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

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Abstract

Sexual to asexual transition is described as a process whereby asexual lineages emerge within sexual species. This phenomenon gives rise to many questions about the maintenance of sexual reproduction and the evolution of asexuality in these organisms. The poplar rust fungus *Melampsora larici-populina* has a complex life cycle, which is typical of rust fungi (Pucciniales). It alternates between a sexual reproduction phase on larch trees (*Larix* spp.) and rounds of asexual multiplication on poplar trees (*Populus* spp.). This study challenges the classic understanding of the *M. larici-populina* life cycle. We conducted a comprehensive population genetic analysis, utilizing 21 microsatellite markers and data from 2,122 individuals gathered over an extended time from various locations in France. Our results demonstrate the existence of many distinct lineages that reproduce asexually through the years, skipping the sexual phase. A 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 over the years. While sexual lineages are predominant throughout France, asexual lineages are more prevalent in the south of the country, due to possible environmental or ecological factors that allow the overwintering of asexual forms. The discovery of variations in the life cycle in this species offers insights into the evolution from sexual to asexual reproduction encountered in many pathogen species. It could serve as a model organism for studying the transition from sexual to asexual reproductive mode.

Keywords: sexual-asexual transition | evolution of reproductive mode | 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). Sexual reproduction, which has benefits and costs, is widespread in eukaryotic organisms (Schurko et al., 2009). The key benefit is increased genetic diversity, purging deleterious mutations, and enhancing adaptation to changing environments (Pierre et al., 2022). However, 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). The offspring is genetically identical to the parent, barring somatic mutations, which renders asexual lineages more vulnerable to the accumulation of deleterious mutations due to a lack of recombination (Lynch et al., 1993; Peck, 1994; Keightley & Eyre-Walker, 2000), and more prone to loose 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. Remarkably, 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; Tilquin & Kokko, 2016; Gladieux et al., 2015). 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*

60 *striiformis* f. sp. *tritici* displays a similar pattern, with different reproductive modes depending on
61 the region. The fungus reproduces sexually in China and Pakistan, which is highlighted by its high
62 genotypic diversity, whereas it reproduces asexually in the rest of the world (Bahri et al., 2009;
63 Mboup et al., 2009; Ali et al., 2014). The rice blast fungus *Magnaporthe oryzae* (Syn: *Pyricularia*
64 *oryzae*) reproduces sexually in a limited area of southeast Asia, its native range, whereas epidemics
65 globally are driven by asexual lineages (Saleh et al., 2012).

66 The coexistence of both reproductive modes in the same geographical environment is still poorly
67 documented, despite it is offering a unique opportunity to decipher the evolutionary and ecological
68 dynamics of sexually or asexually derived lineages. While such studies are rare on fungal
69 biological models, they have been documented in other taxa, such as in aphids (Halkett et al., 2006;
70 Simon et al., 2010), daphnia (Lehto & Haag, 2010), and a cyprinid fish *Carassius gibelio* (Barbuti
71 et al., 2012). The phytopathogenic fungus *Melampsora larici-populina*, causing poplar rust,
72 represents an ideal candidate for elucidating the transition from one reproductive mode to another,
73 as both modes can be found in the same environment. *M. larici-populina* belongs to the taxonomic
74 order of Pucciniales (Steenackers et al., 1996). This species follows a complex life cycle with an
75 obligate sexual reproduction that alternates with rounds of clonal multiplication (Figure S1). Two
76 unrelated host plants are required to complete the life cycle (Hacquard et al., 2011). Clonal
77 multiplication occurs on poplar trees (*Populus* spp.), while sexual reproduction takes place on
78 larch trees (*Larix* spp.) (Lorrain et al., 2019). In the following, lineages that complete the entire
79 life cycle, including the obligate sexual reproduction phase, are referred to as sexual lineages. The
80 sexual reproduction on larch ensures genetic recombination and obliterates all traces of the
81 previous rounds of clonal multiplication (Xhaard et al., 2011; Rouger et al., 2016). The impact of
82 this fungal disease on larch is insignificant, whereas the many rounds of clonal multiplication that
83 occur on poplar leaves from spring to autumn can be responsible for strong epidemics with large
84 nutrient uptake by the fungus and perturbation of photosynthesis (Jiang et al., 2016). Disease
85 symptoms and damage are greatest on cultivated poplars and resemble those of cereal rusts caused
86 by several *Puccinia* species. A previous population genetic study (Xhaard et al., 2011) has
87 provided evidence for some variability in reproductive mode, with an obligate sexual genetic group
88 coexisting with the genetically distant group that displays all hallmarks of long-lasting asexual
89 reproduction. This suggests that some lineages of the poplar rust fungus could survive alone

through clonal multiplication on poplar. Yet, this preliminary study did not provide detailed investigations on the ecological and evolutionary dynamics of this asexual group, largely because it was based on a single year of sampling.

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

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

2 Material and Methods

2.1 Isolate collections

In this study, we examined 2122 individuals of *M. larici-populina* sampled from 61 "départements" across France over 32 years (1992-2024). The dataset (Table S1) includes previously published genotypes from the study by Xhaard et al. (2011), sampled from a countrywide survey conducted in France in 2009 (23 locations | 428 samples), and more recent samples with genotype data original to this study. These recent additions comprise i) samples from a second countrywide survey conducted in 2011 (60 locations | 1374 samples), ii) individuals from the laboratory collection (1992, 1993, 1994, 1996, 1998, 2008, and 2014 | 34 samples), and genotypes from a project monitoring poplar rust epidemics in the Durance River valley (Xhaard et al., 2012; Becheler et al., 2016) that were suspected to belong to the asexual group and thus not included in the previously cited publications (2004, 2008 and 2020 | 246 samples). All these samples were collected in late summer, at the end of the clonal multiplication phase, therefore before leaf fall and lineage engagement into sexual reproduction (Figure S1). In addition, we benefit from the observation of a distinctive poplar stand in Cavalaire-sur-Mer (south of France) that maintains green leaves until mid-winter, where samples were collected in February 2024 (40 samples).

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 amplification size, respectively. The PCR products were then pooled and analyzed using an ABI 3730 Genetic Analyzers (Applied Biosystems) by the company GenoScreen. Fragment sizing

143 utilized a LIZ-1200 size standard, and allele scoring was performed with GENEMAPPER V4.0.
144 Samples that failed to amplify at more than six loci were excluded from further analysis.

145 **2.3 Population genetic and statistical analyses**

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

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

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

171 are found (Kamvar et al., 2014, 2015). The threshold on genetic distance between neighbor
172 genotypes was set using the function `cutoff_predictor` from **poppr**.

173 *Clustering procedure to indirectly identify asexual lineages:* As the asexual reproductive mode
174 freezes genotypes, it retains ancestral allelic states. Asexual lineages can thus rapidly diverge from
175 their sexual counterparts, the latter being still influenced by genetic drift (Rouger et al., 2016). In
176 line with this statement, we previously found large genetic differences between sexual and asexual
177 groups of *M. larici-populina* (Xhaard et al., 2011). Assigning poplar rust individuals would thus
178 be an effective approach to identify asexual lineages without any *a priori*. The main pitfall of this
179 method being that the clustering algorithm should not rely on gathering individuals according to
180 Hardy-Weinberg expectations, which is the common assumption of any Structure-like approach
181 (Pritchard et al., 2000). We thus carry out our clustering analysis using a PCA and discriminant
182 analysis approach based on allelic distribution (Jombart et al., 2010). We used the `find.clusters`
183 function from the **adegenet** v.2.1.10 package to reduce the dimensionality of the dataset.
184 Discriminant analysis of principal components (DAPC) was then used to refine the identification
185 of clusters by maximizing the variance between two clusters, while minimizing within cluster
186 variance (Jombart et al., 2010). Nonetheless, 97.86% of genetic variation among individuals was
187 captured by retaining 90 principal components (n.pca = 90). Individuals were grouped into two
188 clusters using the `dpca` function with K=2. To ensure the reliability of the assignment, only
189 individuals with a high membership coefficient (≥0.8) were retained for further analyses of within-
190 cluster genetic characteristics.

191 *Resampling multilocus lineages (MLLs) as direct evidence for asexual reproduction.* Observing
192 the same genotype over several years is an indication that this lineage survived asexually - unless
193 sexual reproduction could have resulted in the same combination of alleles by chance, which can
194 be ruled out by a *Pidu* analysis (Evett & Weir, 1998; Stoeckel et al., 2024). To track the persistence
195 of MLLs over time, we recorded the years each MLL was detected and calculated its frequency
196 across different years. A lineage that persisted for at least two years was considered asexual. We
197 tested the overlap between the direct and indirect approaches for assessing asexual reproductive
198 mode by performing Fisher's exact test (Clarkson et al., 1993) on the contingency table of MLL
199 sorting according to the direct or indirect approach by using the `fisher.test` function from the
200 **stats** package. In the following, a given MLL is considered asexual if it meets at least one of the

201 two criteria: the assignment to an asexual cluster or the inter-annual resampling over a minimum
202 of two years.

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

212 *Spatial analysis of the repartition of sexual and asexual lineages:* To illustrate the geographical
213 distribution of sexual and asexual individuals across France, the **maps** package v3.4 was utilized.
214 This enabled us to obtain information not only on spatial distribution, but also on the potential
215 coexistence of the two reproduction modes within the same geographical area. Using the `glmer`
216 function from the **lme4** package, a generalized linear mixed model (GLMM) with a binomial
217 distribution and a logit as a link function was employed to assess the regression coefficients
218 (Bolker et al., 2009). To further test the relationship between the reproductive mode variation and
219 the geographic locations, we performed a regression analysis on the count of sexual and asexual
220 lineages per site. The independent variables (fixed effect) were the geographic location represented
221 by latitude and longitude. The effect of the year of sampling was added as a random effect. This
222 analysis was performed on the 2009 and 2011 data originating from the two countrywide surveys,
223 so as not to overrepresent certain locations. To further assess the distribution and relative
224 abundance of asexual MLLs across time and space, we performed Fisher's exact test for fluctuation
225 in MLL abundance across years and locations. Given the large size of the data sets, we employed
226 a Monte Carlo simulation approach with 100,000 replicates to estimate p-values.

227 *Genetic relationships between and among sexual and asexual lineages:* To further elucidate the
228 evolutionary trajectories of sexual and asexual individuals, and to address the question of the
229 dynamics of transition from sexual to asexual reproduction and vice versa, a discrete matrix based

230 on the relative dissimilarity between individuals was calculated by `diss.dist` with the **poppr**
231 package to create a neighbor-joining tree (Saitou & Nei, 1987) using `nj` from the **ape** package
232 v5.8.1.

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

3 Results

3.1 Delineation of multilocus lineages

We identified 1475 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 panmicitic 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 1384 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: 445 individuals were unambiguously assigned to cluster one, while 1676 individuals were assigned to cluster two (Figure S2). 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

269 noteworthy that similar levels of observed heterozygosity were found in both clusters, but a much
270 reduced allelic richness and gene diversity in the asexual cluster 1. Further, the *Pareto β* index was
271 low (~ 0.2), indicating a small number of dominant MLGs, which was consistent with high
272 clonality in cluster 1. In the sexual cluster 2, the *Pareto β* was greater than 1, reflecting a more
273 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	445	64	11	0.142	0.226	4.372	0.525	0.360	-0.461	0.085	0.356
Cluster 2	1676	1411	1373	0.842	1.078	9.529	0.506	0.543	0.070	0.004	0.016
Resampling approach											
Multi-years	488	90	19	0.183	0.248	5.364	0.516	0.390	-0.322	0.129	0.337
Single-year	1633	1385	1365	0.848	1.057	9.700	0.508	0.545	0.068	0.004	0.012
Combination of approaches											
Asex	499	96	25	0.191	0.252	5.414	0.516	0.393	-0.315	0.123	0.327
Sex	1622	1379	1359	0.850	1.051	9.749	0.508	0.544	0.068	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)	434	11
Sex (Clustering approach)	54	1622

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 asexual group determined by the clustering approach revealing a stronger signature of asexual 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 1). 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.

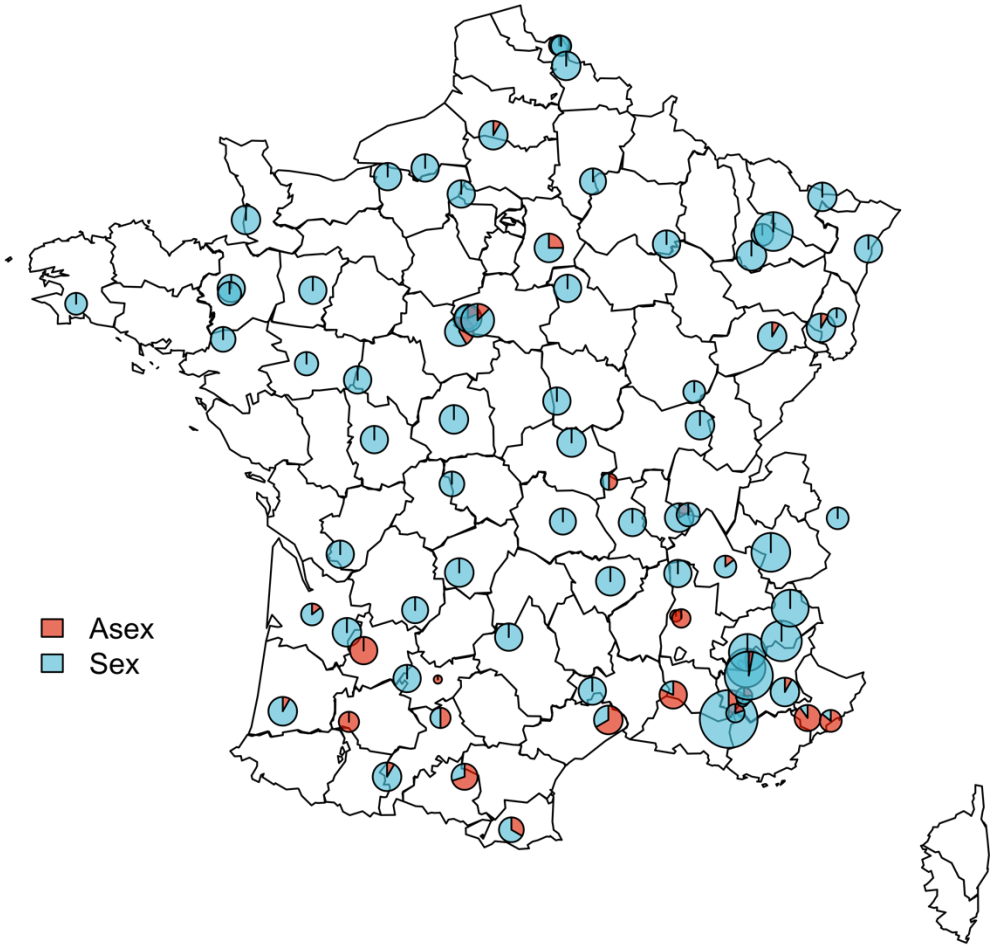

Figure 1

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 S3) 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 = 18.78, sd = 4.333) 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)	-22.99815	2.66959	-8.615	< 2e-16	***
Lat	0.46035	0.04730	9.732	< 2e-16	***
Long	0.09671	0.02912	3.321	0.000896	***

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 two distinct populations, the clonal rate was between 0.93 to 0.99 for the asexual group and between 0 to 0.21 for the sexual group (Figure 2).

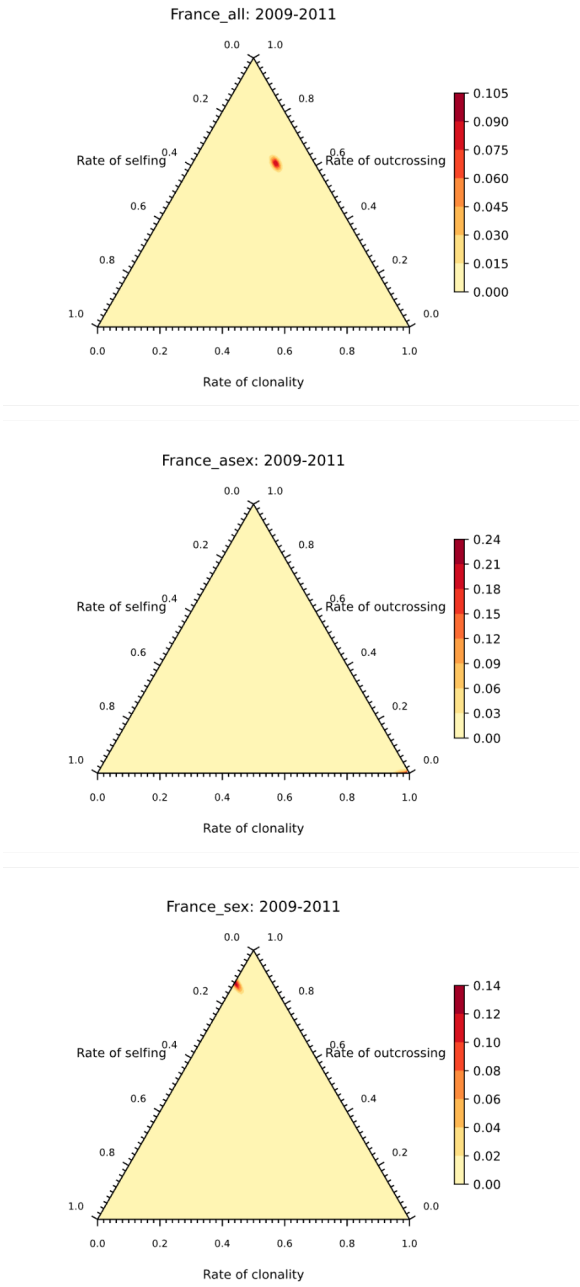

Figure 2

Ternary plot representing the relative proportions of selfing, outcrossing, and clonality rates in three different groups of mixed reproductive, asexual reproductive, and sexual reproductive in France (2009–2011). The color gradient reflects the posterior probability estimates.

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.

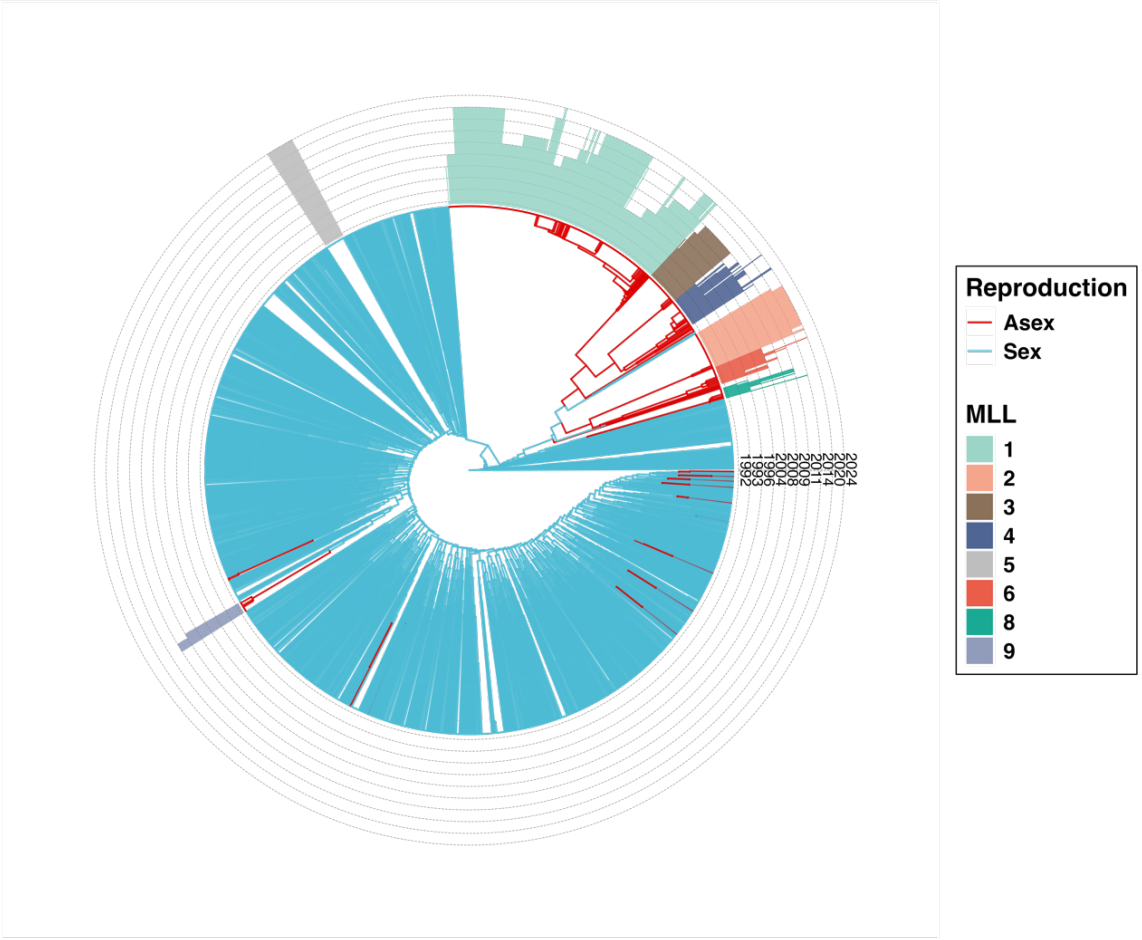

Figure 3

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

3.5 Genetic structure and persistence of asexual lineages

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

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

MLL	*MLG*	N	Year range	R	A_R	H_O	H_E
1	36	280	1992:2024	0.125	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

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

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

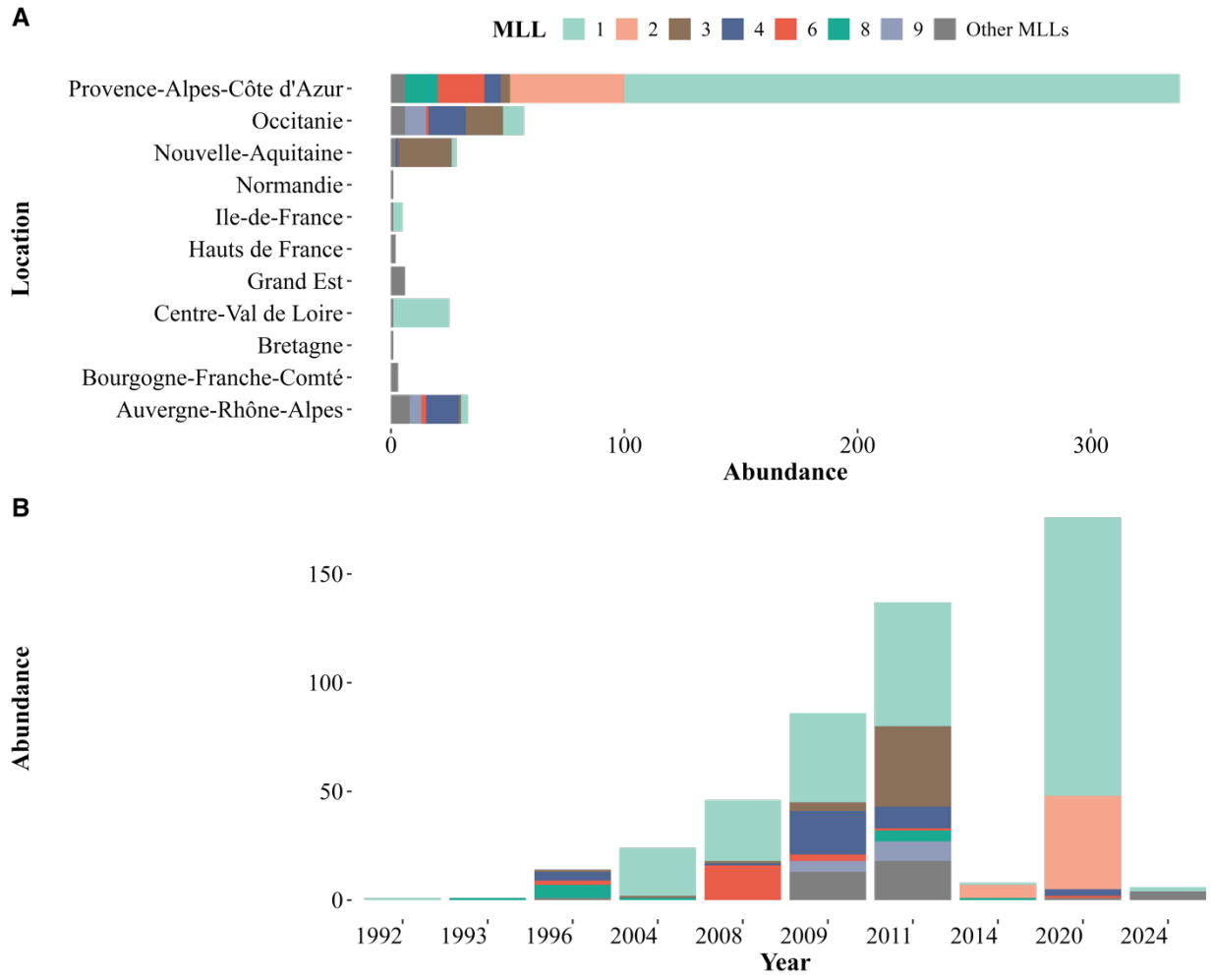

Figure 4

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

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

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

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

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

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

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

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

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

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

521 sampled from 1992 and showing an outbreak in 2020. It was detected in all regions where asexual
522 lineages were sampled, and forms the only contribution to the unexpected occurrence of asexual
523 lineages northward in the center of France, nearby Paris. The genome of a reference asexual isolate,
524 representative of this MLL, has recently been sequenced, and analysis of its genome structure,
525 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 4.2.2. The relevant scripts and data can be accessed at https://github.com/ammarabdalrahem/poplar_rust.git.

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 made 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 and Fabien Halkett prepared the manuscript. All authors revised and approved the manuscript.

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We have used DeepL, Grammarly, and ChatGPT 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

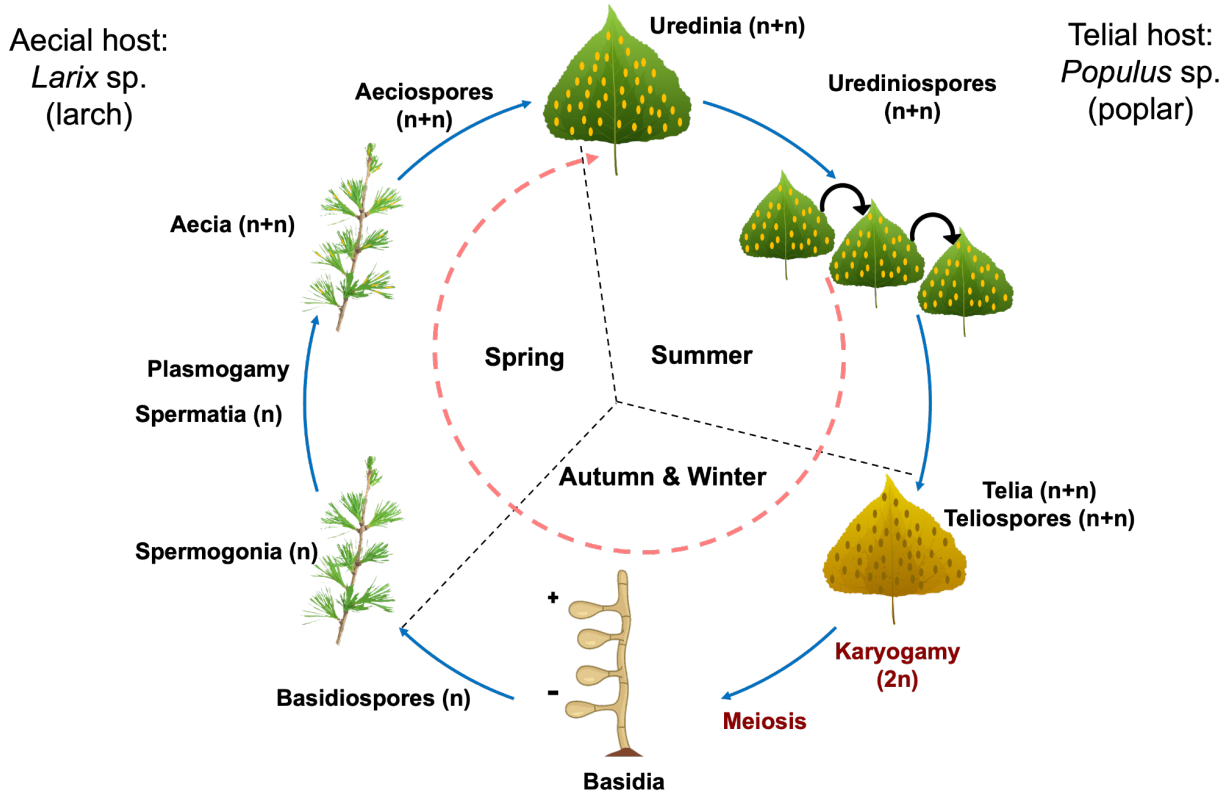

Figure S1

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.

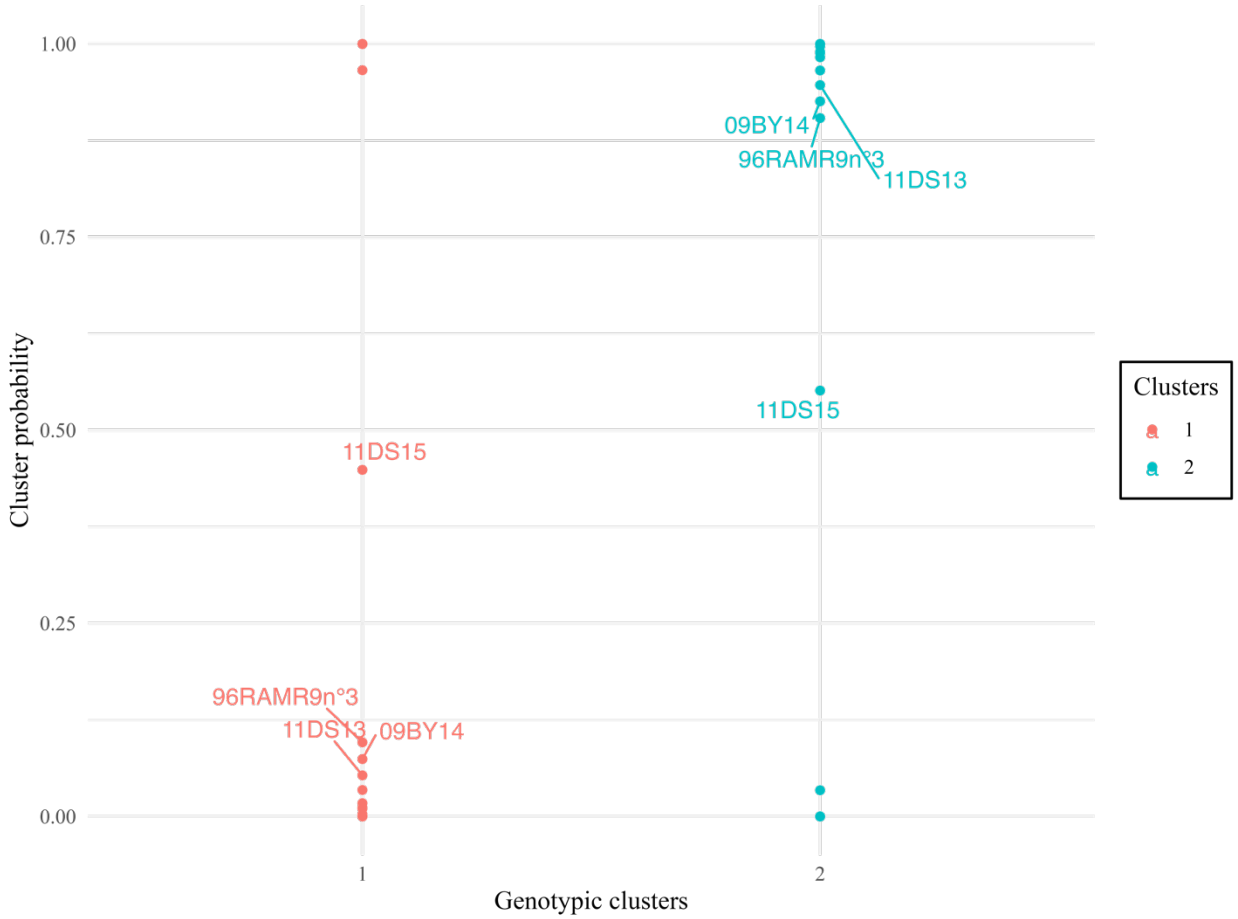

Figure S2

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

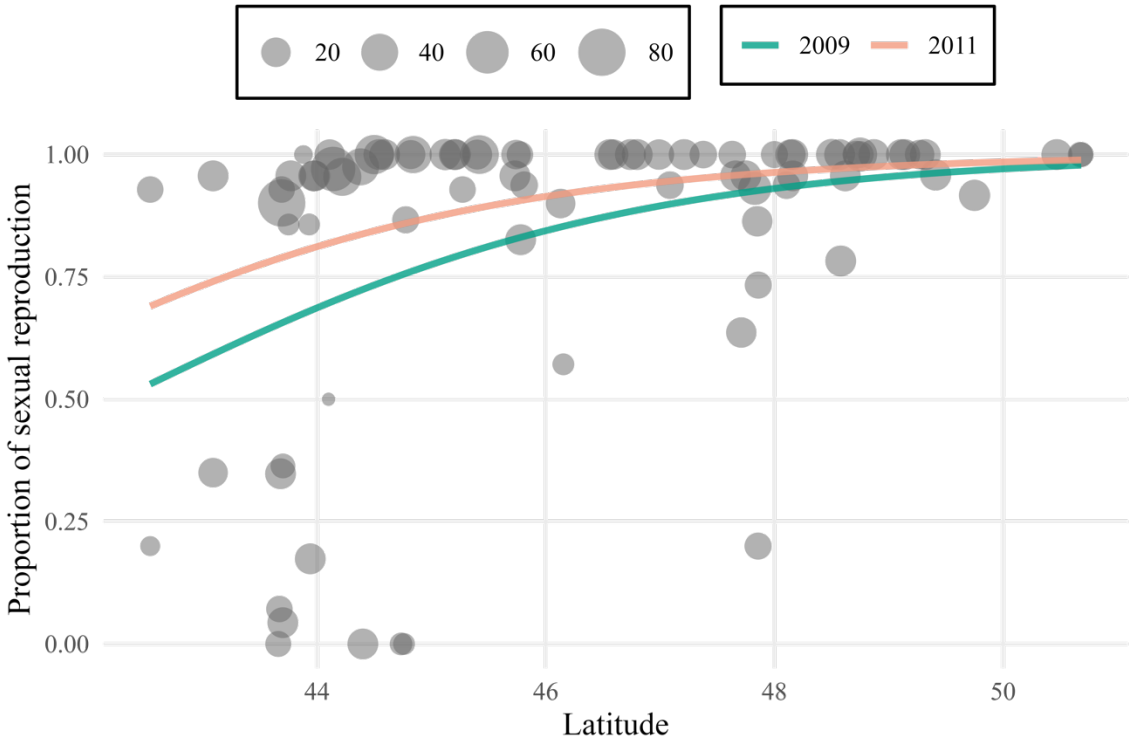

Figure S3

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.