Mycologia, 102(1), 2010, pp. 44–53. DOI: 10.3852/08-098

Puccinia striiformis f.sp. *tritici* presents high diversity and recombination in the over-summering zone of Gansu, China

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Abstract: *Puccinia striiformis* f.sp. *tritici* (PST), a basidiomycota responsible for wheat yellow rust, has a strict clonal behavior and a low genetic diversity in European and Australian populations. On the other hand high diversity has been reported in Chinese populations. Moreover it is thought that in China yellow rust epidemics start recurrently from the western highlands where over-summering occurs. To compare PST genetic diversity in this area to the one described in France seven AFLP primer combinations were used to analyze a sample of 160 isolates collected in 2001 in five counties of Gansu Province. The AFLP data revealed 40 polymorphic bands, discriminating 139 AFLP genotypes. Linkage disequilibrium and phylogeographic analyses support the hypothesis of a reproductive mode that is not strictly clonal. In this regard Chinese isolates from Gansu strongly contrast with the European studies using the same markers. Genetic diversity of this 1 y sampling in Gansu is found to be seven times higher than the one observed in France over 20 y and exhibits lower linkage disequilibrium. The effective population size of the French sample was estimated to be 1000 times smaller than the Gansu population. These results support the hypothesis of large population size as well as the occurrence of genetic recombination, while the importance of Gansu as a main over-summering area requires assessment through larger scale studies.

Key words: effective size, linkage disequilibrium, parasexuality, survival, wheat (stripe) yellow rust

Submitted 28 May 2008; accepted for publication 8 Jun 2009.

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INTRODUCTION

Measuring the importance of recombination in fungi populations remains an intricate task due to both the difficulty of direct morphological observations and the complexity of their life cycle. In fact fungi often combine clonal and sexual reproductive modes, but observations of life cycles can be poorly related to their real weight for populations (Taylor et al 1999, LoBuglio and Taylor 2002). Study of the genetic structure of populations circumvents this difficulty and allows indirect inference on the reproductive behavior of species (for example Brown 1996, Carbone et al 1999). Numerous molecular tools are now available to directly study DNA polymorphism and describe the genetic diversity of populations (Brown 1996, Rafalsky 2002). As an example Burt et al (1996) found molecular evidence for recombination of the human pathogen fungus *Coccidioides immitis* even though mating and meiosis have yet to be reported experimentally. The knowledge of fungal sexuality is especially important for pathogens of economic importance because recombination modulates their adaptive response to cultivar resistances or chemical control (McDonald and Linde 2002). We are interested here in wheat (stripe) yellow rust, which has been described so far as a clonal fungus in numerous studies using various types of molecular markers (Steele et al 2001, Hovmøller et al 2002, Enjalbert et al 2005, Hovmøller and Justesen 2007).

Wheat yellow rust is caused by the biotrophic fungus *Puccinia striiformis* Westend. f. sp. *tritici* Eriks. (PST), Basidiomycota. This major disease of wheat (*Triticum aestivum* L.) on many continents (Asia, Australia, North America, Europe) is prevalent in regions with cool temperatures and humid conditions (Hau and de Vallavieille-Pope 2006). Epidemics develop through the dispersal of urediospores. A single infecting spore is able to colonize wheat leaf and produce numerous aligned pustules harboring

thousands of urediospores. These dikaryotic spores are produced by mitotic cycles, and the reproduction is considered to be clonal (Stubbs 1985) because no sexual (aecidial) alternate host has been described yet (Cummins 1971).

The first genetic argument for a clonal behavior arises from the description of how the genetic structure of this species is affected by specific resistance genes introduced in cultivars. Qualitative resistance genes, which follow the gene-for-gene model from Flor (1956), are found to lose their efficacy few years after their release in wheat cultivars. This is because of the rapid evolution of new virulent races (pathotypes) in the pathogen (Stubbs 1985, Wellings and McIntosh 1990, Bayles et al 2000). Virulence accumulation (i.e. successive fixation of new virulent variants under positive selection) thus is considered the main driving force of yellow rust populations (Stubbs 1985, Wellings and McIntosh 1990). This adaptation mainly results from mutations in a low number of strains; 3–5 major pathotypes are observed in a given year at a regional scale (Bayles et al 2000), thus favoring the hypothesis of stepwise mutation process under clonality.

A second argument supporting clonality arises from molecular studies on *P. striiformis*. Low genetic variation is measured with double-strand RNA (Newton et al 1985), RAPD (Chen et al 1993), repeated genomic sequences (Shan et al 1998) or IGS polymorphism (Roose-Amsaleg et al 2002). More recently the use of linkage disequilibrium tests on AFLP markers confirms this low genetic diversity and a clonal population structure of the fungus in northwestern Europe (Hovmøller et al 2002). Similarly Steele et al (2001) reported a near absence of molecular polymorphism in Australian yellow rust population. Despite this extremely low genetic diversity, an intensive AFLP screening of European pathotypes recently has explained the recent acquisition/loss of virulence factors (Hovmoller et al 2007), giving a more accurate picture of the stepwise mutation process in clonal populations.

However there is suspicion that recombination might exist in some yellow rust populations. First, the occurrence of parasexual recombination in experimental conditions has been reported (Little and Manners 1969). Several papers were published in China in the 1990s that suggested heterokaryosis as a possible mechanism of variation after obtaining strains with new virulence combinations from co-inoculation of an albino isolate and eight Chinese physiological races of yellow rust (Kang et al 1993, 1994a, b; Ma et al 1993). Microscopic observations equally supported these results, as the fusion of nuclei in germ tubes was seen (Ma et al 1993), while Wang et al (2004) reported spores with tri- and tetranuclei at the ratio of 0.42 : 0.55% among naturally collected urediospores. Recombination also was proposed as a possible mechanism of variation in Chinese populations of yellow rust by Shan et al (1998) on the basis of RFLP fingerprints using a repeated sequence, as well as a recent work with microsatellites (Mboup et al 2009). The high genetic diversity described regionally in China (Shan et al 1998, Zheng et al 2000) associated with a low linkage disequilibrium between markers is in contradiction with a strictly clonal reproductive mode. However these results can hardly be compared to the recent AFLP work performed in Europe and Australia because the repeated sequence or microsatellites studied can exhibit higher mutation rate, caused for example by unreciprocal crossing-over (Smith 1976).

The high genetic diversity of Gansu population also can be attributed to fewer summer bottlenecks. Given the favorable over-summering conditions, Gansu is considered the most important source of wheat yellow rust in the autumn in China, which provides yearly the epidemic inocula to the main wheat-growing regions (Wang et al 1963; Wang et al 1965; Liu et al 1984; Xie et al 1990; Li et al 1997; Wan et al 2004, 2007). In the highlands the wheat-growing area is 800–2400 m above sea level. The climatic and agronomic conditions promote summer development of volunteers, while the great variation found in wheat growing stages reduces the host-free gap between harvest and sowing. Thus demographic versus reproductive origins can be advocated to explain the genetic diversity of Gansu populations.

In this study we carried out an AFLP typing of a yellow rust population sampled in 2001 in Gansu with markers identical to those used in Enjalbert et al (2005). The high genetic diversity in Chinese populations was confirmed and compared to that found for European populations with the same AFLP markers. It was shown by linkage disequilibrium analyses that Gansu populations are not strictly clonal, which contradicts previous findings (Stubbs 1985, Wellings and McIntosh 1990, Shan et al 1998, Bayles et al 2000, Steele et al 2001, Hovmøller et al 2002, Chen et al 2002, Wan et al 2004, Hovmøller et al 2007), demonstrating the presence of genetic recombination in yellow rust.

MATERIAL AND METHODS

Fungal sampling.—P. striiformis f.sp. *tritici* samples were collected Oct 2001 in five counties of Gansu Province in China. Infected leaves were taken from different inbred lines in nurseries in a 200 km wide area (FIG. 1, TABLE I). Sporulating leaves were stored individually in paper bags and used to inoculate seedlings of Victo cultivar (de

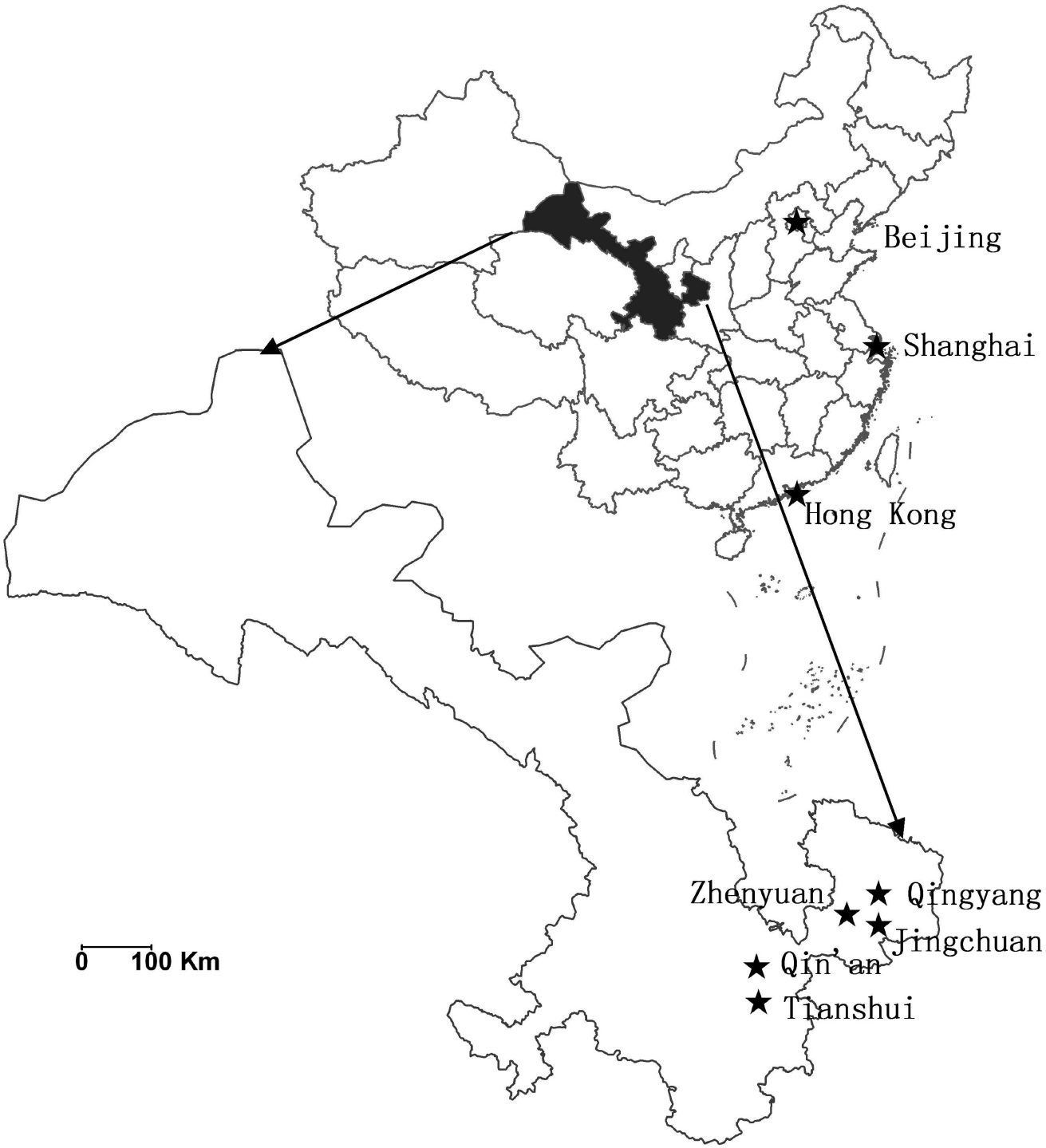

FIG. 1. Sampling locations in Gansu Province, northwestern China.

Vallavieille-Pope et al 1990). Harvested spores were inoculated at low density, and leaves presenting one chlorosis were used to produce single-stripe isolates. One isolate was used to represent one collected leaf, with a total sample of 168 isolates.

AFLP analysis.—A modified CTAB protocol (Enjalbert et al 2002) was used to extract genomic DNA from 10 mg fresh spores. The AFLP protocol (Enjalbert et al 2005) was based on an EcoRI/PstI digestion, a T4 ligation of adapters and two amplifications of the genomic fragments. The first amplification was performed with *Pst* and *Mse* primers with no selective nucleotides, then diluted 1/20 in water and followed by a specific amplification, with two selective nucleotides on both *Mse* and *Pst* primers. Selective primers were used according to Hovmøller et al (2002) (TABLE II).

TABLE I. Geographic origin of the yellow rust samples in eastern Gansu Province, China

Gansu Province	No. of isolates	No. of AFLP genotypes
Tianshui	76	67
Qin'an	9	8
Qingyang	34	28
Zhenyuan	27	24
Jingchuan	14	12

Digestions, ligations and DNA amplifications were performed in an ICycler (Bio-Rad, USA), and PCR products were viewed on a Sequigen apparatus (Bio-Rad) with 6% polyacrilamide silver-stained gels. Polymorphic AFLP fragments (markers) of strong intensity are scored as binary characters for each isolate after checking the polymorphisms on two independent replicates.

Data analysis.—GENETIX software (Belkhir et al 2003) was used to compute the significance of F-statistics and perform a factorial correspondence analysis (FCA) to graphically describe the genetic structure of the populations. To compare diversity between French and Gansu populations, the dataset obtained by Enjalbert et al (2005) was included in the analysis. This dataset was built from 213 isolates representative of a 15 y survey and corresponded to 21 AFLP genotypes and 10 pathotypes. One pathotype from southern France exhibited a major genetic divergence from northern France pathotypes.

To seek out recombination signature in the AFLP genotypes two main tests were performed. Polymorphic bands, except the ones present in a single clone (non-informative for phylogenetic analysis), were included. First, linkage disequilibrium in the dataset was described by the standardized association index (r_d, Maynard Smith 1993) and its distribution in randomized datasets. These values were computed with the MULTILOCUS program (Agapow and Burt 2001). We also tested a phylogenetic method, the parsimony tree length permutation test (PTLPT, Burt et al 1996), by comparing the length of the most parsimonious tree (DNAPARS, PHYLIP package, Felsenstein 1989) of the data to the distribution of tree length obtained in randomized datasets (SEQBOOT, PHYLIP). The dataset was reduced to one isolate for each genotype detected and to one locus for all redundant polymorphic bands. This led to discard duplicates to obtain more conservative tests in clonal species.

The most parsimonious tree was obtained with DNAPARS (PHYLIP) and visualized using TreeView software. NONA software (www.cladistics.com) was used to perform bootstraps and test the significance of tree branching.

An estimate of nucleotide diversity π was performed to compare the observed genetic diversity in Europe and Gansu Province. According to Innan et al (1998), the average proportion of shared bands between isolates can be calculated as follows:

$$\tilde{F} = 1 - \left(H \frac{m_p}{m_t} \right)$$

with m_p being the number of polymorphic bands, m_t the total number of bands and H the average probability for two isolates to be different at a polymorphic band. A program provided by the author was used to estimate π from the observed number of AFLP band as well as their size range and $\tilde{F}$.

RESULTS

During band scoring attention was given to the polymorphisms described in European populations (Hovmøller et al 2002, Enjalbert et al 2005) to compare the genetic diversity of Gansu and European populations. Within Chinese isolates a total of 40 polymorphic bands were revealed by the seven AFLP primer combinations. Thirty polymorphisms were selected for their stability in replicate experiments. Out of the 168 isolates studied 160 were kept for the reliability of their genotype. When the Gansu sample was compared to European genotypes 25 more polymorphisms between the two groups were scored (TABLE II). The AFLP differentiated 139 multilocus genotypes in the Gansu population, with no multilocus genotype (clone) sampled more than three

TABLE II. Primer combinations used for AFLP analysis and their polymorphism

Primer combinations[a]	No. of polymorphic bands		
	Within Gansu	Within France	France-Gansu specific
P(AC)M(AC) - P12M12	3	5	1
P(AC)M(AG) - P12M13	4	3	2
P(AC)M(TT) - P12M26	3	4	2
P(GA)M(TC) - P19/M24	1	3	1
P(GC)M(AA) - P20M11	7	8	4
P(GT)M(AG) - P22/M13	7	3	2
P(GT)M(CG) - P22/M17	5	3	3

[a] P(AC) corresponds to a Pst primer (5′GTAGACTGCGTACATGCAG) plus two selective nucleotides (AC), and M(AC) to a Mse primer (5′GACGATGAGTCCTGAGTAA) plus two selective nucleotides (AC). P12M12 corresponds to the key-gene code.

TABLE III. Genetic differentiation between sampled populations (F_{ST})

FST	Jingchuan	Qingyang	Tianshui	Zhenyuan
Qin'an	0.093*	0.096^{ns}	−0.020^{ns}	0.070**
Jingchuan		0.084^{ns}	0.082**	−0.001^{ns}
Qingyang			0.075**	0.055**
Tianshui				0.067**

^{ns} Non significant.
* significant with P value $< 5\%$.
** significant with P value $< 0.5\%$.

times. A low genetic differentiation was found among sites (low F_{st} values, TABLE III), confirmed by the absence of subgroups in the AFC analysis (FIG. 2). The joint AFC analysis of European and Gansu data revealed a clear divergence among genetic groups in Gansu, southern France and northern France (FIG. 3).

The distribution of the 30 bands among the 139 AFLP genotypes was analyzed to test for the presence of recombination in the population. By eliminating duplicates in the individuals and the polymorphic sites, we applied conservative conditions to assess recombination events during the genesis of genotypes. The standardized association index r_d of the observed dataset is 0.021, significantly out of the distribution range obtained for the randomized dataset (−0.003–0.005, data not shown). Similarly the tree estimated from the observed AFLP dataset is 356 steps long, which is significantly shorter than the trees generated from the randomized dataset (443 steps on average, FIG. 4) but longer than the length expected under strict clonality (30 steps). Both tests let us discard the null hypothesis of free recombination in the population (or linkage equilibrium between markers), and confirmed the importance of clonal reproduction in the Gansu population.

When analyzing the genetic structure between the five sampled locations, low (0.05–0.08) but significant F_{st} were observed in six over 10 pair-wise comparisons (TABLE III). We produced A UPGMA cladogram of the populations (FIG. 5). Because this geographic differentiation could contribute to the linkage dis-

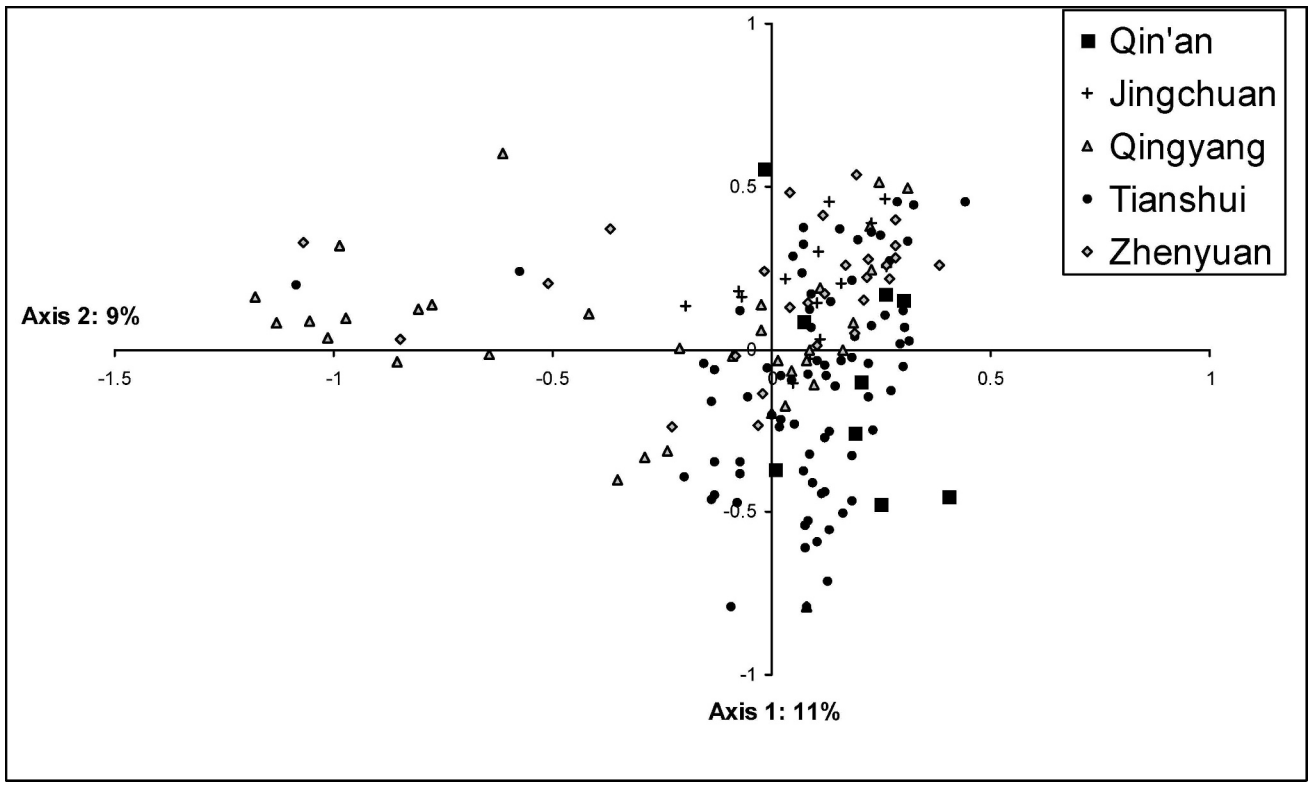

FIG. 2. Factorial analysis over AFLP data of Gansu population.

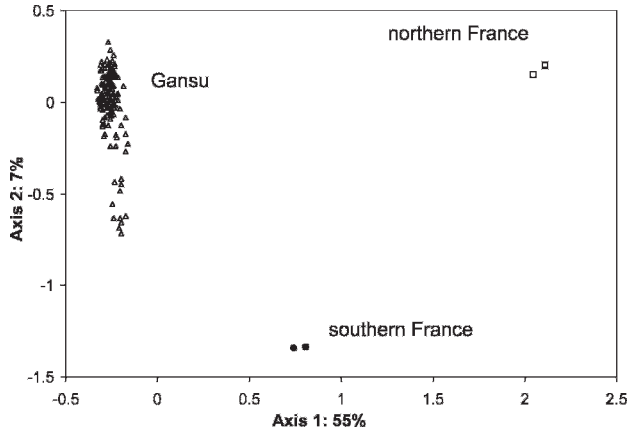

FIG. 3. Joint factorial analysis over French and Gansu AFLP data.

equilibrium between markers additional association index and PTLPT tests were performed on nondifferentiated locations (e.g. Tianshui and Qingyang). These analyses slightly lowered the r_d value and raised the PTLPT value, increasing the evidence of recombination without affecting the significance level of the tests.

We calculated diversity indices, as well as the estimates of nucleotide diversity obtained for the Gansu and the European populations (TABLE IV). The diversity measured in the Gansu population is approximately seven times higher than the one measured in the French population, as shown by the estimated population size (TABLE IV). Multilocus genotype diversity is much higher in the Chinese sample because of weaker linkage disequilibrium between bands. Moreover a higher number of polymorphic bands were in the Chinese sample, as shown by the nucleotide diversity estimates. No

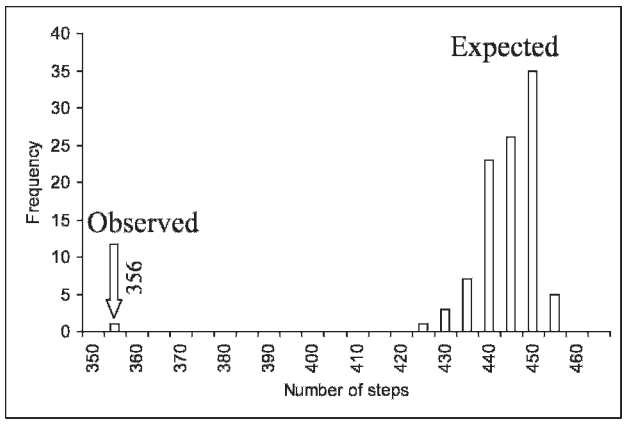

FIG. 4. Randomized distribution and observed value of step number in most parsimonious tree. The distribution of step number under the null hypothesis (no association between alleles) was obtained after 100 randomisations of alleles in the individuals (Burt et al 1996).

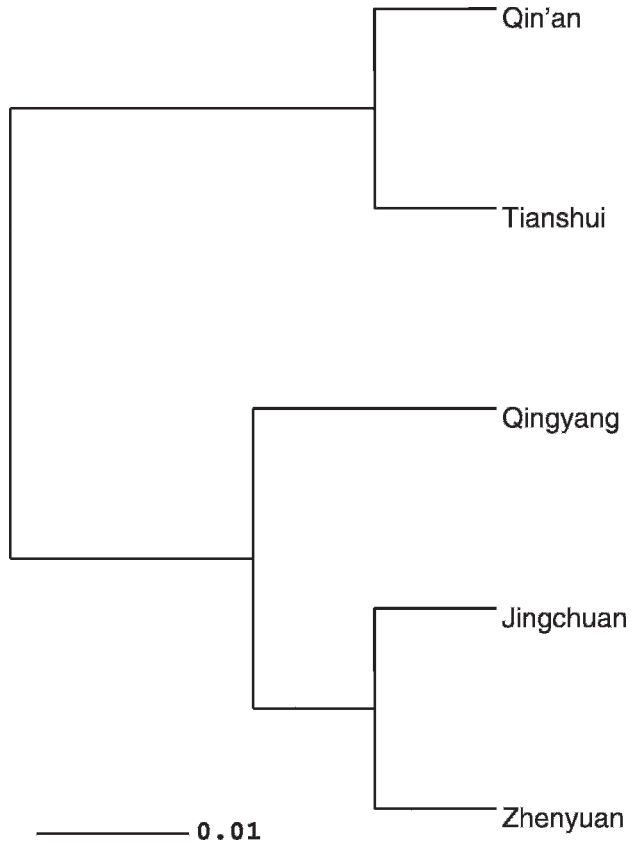

FIG. 5. Cluster analysis of the five sampled locations in Gansu, China (UPGMA on Nei distances with AFLP data).

dominant genotype thus is found in the Gansu population within a single-year survey, in strong contrast with the highly redundant structure of European population (Hovmøller et al 2002, Enjalbert et al 2005).

DISCUSSION

This analysis of molecular diversity in Gansu Province confirms the knowledge of high diversity of yellow rust population in China and in Gansu in particular (Shan et al 1995, Zheng et al 2000). Comparison can be made for genetic diversity and recombination tests among Gansu and European populations because the same markers are used.

Evidence for recombination.—The hypothesis of a random association of polymorphic bands in the sampled individuals is rejected by tests based on linkage disequilibrium and parsimony, in accordance with the clonal urediospore cycle of PST. However even if panmixia is rejected in all tests a key result here is also that the genetic structure negates a strictly clonal reproductive behavior. The best illustration can be found in the phylogenic test (FIG. 4) where

TABLE IV. Nucleotide diversity estimates of Gansu and French populations

	Gansu population	French population (Enjalbert et al 2005)
Sample size	160	213
Number of AFLP loci	30	16
Nucleotide diversity	0.297	0.041
$\Pi = \theta = 4N_e\mu$	0.0077	0.00014
Effective size[a]	1.9×10^6	3.5×10^4

[a] Estimate of the population effective size under the assumption of a nucleotide mutation rate of 10^{-9} per site.

356 steps are found in the best parsimonious tree, whereas the random distribution is centered on a 443 value. If there is no recombination and no homoplasy (independent apparition of the same allele in two individuals) a number of steps equal to the number of polymorphisms (i.e. 30) are expected. By comparison all most parsimonious trees for French isolates present 49 steps, just a little over the 30 steps expected under clonality (Enjalbert et al 2005). The value for Gansu samples (356 steps) is distant from the expected value under recombination (443) but also much further away from the length expected under clonality (30 polymorphisms); recombination must have occurred in addition to the epidemic clonal behavior of this species. It should be mentioned that AFLP markers are likely to present homoplasy (Vekemans et al 2002, Caballero et al 2008) and that part of the extra steps might be due to independent mutations leading to same-band variations in AFLP profiles. However if all the extra steps were due to homoplasy the high rate of nucleotidic mutation thus necessary would not be in accordance with the majority of monomorphic bands observed in each AFLP profile. Effectively a more even distribution of polymorphisms in the AFLP profiles would be expected in such case. The lowest proportion of homoplasy in the French dataset thus leads to the conclusion that recombination is the main origin of the observed genetic structure in Gansu.

The detection of recombination in the Gansu population agrees with a recent study focused on Tianshui area (Mboup et al 2009), as well as with experimental studies addressing the question of parasexuality in PST. Authors have described both fusion of germ tubes of urediospores and transfer of nuclei from one hyphal cell to another (Little and Manners 1969, Taylor 1976, Wright and Lennard 1980, Kang et al 1994a). Based on the detection of new pathotypes, heterokaryosis was assumed, even if nucleus recombination (parasexuality) remains unproven.

Genetic variability in French and Gansu populations.—First, the two populations were contrasted for their genetic structure, as higher genetic diversity, less linkage disequilibrium, and thus a higher genotypic diversity was found in Gansu. Almost no resampling of clones was observed in Gansu, strongly contrasting with the redundancy of samples observed in northwestern European populations. Second, the higher diversity of Gansu population was reflected by the number of polymorphic bands detected. A difference of nucleotide diversity of 20 between the two populations was found (TABLE IV). Hovmøller et al (2002) screened more than 250 combinations to find only 16 polymorphic primer combinations in northwestern European pathotypes. The same primers used with the Gansu population revealed 30 polymorphisms.

Because there is a primer selection bias for the European isolates, the genetic and genotypic diversity of the Gansu populations are most likely at least two orders of magnitude higher than the French ones. To better compare these diversities we estimated from AFLP data the effective size of populations (i.e. the size of an ideal panmictic population showing the same genetic diversity as the studied population) (TABLE IV). In a population submitted to temporal variations of its census size, the effective size, *Ne*, corresponded to the harmonic mean of the population sizes over all generations (Sjodin et al 2005). When populations are submitted to bottlenecks their effective size then will be near their minimum size. Thus in rust population subjected to recurrent bottlenecks in summer and winter *Ne* corresponds more or less to the number of individuals surviving these seasons. Assuming the same mutation rate in the two populations, the effective population size of European sample appeared to be 40 times smaller than the Gansu population and about 1000 times smaller when corrected for the initial bias in AFLP primer selection. Here the emphasis should be placed on the difference in sampling schemes used on the two continents. The description of the French population was based on a multi-annual sampling on a national scale and can be considered as representative of the genetic diversity of the northwestern European population over a 10 y period (Enjalbert et al 2005). The description of the Gansu population was based on a regional, single-year sampling. Thus the real difference in effective size of

French and Gansu PST populations most certainly was underestimated. Gansu has the highest diversity of pathotype compared to other provinces and is at the origin of most of new pathotypes (Wan et al 2004). However the higher polymorphism expected in all of China might further increase the contrast between European and Chinese difference in diversity at a continental scale.

When studying the genetic divergence between Gansu and French populations the presence of shared polymorphisms places the southern France isolates at an intermediate distance between Gansu and northernern France isolates. Because the southern French isolates were assumed to belong to a Mediterranean and Middle East genetic group (Stubbs 1985, Enjalbert et al 2005) this genetic differentiation can be related approximately to the geographic position of the populations. The genetic differences among these three origins will need further molecular and statistical analysis to provide better information on the time and scheme of divergence. Whether these origins are related to bread wheat diffusion during the initial spread of agriculture in the world or to secondary human migrations or trade, such as along the Silk Road between Europe and Asia (Stubbs 1985) remains to be determined.

Origin of the genetic diversity of Gansu population.—The high diversity of Gansu PST population can result from three different origins: year-around availability of wheat, presence of recombination and possible existence of an unknown sexual cycle. A single or a combination of several of these mechanisms can explain the amount of diversity found in Gansu.

First, Gansu is a highland where conditions favor the multiplication of rust in the summer. This occurs because of mild temperatures, high hygrometry and precipitation, and overlapping periods of cultivation in fields distributed at different altitudes and orientations (Wan et al 2007). This diversity of wheat developmental stages provides a green bridge (Zadoks 1984) that fosters rust survival in the summer. A higher effective population size during the summer, as opposed to the major bottleneck assumed in NW Europe, thus could explain the higher genetic diversity.

Second, the existence of recombination could lower the strength of the selective sweeps occurring in clonal populations. Sweeps are especially strong when there is a breakdown of a newly introduced specific resistance in cultivars. Note that the diversity of cultivars in Gansu also might favor the diversity of pathogen population as a result of disruptive selection, as opposed to the strong directional selection present in Europe (Bayles et al 2000, Hovmøller et al 2002). This host diversity on a small geographic scale also might favor parasexuality, aiding the encounter of different PST strains on a same leaf. Both low PST diversity and uniformity of host population then could explain why parasexual recombination has not been detected in European populations.

Third, if sexual reproduction is present in Gansu the great longevity of teliospores in rusts and the presence of a putative aecidious host could improve survival during the dry periods and maintain large populations. This would result in reducing the strength of the demographic bottleneck during over-summering. However the observation of an aecidian host for PST has not been reported so far and thus this hypothesis remains speculative.

Gansu is the most important over-summering zone for yellow rust in China on the basis of epidemiological studies (Wang et al 1963, 1986; Wang et al 1965; Xie et al 1994; Li et al 1997). The results reported here are in accordance with the hypothesis of a large effective size of the PST population sampled in this province, as well as with the presence of gene flows between the different areas. The question whether this diversity results mainly from the strong local survival in Gansu or from genetic exchanges with other areas conducive to PST survival needs to be confirmed through genetic studies at larger geographic scales. Molecular analyses also revealed that partial recombination may occur in this region even though the biological mechanisms for recombination remain to be documented. Note that asexual recombination is presently the leading hypothesis, due to the reports of heterokaryosis in PST (Kang et al 1993, 1994a, 1994b; Ma et al 1993; Wang et al 2004). Measuring the relative importance of the green bridge versus the existence of parasexuality is an important factor in the construction of integrated disease management in China.

ACKNOWLEDGMENTS

We thank Drs Jin Shelin and Cao Shiqin for their help in collecting rust samples as well as L. Gerard and N. Galet for their technical help during the spore propagation. The work was supported by the European BIOEXPLOIT FOOD-CT-2005-513959 project and in part by PRA and the National Basic Research Program (2006CB100203) of China.

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