGs. In contrast, MLL5 (a most prevalent MLL but classified as sexual) consists of individuals sampled only in 2024 that have exactly the same genotype. These observations underscored the evolutionary relationships and temporal dynamics between reproductive modes within and among *M. larici-populina* lineages.



344 **Figure 3**

345 Circular Neighbor-joining (NJ) tree based on microsatellite data (21 markers) illustrating the genetic
346 relationships among *M. larici-populina* individuals. The color of the branches indicates the reproductive mode,
347 with blue representing sexual and red representing asexual modes. The outer rings represent the year of sample
348 collection, with each height corresponding to a year. The nine most abundant multilocus lineages (MLLs) are
349 color-coded.

350 **3.5 Genetic structure and persistence of asexual lineages**

351 A more detailed analysis of the asexual group identified several distinct genetic lineages. Among
352 these, seven lineages were found to be the most abundant. The dominant lineages exhibited varying
353 levels of persistence over the years, as indicated by their range of collection years (Table 4). In
354 particular, MLL1 was consistently detected in almost all collection years and across all regions,
355 highlighting its long-term persistence and widespread occurrence.

356 **Table 4.** Characteristics of the seven most abundant asexual multilocus lineages (MLLs) of *M.*
357 *larici-populina*.

MLL	MLG	N	Year range	R	A_R	H_O	H_E
1	36	279	1992:2024	0.122	1.274	0.519	0.274
2	3	49	2014:2020	0.042	1.290	0.571	0.287
3	4	44	1996:2009	0.070	1.316	0.619	0.312
4	9	38	1996:2020	0.216	1.266	0.499	0.263
6	6	23	1996:2020	0.227	1.196	0.354	0.192
8	5	14	1993:2014	0.308	1.306	0.575	0.296
9	3	14	2009:2011	0.154	1.189	0.350	0.183

358 *MLL:* number of multilocus lineages, *MLG:* number of multilocus genotypes, N: number of individuals, *R*:
359 Genotypic diversity, A_R: Allelic richness, H_O: Observed heterozygosity, H_E: Expected heterozygosity.

360

The distribution of these lineages in France differed with some lineages being widespread and others more restricted to some areas (Figure 4).

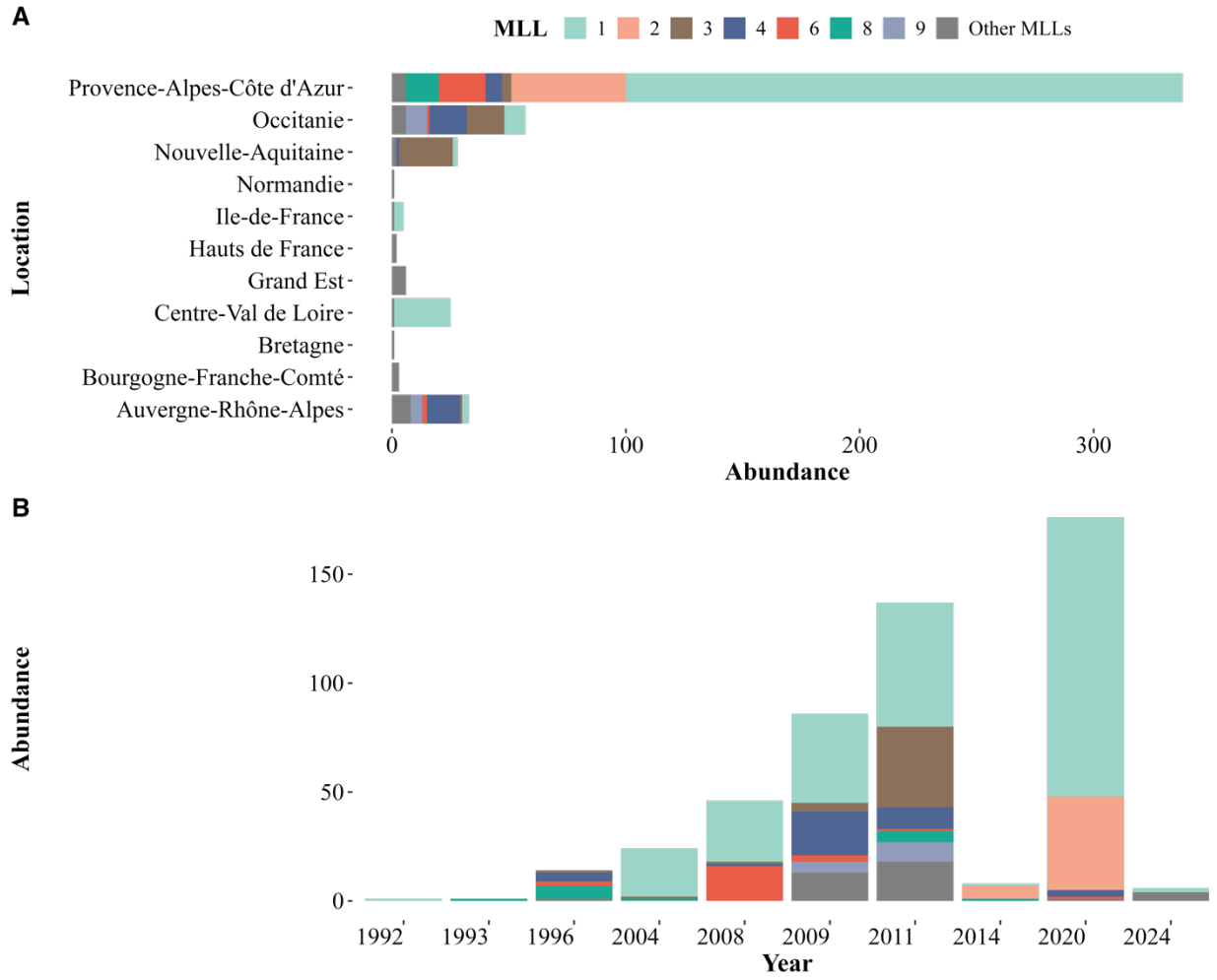


Figure 4

Distribution of asexual lineages over time and across locations. (A) The abundance of asexual MLLs in different French administrative regions. (B) The abundance of asexual MLLs in different years.

Fisher's exact test revealed a highly significant association between MLL distribution and both temporal and spatial factors (P = 1e-05). The analysis revealed that certain MLLs were repeatedly observed in the same geographic regions and over multiple sampling years, suggesting a pattern of persistence. Conversely, other MLLs appeared sporadic.

4 Discussion

The poplar rust fungus *M. larici-populina* is commonly regarded as an obligate sexual species that needs the presence of both poplars and larches to perform its annual life cycle (Pinon & Frey, 2005; Hacquard et al., 2011). However, a previous population genetic study suggested the existence of a distinct group of individuals that would survive asexually only on poplar hosts (Xhaard et al., 2011). Here i) we characterized the existence of an asexual group maintaining on the poplar hosts over the years, ii) we found that this asexual group is made up of several genetic lineages. We documented that some of these asexual lineages have persisted for nearly three decades despite their coexistence with sexual lineages in some places. These findings challenge conventional assumptions about the evolutionary dominance of sexual recombination in this species and offer a unique opportunity to investigate the transitions from sexual to asexual reproduction in a plant pathogenic fungus.

In the present study, two complementary approaches were employed to reveal and characterize asexual individuals. A clustering approach based on differences in allelic frequency and probability of membership was combined with a resampling approach aiming to identify the persistence of lineages over the years. We observed a strong overlap between these indirect and direct approaches, which enabled a sound delineation of asexual lineages among *M. larici-populina* individuals. This result further supports the reliability of the clustering approach performed in the initial study of (Xhaard et al., 2011) to identify asexual lineages indirectly. Applying such an individual based clustering approach is all the more relevant that sexual and asexual lineages can be admixed in the same location (Halkett et al., 2006). Consequently, the clustering approach can be regarded as a robust and independent method for detecting cryptic population genetic structures. In addition to the genetic differentiation between sexual and asexual clusters caused by the lack of gene flow, several genetic signatures clearly distinguished asexual lineages thereby reflecting the fundamental differences in evolutionary trajectories between these reproductive modes (Balloux et al., 2003; Halkett et al., 2005; Rouger et al., 2016). Sexual reproduction ensures the convergence toward Hardy-Weinberg equilibrium (HWE) with recombination among loci that reduces linkage disequilibrium (LD). Asexual reproduction, on the other hand, distorts both allelic and genotypic frequencies mostly because it freezes genotypes (Stoeckel & Masson, 2014; Rouger et al., 2016). As a result, the best way to identify asexual

402 lineages is to apply a multi-criteria method (Kronauer et al., 2012) in which the (diploid)
403 population displays the following hallmarks (Halkett et al., 2005; Arnaud-Haond et al., 2007): a
404 reduced genotypic diversity, combined with a significant excess of heterozygotes (negative F_{IS}
405 values). It should be noted that we also observed a very high variance of F_{IS} across loci, as expected
406 when some recombination events seldom occur (Balloux et al., 2003; Stoeckel & Masson, 2014;
407 Stoeckel et al., 2021). Here, the values found for these indices are consistent with a very high
408 asexuality rate as inferred by ClonEstiMate analysis. The asexual group also displayed significant
409 linkage disequilibrium among loci, which is often reported in asexual and partially asexual species,
410 despite not being consistently expected in neutrally evolving populations (Stoeckel et al., 2021).
411 Linkage disequilibrium was also observed in the sexual group along with a positive F_{IS} value as
412 the result of a Wahlund effect. Indeed, the sexual group encompasses different genetic subgroups
413 that have been identified by previous population genetics studies (Xhaard et al., 2011; Persoons et
414 al., 2017) and which are not considered here. We observed clone mates in the sexual group too, as
415 these can form lineages as abundant as those in the asexual group, with the notable difference that
416 they occur always at a unique sampling site and that, above all, they are never resampled over the
417 years in spite of their local abundance. This local abundance of clone-mate lineages is in
418 accordance with the sampling mostly done in the autumn, at the end of the clonal epidemic phase
419 of *M. larici-populina* life cycle. With regards to this, it should be recalled that the mandatory sexual
420 reproduction that takes place on larch resets the population genetic indices in the sexual group
421 each year (Xhaard et al., 2011; Rouger et al., 2016).The contrast in population genetic indices
422 provide a sound framework for distinguishing reproductive modes, supporting the reliable
423 assignment of individuals into either sexual or asexual groups and providing the first insights into
424 their respective evolutionary history (Xhaard et al., 2011).

425 In accordance with the previous study of Xhaard et al. (2011), we found that the asexual group
426 was unevenly distributed across the country, and mostly occurred in southern France. Asexual
427 lineages have often been described as distributed at the margins of a species' natural range (Simon
428 et al., 1999; Halkett et al., 2005; Drenth et al., 2019; Pereyra et al., 2023), a phenomenon called
429 the geographic parthenogenesis (Peck et al., 1998). Poplar rust is intimately dependent upon the
430 presence of its two hosts. Although the native range of European Larch (*Larix decidua*) is restricted
431 to the Alps and some other Central-European Mountain ranges, it has been widely planted in many

432 parts of France in mountains and plains since the 18th century. Nevertheless, it is less frequent in
433 the Mediterranean area, which can therefore be considered as a margin for the poplar rust fungus.
434 If we compare our results with those of other rust fungi, particularly those infecting wheat, we can
435 see a larger spatial segregation between the sexual lineages found only in the disease's center of
436 origin and the asexual lineages that have invaded the crops worldwide. Beyond rusts, this
437 observation is common to many pathogenic species as for the ascomycete fungus responsible for
438 rice blast (Saleh et al., 2014), or for the oomycete responsible for potato late blight (Ristaino,
439 2002). Interestingly, asexual lineages in these cases are specialized on different cultivars, and
440 therefore on different host genotypes (Goyeau et al., 2007), and the relative abundance of asexual
441 lineages therefore fluctuates over time with the use or disuse of these cultivars (Fontyn et al.,
442 2022). This shows to which extent the asexual evolution of these plant pathogen species is
443 associated with human practices. By contrast, in *M. larici-populina*, asexual lineages are sampled
444 mostly on wild poplar trees and are thus found on different host genotypes. We thus can argue that
445 this rust species enables the incipient stage of sexual to asexual transition to be documented.

446 A puzzling observation is that asexual lineages of *M. larici-populina* coexist with their sexual
447 counterparts in most sampling sites. The absence of larch trees in some areas can be compensated
448 for by the very high dispersal abilities of this species (Barrès et al., 2008), which can be wind
449 dispersed over hundreds of kilometers in a year (Saubin et al., 2024).This can readily explain why
450 sexual and asexual lineages are admixed even if no larch tree exists in the close vicinity of the
451 sampling site. Another non-exclusive hypothesis to account for the long-lasting coexistence of
452 sexual and asexual lineages is that of a temporal fluctuation in the environmental conditions that
453 favor the ones over the others. This temporal fluctuation could be responsible for the fluctuations
454 in abundance we observed, with almost half of the asexual lineages sampled in the year 2020 alone.
455 These fluctuations in relative sexual vs. asexual abundances could be linked to peculiar
456 meteorological events that would desynchronize the alternation between poplar and larch during
457 the sexual life cycle and disadvantage sexual lineages that have to complete this complex life cycle.
458 An outstanding example of the effect of a hazardous event on the fate of sexual and asexual
459 lineages was documented in the red alga *Agarophyton chilense* along the Chilean coasts (Becheler
460 et al., 2020). This study monitored wild (mostly sexual) and cultivated (asexually propagated)
461 populations of this red alga before and after a devastating earthquake. Although sexual populations

462 paid a large tribute immediately after the catastrophe, the artificial maintenance of an asexual
463 population caused the algae culture to collapse in the long term, suggesting that each mode of
464 reproduction plays a role in maintaining a dynamic equilibrium.

465 The distribution of asexual lineages of *M. larici-populina* prevailing in southern France points to
466 the first mechanism that can explain their maintenance and diversification. In milder climates the
467 asexual survival could be facilitated by a longer growing period of poplars, creating a so-called
468 green bridge that would enable the rust lineages to jump over the winter from poplar leaves to
469 poplar leaves. According to this hypothesis, the asexual lineages would passively bypass the sexual
470 phase on larch, without the need for any genetic determinism. In support of this hypothesis, it
471 should be noted that *M. larici-populina* urediniospores can survive for 10 months and still be able
472 to infect poplar leaves (Taris, 1968). But in these experiments, rust-infected poplar leaves were
473 stored either in a greenhouse, or in a wire cage 2 meters above ground level, which does not mimic
474 real natural overwintering conditions. According to this neutral hypothesis, both the onset and the
475 survival of asexual lineages would be a matter of chance only. In support of this hypothesis, we
476 observed that some asexual lineages branched among the sexual cluster and that these lineages
477 have only been revealed through the resampling over years, indicating they are in very close
478 genetic proximity with the lineages that undergo obligate sexual reproduction each year.

479 The persistence of several asexual lineages over decades, attesting to their evolutionary
480 maintenance and relative success, could also be indicative of some genetic determinism favoring
481 their survival over winter. In support of this hypothesis, the trade-off between overwintering ability
482 and multiplication during the epidemic season has been documented in other fungal species
483 (Hamelin et al., 2016; Suffert et al., 2018), and proven to account for the coexistence and
484 evolutionary maintenance of sexual and asexual lineages (Castel et al., 2014). It is also likely that
485 those long-lasting asexual lineages could have lost, at least to some degree, their ability to invest
486 in sexual reproduction, maybe to the benefit of greater asexual survival. Our population genetic
487 analysis, and especially the high variance of F_{IS}, would nonetheless indicate that recombination
488 does not occur among asexual lineages and thus that they do not evolve under strict asexuality but
489 rather as a succession of clones maintaining on poplar leaves over winter, renewed over the years.
490 Furthermore, the rare engagement of asexual lineages into the sexual reproduction can bring
491 additional explanations for the diversity of asexual lineages we observed. In line with the concept

492 of contagious asexuality observed in other organisms with mixed reproductive modes, such as
493 aphids and daphnia (Simon et al., 2002, 2003; Halkett et al., 2006, 2008), asexual lineages that
494 invest in sexual reproduction could be able to produce offspring that inherit their propensity to
495 survive asexually (Halkett et al., 2008). Deciphering further the history and evolutionary success
496 of these asexual lineages calls for a dedicated experiment to test their performance and investment
497 throughout the life cycle. While the complex life cycle of *M. larici-populina* can be implemented
498 in the laboratory (Pernaci et al., 2014), it remains a challenging experiment that is still ongoing.

499 The main original result of our study was to document the diversity among asexual lineages in *M.*
500 *larici-populina*, with some lineages persisting over decades and others appearing more
501 sporadically. The fact that two sampling campaigns covered the entire French territory, and that
502 other sites were sampled regularly over more than 30 years is evidence for this pattern not being
503 due to sampling bias. Notably, differences in abundance correlate with differences in the genotypic
504 structure between lineages. We documented that asexual MLLs can consist of different genotypes
505 (MLGs), unlike lineages within the sexual group that primarily consist of a singular repeated MLG.
506 While some persistent asexual lineages, such as MLL1, showed relatively high genotypic diversity
507 over time. Here it is important to state that only slight differences distinguish MLGs within asexual
508 MLLs, in the vast majority of cases up to three loci differ only. Moreover, the changes consist in
509 either heterozygous to homozygous conversion or to increase or decrease in allele length by a one
510 step mutation. This regular pattern of allele changes, found across all MLGs within MLLs,
511 supports the hypothesis that the asexual lineages evolved through single mutation steps. Although
512 mutation rates for the microsatellite markers used in *M. larici-populina* have not yet been
513 estimated, this regular pattern could present an opportunity to apply a tip calibration approach of
514 molecular clock analysis to estimate the timing of the emergence and persistence of these asexual
515 lineages (Rieux & Balloux, 2016). Yet, the differential abundance and genotypic diversity among
516 asexual lineages reflects heterogeneity within asexual population dynamics and emphasizes the
517 necessity to consider each lineage independently, rather than presuming that the whole asexual
518 population follows the same evolutionary trajectory. The most parsimonious hypothesis is that this
519 diversity has been generated by the repeated emergence of asexual lineages through the
520 evolutionary mechanisms previously discussed (chance of survival or contagious asexuality).
521 Among all asexual lineages, MLL1 stood out as the most abundant lineage, being repetitively

522 sampled from 1992 and showing an outbreak in 2020. It was detected in all regions where asexual
523 lineages were sampled, and forms the only contribution to the unexpected occurrence of asexual
524 lineages northward in the center of France, nearby Paris. The genome of a reference asexual isolate,
525 representative of this MLL, has recently been sequenced, and analysis of its genome structure,
526 polymorphisms, and gene content will further document the tale of this dominant asexual lineage.

Data

To ensure reproducibility, all analyses were executed using R version 4.4.1. The relevant scripts and data can be accessed at https://doi.org/10.5281/zenodo.15100450 (Abdalrahem et al., 2026).

Author Contributions

Sébastien Duplessis, Pascal Frey, and Fabien Halkett conceived and designed the study. Axelle Andrieux designed and performed most of the molecular experiments. Ronan Becheler, Ammar Abdalrahem, Axelle Andrieux, and Fabien Halkett performed the manual curation of genotyping results. Axelle Andrieux, Ronan Becheler, and Fabien Halkett did the preliminary analyses of the data. Ammar Abdalrahem performed the final data analyses with help and advice from Benoit Marçais, Ronan Becheler, Solenn Stoeckel, and Fabien Halkett. Ammar Abdalrahem, Kadiatou Schiffer-Forsyth, and Fabien Halkett prepared the manuscript. All authors revised and approved the manuscript.

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We have used AI tools to proofread, suggest synonyms, check grammar and spelling, and debug R script. All suggestions made by these tools have been scrutinized and cross-checked by the authors.

Conflicts of Interest

The authors declare no conflicts of interest

Benefit-Sharing Statement

The advantage of this study is that its analysis code is an automated workflow and data are publicly accessible.

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Supplementary figures

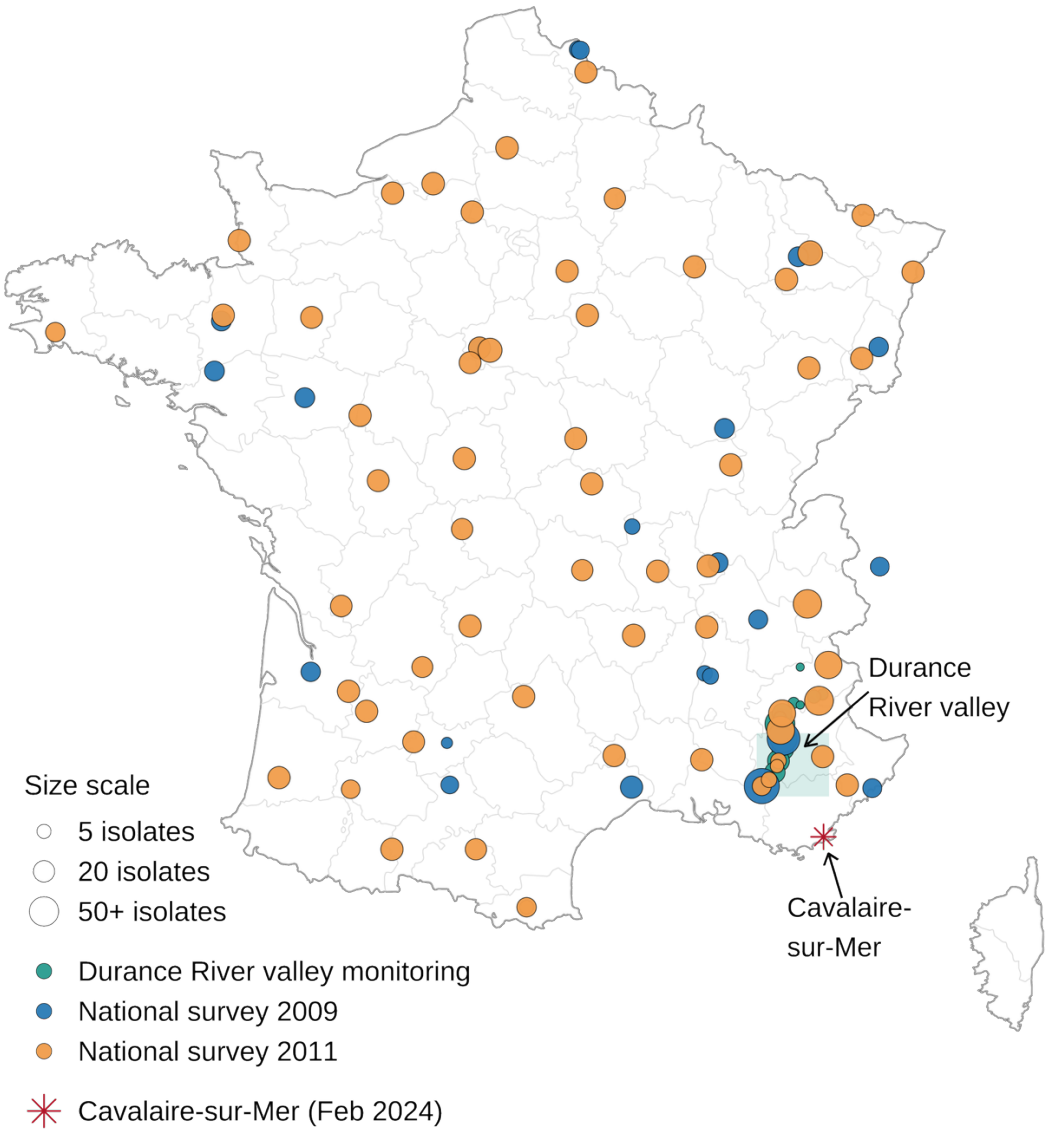

Figure S1

Geographic distribution of *Melampsora larici-populina* isolates collected across France. Each circle represents a sampling site, with size proportional to the number of isolates collected (5, 20, or 50+ isolates). Colors indicate the sampling campaign: blue for the 2009 national survey, orange for the 2011 national survey (n = 45 sites), and teal for the long-term monitoring program in the Durance River valley (surveys conducted in 2004, 2008, and 2020). The red asterisk marks the collection at Cavalaire-sur-Mer in February 2024. Historical isolates collected before 2004 are not shown on this map but were included in the population genetic analyses.

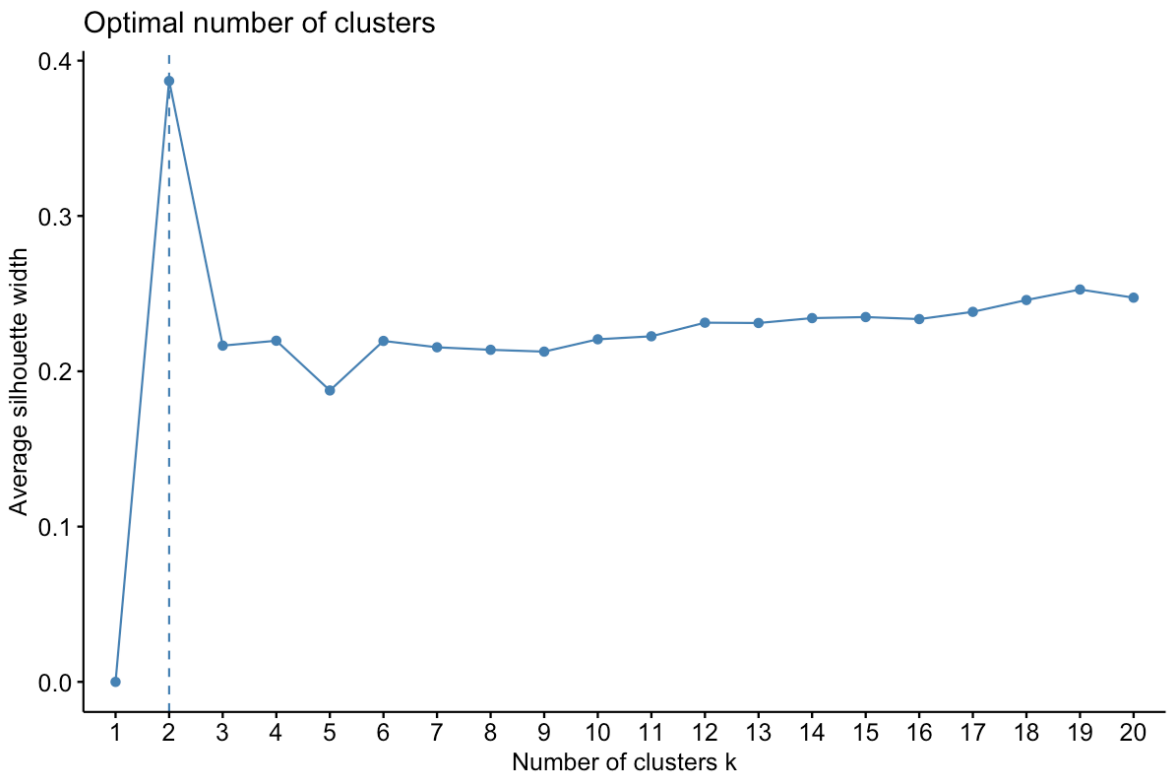

Figure S2

Silhouette analysis for determining the optimal number of genetic clusters (k) in *Melampsora larici-populina*. Average silhouette width was computed for k = 1 to 20 using K-means clustering applied to the first 10 principal components of the microsatellite allele frequency matrix. The dashed vertical line indicates the optimal k = 2, which achieved the highest average silhouette width (≈ 0.4), indicating the most likely distinct and well-separated cluster structure.

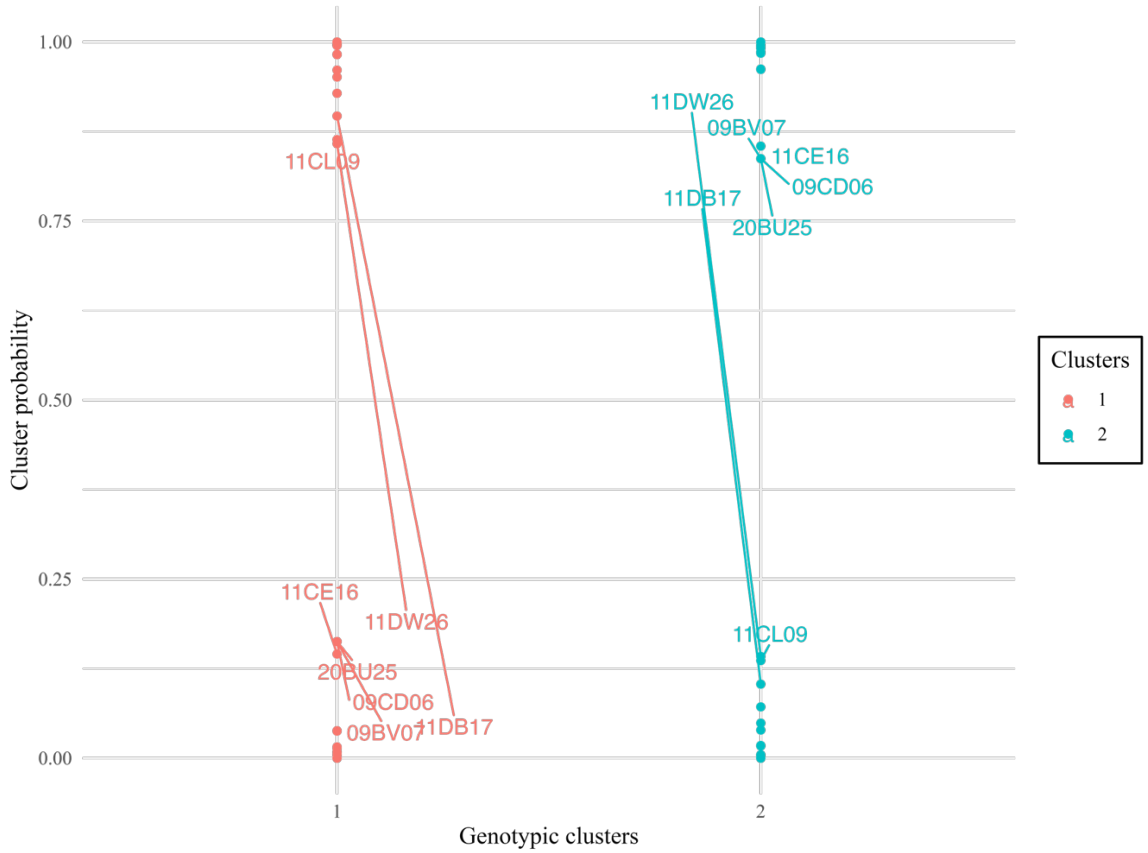

Figure S3

Scatter plot illustrating the membership probability for individuals within each genotypic cluster, with the names of individuals of lower probability.

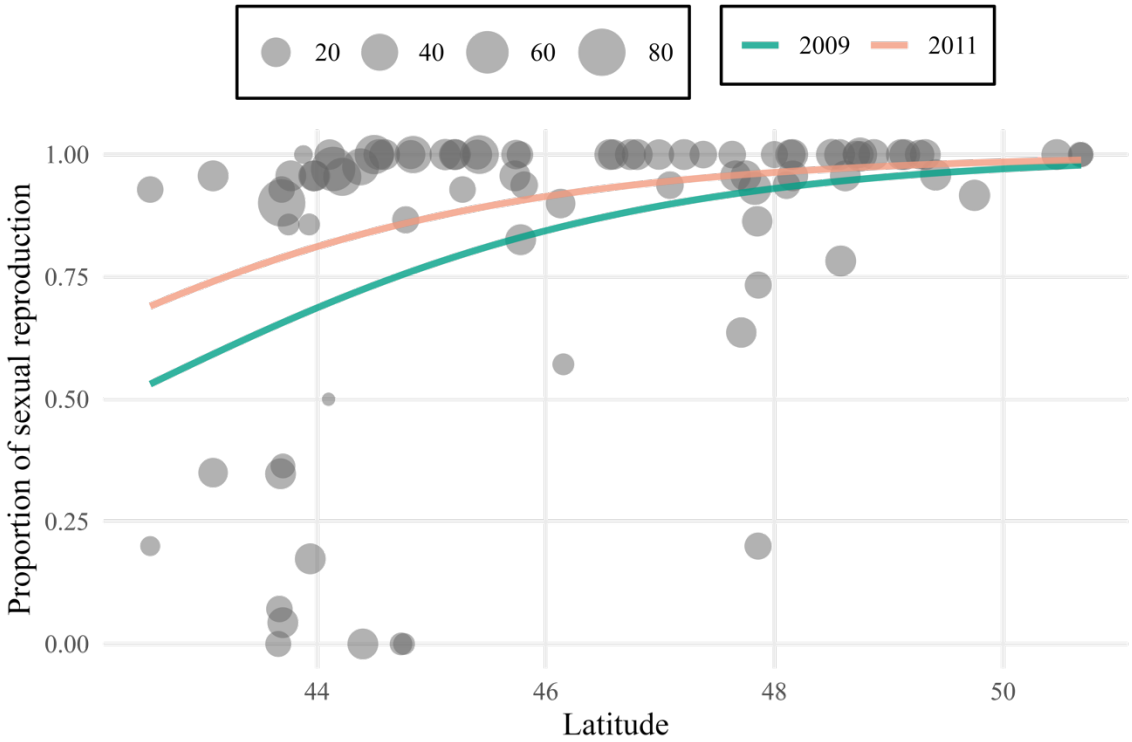

Figure S4

The regression plot illustrates the relationship between latitude and the proportion of sexual reproduction. This relationship was analyzed using a binomial generalized linear mixed model (GLMM). It was based on data from 2009 and 2011. The size of the points represents sample sizes.

Supplementary Tables

Table S1. The data set for 2,122 *Melampsora larici-populina* individuals used in population genetic analyses includes collection year, geographic location, and genotypes across 21 microsatellite markers.