Theor Appl Genet (1998) 97: 211–219

A. Gallois · J. C. Audran · M. Burrus

Assessment of genetic relationships and population discrimination among *Fagus sylvatica* L. by RAPD

Received: 9 January 1998 / Accepted: 25 February 1998

Abstract We assessed the genetic relationships between members of the *Fagaceae* family by RAPDs in order to better ascertain the taxonomic status of a very particular population of *Fagus sylvatica*, the 'tortuosa' variety. Intra- and inter-population Nei and Li's mean genetic distances were compared, and the genetic relationships between individuals were clarified on dendrograms by the Neighbor joining method. RAPD analysis was first conducted on three species from three genera, *Quercus petraea*, *Castanea sativa*, and *Fagus sylvatica*, in order to develop an efficient RAPD protocol. The variety level was then studied, and a general tendency of the individuals to cluster by variety was observed. Individuals also clustered by geographic locations, but the genetic distances between populations were not correlated to the distances between sites. Finally, we compared the common beech and 'tortuosa' varieties from two different locations, Verzy and Süntel. Both populations from one location were closer than the same variety from two sites. This last result is in agreement with those previously obtained with isozymes. Hypotheses concerning the origin of the 'tortuosa' variety are discussed.

Key words *Fagaceae* · *Fagus sylvatica* L. · RAPD analysis · Classification of individuals · Genetic relationships

Introduction

The 'tortuosa' beech (Pépin 1861) (Fig. 1a), a particular variety of *Fagus sylvatica* L., also called twisted beech (Thiébaut et al. 1993) or winding beech (Démesure et al. 1995), can only be found naturally in three places in the world: in Verzy near Reims (France, 49°14′N, 3°59′E, alt: 288 m.), in the Süntel mountains near Hanover (Germany, 52°12′N, 9°17′E, alt: 170–250 m.), and in Dalby-Söderskogs in southern Sweden (55°38′N, 13°19′E). These '*tortuosa*' trees exhibit a unique phenotype with a winding ramified trunk and twisted falling branches which form an umbrella-like crown. Common beech trees (Fig. 1b), on the other hand, have a vertical erected trunk that often remains undivided until the crown (Thiébaut et al. 1992, 1993). At all three sites, both populations of *F. sylvatica* coexist while keeping their morphological features constant, although intermediate forms have also been seen.

Though this unique phenotype has been studied for decades, little information is available on its genetic bases. The winding trait is genetically transmitted to the progeny: according to Mathieu (1863), cited by Rol (1955) and Boureux (1992), of the seeds harvested from a 'tortuosa' tree 60% give rise to 'tortuosa' forms and 40% to common and intermediate forms. The genetic diversity within *Fagus* has been investigated using allozymes (Démesure 1991; Démesure et al. 1992, 1995). The latter analyzed the genetic variation at 12 enzyme loci within and between different natural populations of common beech, 'tortuosa' beech, and other *Fagus* species. Their molecular results confirmed previous varietal identification based on morphology (Pépin 1861) and enabled a better description of the genetic structure of the population within *Fagus*, thus contributing to a better understanding of the evolution of the 'tortuosa' variety.

When compared to isozyme analysis, DNA-based strategies have the advantage of generating an almost unlimited quantity of markers that are independent of environment and developmental stages. It has been suggested that random amplified polymorphic DNA (RAPD) analyses (Williams et al. 1990; Welsh and McClelland 1990) generally show similar or higher

Communicated by P. M. A. Tigerstedt

A. Gallois · J. C. Audran · M. Burrus (✉)
Laboratoire de Biologie et Physiologie Végétales (EA 2069), URCA, BP 1039, 51687 Reims cedex 2, France
Fax: +33.3.26.05.32.79
e-mail: monique.burrus@univ-reims.fr

levels of polymorphism than other types of markers, like isozymes (Liu and Furnier 1993; Kremer et al. 1994). This can be explained by the more random and efficient genome sampling provided by RAPDs than by isozymes which are restricted to genes coding for proteins. Thus, RAPDs may be more useful than isozymes in detecting population differentiation and of greater value in taxonomic studies on species relationships (Demeke et al. 1992), closely related taxa (Kump and Javornik 1996; Kremer et al. 1994) and for genotype comparisons. They are also useful for detecting chromosomic mutations, such as inversion, deletion, or translocation, and genic mutations in clones regenerated from *in vitro* cultures (Brown et al. 1993; Yang and Schmidt 1994; Rani et al. 1995).

In the work described here, we have developed a RAPD protocol for members of the *Fagaceae* family, and we have studied RAPD variation among several populations of *Fagus sylvatica* in order to detect intraspecific polymorphism and to (1) distinguish among diverse *F. sylvatica* varieties and examine their genetic relationships, (2) assess the genetic diversity between different local populations of common beech, (3) compare the regional genetic divergence within populations of common and 'tortuosa' beech. This study will allow us to better ascertain the taxonomic status of the 'tortuosa' population among the *F. sylvatica* species.

Fig. 1 **a** A 'tortuosa' beech tree in the Verzy forest. **b** A common beech tree (*Fagus sylvatica* L.) in the Verzy forest. *Bar*: 2 m

Materials and methods

Plant material

Ten different populations of the *Fagaceae* family were analyzed (Table 1). Samples of *Quercus petraea* (Mattuschka) Liebl. and *Castanea sativa* Mill. were collected in Northern France (Verzy), as were samples of the 'purpurea' and the 'pendula' varieties of *F. sylvatica* (Reims) (Fig. 2). The populations of 'tortuosa' beech were collected in Verzy (Northern France) and in Süntel (Germany) (Fig. 2). Samples of common beech were collected in Northern France (Verzy and Hautvillers), in Central France (Tronçais), and in Germany (Süntel) (Fig. 2). For each population, 5 randomly chosen individuals were collected.

Fresh leaves or dormant buds from individual trees were collected in natural conditions, rinsed, frozen in liquid nitrogen, and stored at $-80°C$ until DNA extraction.

DNA isolation

The DNA for templates was isolated from the frozen material of individual plants using a CTAB-based method (Rogers and Bendich 1988) adapted to our material. Approximately 0.25–0.5 g of leaves or buds was ground in liquid nitrogen and transferred into a 25-ml centrifuge tube with 4 ml of extraction buffer (100 m*M* Tris-HCl, pH 8.0; 20 m*M* EDTA pH 8.0; 1.4 *M* NaCl; 2.5 µl/ml β-mercaptoethanol) and 1 ml of 10% CTAB. After a 30-min incubation at 65°C, the mixture was treated with an equal volume of cold chloroform and centrifuged at 10,000 *g* at room temperature for 20 min. The DNA was precipitated from the aqueous phase by mixing with 2.5 volumes of 95% ethanol ($-20°C$), pelleted by centrifugation (10,000 *g*; 10 min; $+4°C$), air-dried, and resuspended in 10 m*M*

Table 1 List of the different populations, their code, and their localization

Code	Abbreviation	Species	Variety	Geographical localization	Location on map
1	Qp	*Quercus petraea*		Verzy (Marne-France)	V
2	Cs	*Castanea sativa*		Verzy (Marne-France)	V
3	Fs	*Fagus sylvatica* L.		Verzy (Marne-France)	V
4	CoS	*Fagus sylvatica* L.		Süntel (Germany)	S
5	CoH	*Fagus sylvatica* L.		Hautvillers (Marne-France)	H
6	CoT	*Fagus sylvatica* L.		Tronçais (Allier-France)	T
7	Pu	*Fagus sylvatica* L.	'purpurea'	Reims (Marne-France)	R
8	Pe	*Fagus sylvatica* L.	'pendula'	Reims (Marne-France)	R
9	To	*Fagus sylvatica* L.	'tortuosa'	Verzy (Marne-France)	V
10	ToS	*Fagus sylvatica* L.	'tortuosa'	Süntel (Germany)	S

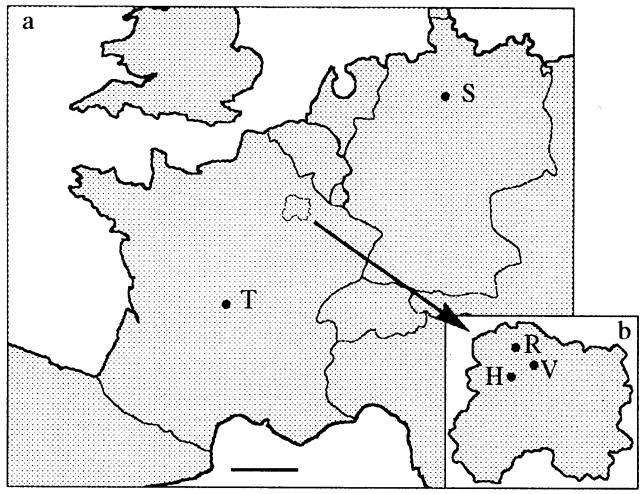

Fig. 2a, b Sampling locations in Western Europe (**a**) and in the department of Marne (France) (**b**). The geographical sites are Verzy (*V*), Hautvillers (*H*), Tronçais (*T*), Reims (*R*), and Süntel (*S*). *Bar*: 200 km

ammonium acetate. The DNA precipitation was repeated once. Finally, the DNA pellet was rinsed twice with cold 70% ethanol, air-dried, and resuspended in sterile distilled water.

The DNA concentration was estimated by spectrophotometry at 260 nm, and the purity was by the ratio of the absorbances at 260 and 280 nm.

DNA amplification

Primers were obtained from Bioprobe (same codes as Operon Technologies). In a final volume of 10 µl, amplification mixtures contained 10 m*M* Tris-HCl (pH 9.0), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.1% Triton X-100, 0.2 g/l gelatin, 0.1 m*M* of each dNTP, 0.3 µ*M* of a single primer, 0.13 unit of *Taq* polymerase (Appligène, France), and 20 ng of genomic DNA. The mixture was overlaid with 2 drops of mineral oil. The polymerase chain reaction (PCR) was run in a Thermal Cycler (Crocodile III, Appligène), the optimal program starting with 5 min at 93°C, followed by 40 cycles of 30 s at 91°C, 30 s at 36°C, 1 min at 70°C. The program terminated in one 5-min step at 70°C. Amplified samples were kept at 4°C until they were loaded, electrophoresed on 2.2% agarose ("Routine", Eurogentec) gels, and stained with ethidium bromide. Negative controls, i.e., the reaction mixture without template DNA, were run with each amplification. In all cases, pGEM Marker (Promega) was used as the size marker.

All primers used in this study were random sequence, 10-mer oligonucleotide primers with G + C contents of up to 60%. A total of 100 primers (Oligo Sets A, B, H, F, and S) were initially screened for amplification on *F. sylvatica*, using a four-individual sample of common and 'tortuosa' beech. Seventy-three primers which gave rise to amplified products and potential polymorphism were further studied.

Data scoring and analysis

We only compared RAPD profiles resulting from the same experiment (same primer and reaction mix, same amplification reaction and electrophoresis). For intraspecific variation studies, the DNA from all five randomly chosen individuals of the eight *F. sylvatica* populations were simultaneously amplified and run together on one agarose gel. Band scorage was carried out for all the individuals, but data concerning each survey were separately considered.

For each individual and for each primer that yielded a clear pattern, amplification products were scored as present (1) or absent (0). Pairwise comparisons of all the genotypes considered in each survey were used to estimate the genetic distance of Nei and Li (1979): $D = 1 - (2\ N_{xy}/N_x + N_y)$, where N_x and N_y are the number of fragments present in individuals (x) and (y) respectively, and N_{xy} is the number of fragments shared by both individuals.

In each survey, different populations were compared (Table 2). For each population of 5 individuals, the intra-population Nei and Li's mean genetic distance ($\pm$ standard deviation) was calculated; it corresponded to the mean genetic distance calculated between all individuals of a given population and was called intra-population distance. In the same way, the inter-population Nei and Li's mean genetic distances ($\pm$ standard deviation) were calculated for all the pair-wise populations of the survey. For each pair this consisted of calculating the mean of all genetic distances between the individuals of the first population and those of the second population; it was called inter-population distance for simplification purposes. In order to verify whether two populations were genetically distant, the intra-population distances of both populations were compared to the corresponding inter-population distance using the statistical *t*-test. If two intra-population distances were significantly smaller than the corresponding inter-population distance, then both populations were significantly genetically distant.

Dendrograms were constructed using the Neighbor-joining method (NEIGHBOR software-PHYLIP package) and the Nei and Li's distance matrix.

Primer selection

Primer selection was done in experiments C and D (Table 2) and was based on two criteria, as previously suggested by Moreau (1993):
1) primers that allowed the generation of one or more "discriminating fragments". When analyzing two populations (a) and (b), we called "discriminating fragments" the fragments for which: $|n_a - n_b| \geq 4$, with: n_a = number of individuals of population (a) that possess the fragment, n_b = number of individuals of population (b) that possess the fragment.
2) primers which were the "most discriminant". For each primer and for each comparison of four populations (Table 2, experiments C and D), we calculated the four intra-population distances and the six inter-population distances as previously described. We compared each intra-population distance with the corresponding inter-population distances. Twelve comparisons were made. When, in at least 9 out of the 12 comparisons, the intra-population distances were smaller than the corresponding inter-population genetic distances, the primer was considered as being discriminating and was selected for further analysis.

Selection was performed using either one or both criteria, depending on the best clustering of the populations.

Results and discussion

RAPD analysis of three members of the *Fagaceae* family

In order to evaluate the ability of RAPD to assess genetic diversity and to distinguish among *F. sylvatica* varieties, we primarily worked on the establishment of standard experimental conditions that would allow reliable separation of distant members of the *Fagaceae*

Table 2 Intra- and inter-population mean distances of Nei and Li calculated from pairwise comparisons of all corresponding individuals. Twenty-three primers were used for species comparison and 59 primers for all the other comparisons

Code	Species	Mean Nei and Li's distance (± SD)	Number of primers used
A. Species comparison			
1	*Quercus petraea*	0.320 (± 0.047)	23
2	*Castanea sativa*	0.181 (± 0.027)	
3	*Fagus sylvatica* L.	0.275 (± 0.059)	
1/2	*Quercus*/*Castanea*	0.756 (± 0.032)	
1/3	*Quercus*/*Fagus*	0.833 (± 0.020)	
2/3	*Castanea*/*Fagus*	0.807 (± 0.021)	
B. Variety comparison			
3	*Fagus sylvatica* L. Verzy	0.230 (± 0.044)	59
6	*Fagus sylvatica* L. Tronçais	0.228 (± 0.022)	
9	*Fagus sylvatica* L. 'tortuosa'	0.228 (± 0.022)	
7	*Fagus sylvatica* L. 'purpurea'	0.188 (± 0.020)	
8	*Fagus sylvatica* L. 'pendula'	0.166 (± 0.033)	
3/6	*Fagus sylvatica* Verzy/*Fagus sylvatica* Tronçais	0.250 (± 0.026)	
3/9	*F. sylvatica* Verzy/*F. sylvatica* 'tortuosa'	0.250 (± 0.026)	
3/7	*F. sylvatica* Verzy/*F. sylvatica* 'purpurea'	0.245 (± 0.020)	
3/8	*F. sylvatica* Verzy/*F. sylvatica* 'pendula'	0.285 (± 0.026)	
6/9	*F. sylvatica* Tronçais/*F. sylvatica* 'tortuosa'	0.254 (± 0.028)	
6/7	*F. sylvatica* Tronçais/*F. sylvatica* 'purpurea'	0.228 (± 0.027)	
6/8	*F. sylvatica* Tronçais/*F. sylvatica* 'pendula'	0.283 (± 0.040)	
9/7	*F. sylvatica* 'tortuosa'/*F. sylvatica* 'purpurea'	0.242 (± 0.019)	
9/8	*F. sylvatica* 'tortuosa'/*F. sylvatica* 'pendula'	0.290 (± 0.023)	
7/8	*F. sylvatica* 'purpurea'/*F. sylvatica* 'pendula'	0.262 (± 0.032)	
C. Location comparison			
3	Verzy	0.230 (± 0.044)	
4	Süntel	0.254 (± 0.024)	
5	Hautvillers	0.198 (± 0.019)	
6	Tronçais	0.222 (± 0.054)	
3/4	Verzy/Süntel	0.267 (± 0.023)	
3/5	Verzy/Hautvillers	0.225 (± 0.027)	
3/6	Verzy/Tronçais	0.254 (± 0.036)	
4/5	Süntel/Hautvillers	0.256 (± 0.019)	
4/6	Süntel/Tronçais	0.266 (± 0.029)	
5/6	Hautvillers/Tronçais	0.232 (± 0.033	
D. Verzy/Süntel comparison			
3	*Fagus sylvatica* L. from Verzy	0.230 (± 0.044)	
4	*Fagus sylvatica* L. from Süntel	0.254 (± 0.024)	
9	*Fagus sylvatica* L. 'tortuosa' from Verzy	0.228 (± 0.022)	
10	*Fagus sylvatica* L. 'tortuosa' from Süntel	0.227 (± 0.014)	
3/4	*F. sylvatica* L. Verzy/*F. sylvatica* L. Süntel	0.267 (± 0.023)	
3/9	*F. sylvatica* L. Verzy/'tortuosa' Verzy	0.250 (± 0.026)	
3/10	*F. sylvatica* L. Verzy/'tortuosa' Süntel	0.278 (± 0.019)	
4/9	*F. sylvatica* L. Süntel/'tortuosa' Verzy	0.266 (± 0.021)	
4/10	*F. sylvatica* L. Süntel/'tortuosa' Süntel	0.248 (± 0.025)	
9/10	'tortuosa' Verzy/'tortuosa' Süntel	0.273 (± 0.022)	

family: *Quercus petraea* Liebl., *Castanea sativa* Mill., and *Fagus sylvatica* L. (populations nos. 1, 2 and 3, Table 1).

We tested 39 primers from the A, B, H and S kits, of which 23 generated PCR products with a clear pattern for all of the 15 individuals studied. A typical amplification pattern is shown in Fig. 3. RAPD fragments generated in each genus with each primer were so similar from individual to individual within one genus and so different from genus to genus that, just by looking at the gels, we could identify all the individuals by genus even without any dendrogram.

In order to establish the genetic relationships between the species, we pooled data from all the 23 primers, which allowed the generation of 350 amplification fragments. Among these, 2 (0.6%) were monomorphic and 75 (21.4%) seemed specific to one population, i.e., they were amplified in all the individuals of one population but not elsewhere (Fig. 3). Twenty-seven fragments (7.7%) appeared to be specific to *Fagus*

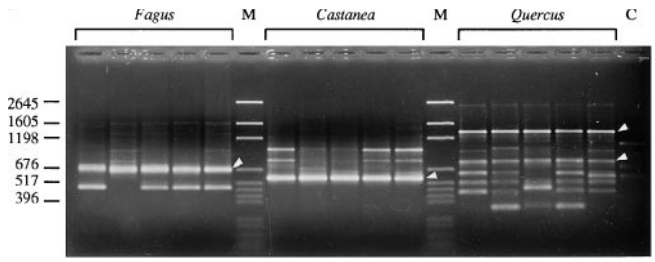

Fig. 3 Typical ethidium bromide-stained agarose gel of amplification products. This profile was obtained with primer H12 and 5 individuals each of *Fagus sylvatica* L., of *Castanea sativa* Mill., and of *Quercus petraea* Liebl. (populations nos. 1, 2, 3). *Arrows* Fragments specific to the species, *C* negative control, *M* DNA molecular-weight-marker pGEM Markers (Promega)

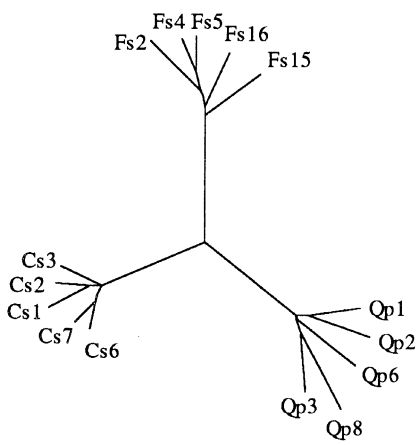

Fig. 4 Dendrogram showing the genetic relationships between *Fagus sylvatica* L. (*Fs*), *Castanea sativa* (*Cs*), and *Quercus petraea* (*Qp*) classified by banding pattern using 23 primers (populations nos. 1, 2, 3)

sylvatica, 26 fragments (7.4%) to *Castanea sativa*, and 22 fragments (6.3%) to *Quercus petraea*. Within each taxon, the genetic divergence was limited, as indicated by the low intra-species distances that ranged from 0.181 ± 0.027 to 0.320 ± 0.047 (Table 2). The inter-species distances, varying from 0.756 ± 0.032 to 0.833 ± 0.020, were always significantly higher than the corresponding intra-species distances ($P = 0.001$). These populations could be considered as being significantly different. Actually, on the dendrogram (Fig. 4), each species constituted a cluster without overlap and separated by a great distance.

The protocol developed in this preliminary study was efficient in enabling easy discrimination of the species of three different genera with a low number of primers. In the same way, with 14 primers, Whitty et al. (1994) discriminated various species belonging to three genera of the *Cassiinae* tribe. In yet another comparison of higher plant taxa, Graham and McNicol (1995) using 10 primers separated 13 different species of *Rubus* into three known subgenera.

RAPD analysis among varieties of *Fagus sylvatica*

In many cases, RAPDs allow discrimination between varieties within a species (see Morell et al. 1995) and are useful for the establishment of genetic relationships at this taxonomic level. In order to determine the position of the 'tortuosa' population among the *Fagus sylvatica* species, we compared the distances between the common beech, the 'tortuosa', the 'purpurea' and the 'pendula' varieties with 59 primers that gave a clear scorable pattern. The common beech populations of Verzy and of Tronçais (France) were studied in order to highlight possible gene exchange between the common beech and the 'tortuosa' populations of Verzy.

A total of 692 fragments were generated; 116 fragments (16.8%) were monomorphic and 2 (0.3%) seemed to be population-specific. One was exclusively observed in the common beech of Verzy (fragment H02-$_{435}$), and 1 in the 'pendula' variety (H12-$_{428}$). Confirmation, however, should be made before they are used as variety markers.

Table 2 presents genetic distance estimations using all the 59 primers. The intra-population distances varied from 0.166 ± 0.033 for the 'pendula' variety to 0.230 ± 0.044 for the common beech population of Verzy. The inter-population distances varied from 0.228 ± 0.027 to 0.290 ± 0.023. The *t*-test analysis indicated that the distances between the 'pendula' variety and all four other populations were significantly higher than the corresponding intra-population distances ($P = 0.001$). A similar observation was made between the 'tortuosa' population and the common beeches of Tronçais. On the contrary, the genetic distances between the other populations were not significantly different from their intra-population distances.

A clear discrimination between several populations was observed on the dendrogram made with 59 unselected primers (Fig. 5). The 'pendula' population formed a group well apart from the other populations without any overlap. Another cluster was made of two distinct groups, one with 4 Verzy common beeches and the second with 4 'tortuosa' individuals. Four 'purpurea' trees also clustered, as well as 4 common beeches of Tronçais. Three individuals formed the last cluster although from different populations, a common beech tree of Verzy (Fs 15) with a 'tortuosa' tree (To 1), and a common beech tree of Tronçais (CoT 5). Selection of the most discriminant primers, here 35, led to the same clustering and did not change the topography of the dendrogram (data not shown).

This study indicates that the 'pendula' variety is effectively the most distant one of the five populations, whereas the common beech, the 'purpurea' and the 'tortuosa' varieties share high similarities. It also shows that the common beech populations of Verzy and of Tronçais do not form a homogenous group. The common beech trees of Verzy appear closer to the 'tortuosa' population than to the Tronçais population, although

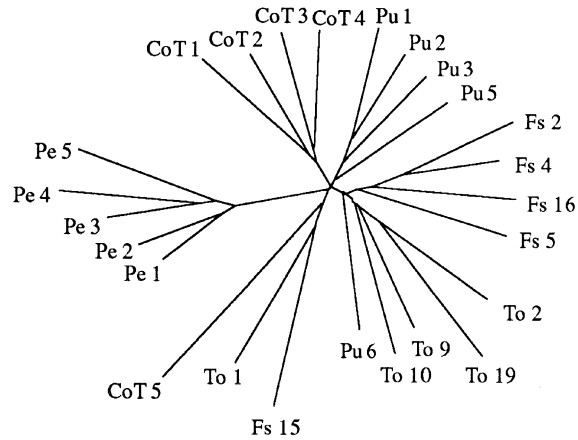

Fig. 5 Dendrogram showing the genetic relationships between varieties of *Fagus sylvatica* classified by banding pattern using 59 primers. *Pe* 'pendula', *CoT* common beech of Tronçais, *Pu* 'purpurea', *Fs* common beech of Verzy, *To* 'tortuosa' (populations nos. 3, 6, 7, 8, 9)

both populations belong to the same taxon. It is interesting to note the clustering of Fs 15 with To 1: 'tortuosa' trees are known to produce progeny of the common beech phenotype as well as of the 'tortuosa' phenotype (Matthieu 1865 cited by Rol 1955; Lange 1974). Fs 15 could have been part of the progeny of a 'tortuosa' tree; it would then have a higher similarity with the gene pool of the 'tortuosa' variety while still presenting the 435-bp band found previously in all five common beeches of Verzy and no morphological differences with *F. sylvatica* L.

The clustering of these populations required at least 35 discriminant primers. This number is rather high when compared to studies on other species. For instance, Novy et al. (1994) could discriminate 17 cranberry varieties with 22 primers, and Tschammer and Zyprian (1994) could differentiate most of the 22 grapevine varieties with 20 primers; Iqbal et al. (1995) confirmed the classification of *Rhododendron* species and varieties with only 10 primers. The high number of discriminant primers required in our study may be explained by the very tight relationships between the 'tortuosa' population and the common beech group compared to those between the other varieties. By eliminating the common beech population of Verzy from our survey, we were able to discriminate among the remaining four populations with only 22 primers (data not shown). Furthermore, it is not general practice to analyze five individuals within each population; the general tendency which is found in the literature is 1 specimen per variety or bulked DNA. Although studying populations of 5 specimens increases the variability within each population and tends to complicate variety clustering, these clusters fit better to reality.

From this analysis, we may conclude that there seems to be a geographical effect of Verzy that overcomes the distinction between the 'tortuosa' variety and the common beech.

RAPD analysis of *F. sylvatica* L. from different geographical sites

In order to further investigate the geographical effects, we compared specimens of *F. sylvatica* L. sampled from four different locations: Tronçais, Verzy and the neighboring site of Hautvillers chosen because of its absence of any 'tortuosa' tree, and Süntel (Germany), which also has mixed populations of common beech and 'tortuosa'. The study was carried out with the same 59 primers used previously. These generated 687 fragments: 121 (17.6%) were monomorphic and none was specific to one geographical site.

Genetic distances obtained with all 59 primers are presented in Table 2. Intra-population distances ranged from 0.198 ± 0.019 for the population of Hautvillers to 0.254 ± 0.024 for the German population, and inter-population distances ranged from 0.225 ± 0.027 to 0.267 ± 0.023. As expected, geographical effect was less important than variety effect: the *t*-test analysis showed no significant difference between intra- and inter-population distances ($P = 0.05$), probably because of the high intra-population distances and high standard deviations of several populations, Süntel for instance, compared to the inter-population distances.

Genetic relationships are theoretically expected to fit reality better when established with RAPD data obtained from all informative primers rather than from selected primers. Therefore, the calculation of genetic relationships between the four geographical sites was performed with all 59 primers, but proper site discrimination required primer selection because of the tight relationships between the studied populations. Thirty-one primers that allowed the generation of at least 1 discriminating fragment (criterion no. 1) were kept for site analysis, these generated 469 fragments. Figure 6 shows the dendrogram obtained in these conditions. A clustering of all the trees of Tronçais was observed, and this clustering was well apart from the other populations. In this group, 1 individual, CoT 5, appeared relatively distant from the other trees of Tronçais, thus increasing the values of intra-population distances and standard deviation of this population (Table 2). The 5 trees of Hautvillers also clustered. The two Tronçais and Hautvillers groups were the most distant ones on the dendrogram. Four trees of Verzy formed a cluster, and 3 individuals of Süntel (CoS 4, CoS 6 and CoS 2) another one. The other trees remained unclustered (CoS 1, Fs 15 and CoS 8). The tendency of clustering by geographical site was significant when considering Hautvillers, Tronçais and, to some extent, Verzy, but it was less important for Süntel. The grouping of Süntel was not clear because this population was

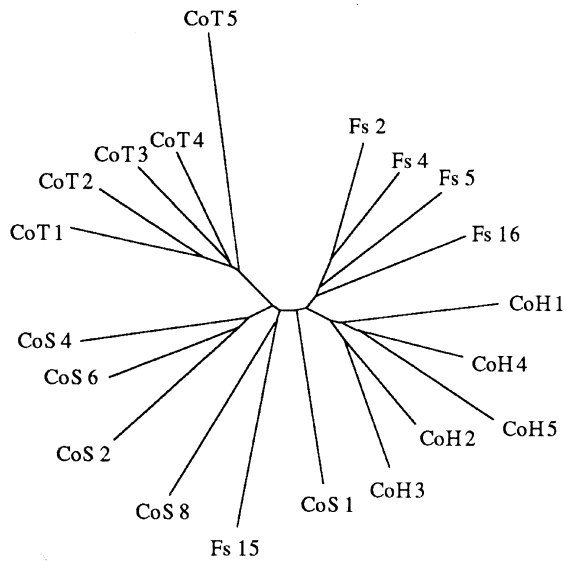

Fig. 6 Dendrogram for four populations of *Fagus sylvatica* L. sampled in different locations and classified by banding pattern using 31 primers. *CoT* Common beech of Tronçais, *CoS* common beech of Süntel, *Fs* common beech of Verzy, *CoH* common beech of Hautvillers (populations nos. 3, 4, 5, 6)

heterogeneous and the individuals were further apart from each other, as already shown by the intra-population distance.

Grouping germplasm according to geographical origin has often been achieved through RAPDs (M'Ribu and Hilu 1994; Balakrishna 1995; Kump and Javornik 1996) and, generally, RAPDs permit populations from different geographical sites to be discriminated even if few or no specific fragments are found. In our study, some geographical clustering was observed after primer selection, but we also observed that the dendrogram constructed with 59 informative primers (data not shown) was not much different from the dendrogram constructed from 31 selected primers: primer selection modified the position of only 2 out of 20 individuals, which were initially misclustered from 59 primers. Similarly, when studying two oak species, Moreau (1993) observed the same clustering using data from all informative primers or from the most discriminating selected primers.

It is striking that the geographical distance is not correlated with the genetic distance. On the dendrogram, the populations of Verzy and of Hautvillers, less than 12 km distant from each other, clustered separately without any overlap. Surprisingly, the genetic distance between the populations of Verzy and of Süntel, separated by approximately 600 km, was not really higher than the genetic distance between Verzy and Hautvillers. The genetic distance between Tronçais and Verzy was higher than the genetic distance between Verzy and Süntel, although Verzy and Tronçais are 300 km apart. A common characteristic of Verzy and Süntel is the presence of 'tortuosa' individuals within the common beech populations, whereas, in Hautvillers and Tronçais, there is no known 'tortuosa' tree. The tight relationship between Verzy and Süntel could be explained by:

1) cross-pollination and hybridization between 'tortuosa' and common beech trees, cross-pollination being the most frequent mode of fertilization in *F. sylvatica* (Thiébaut and Vernet 1981), leading to some genetic information in common between them, as obvious in the Fs 15 tree;

2) when considering the independent occurrence of the 'tortuosa' variety at both sites (Démesure et al. 1995), a similar gene plasticity and a closer gene pool, leading to the emergence of the same mutational event. The impact of the 'tortuosa' genetic background in the common beech population of Verzy and of Süntel should be verified in the third site with mixed populations of common beech and 'tortuosa' trees, Dalby-Söderskogs (Sweden).

Comparison between the common beech and the 'tortuosa' variety of Verzy and those of Süntel

In order to further clarify the genetic relationships between the common beech and the 'tortuosa' trees, we more precisely compared the populations originating from Verzy and Süntel, both sites being known for their mixed populations. Fifty-nine unselected primers gave 685 scorable fragments, of which 118 (17.2%) were monomorphic and none was specific to any population.

Results of the genetic distances are in Table 2. Intra-population distances varied from 0.227 ± 0.014 for the 'tortuosa' population of Süntel to 0.254 ± 0.024 for the common beech population of Süntel. Inter-population distances varied from 0.248 ± 0.025 to 0.278 ± 0.019. The statistical analysis showed that only twice were the inter-population distances significantly different from the corresponding intra-population distances: the 'tortuosa' population of Verzy was significantly different from the 'tortuosa' population of Süntel ($P = 0.001$) and the 'tortuosa' group of Süntel was different from the common beech group of Verzy ($P = 0.001$). With all the 59 initial primers in the other cases, no significant difference was observed between the populations. In particular, no difference was observed between the common beech and the 'tortuosa' populations within a same site.

In order to improve the population separation, we selected 25 primers according to both criteria described in the Materials and methods: 358 scorable fragments were obtained. The results are presented on Fig. 7. Three major clusters were observed. All the five 'tortuosa' individuals of Süntel clustered well apart in the dendrogram. Far distant from this group were two other clusters, one made of four 'tortuosa' trees of Verzy and, nearby, the second one made of four common beech trees of Verzy. For the remaining individuals, the situation was less clear: three common

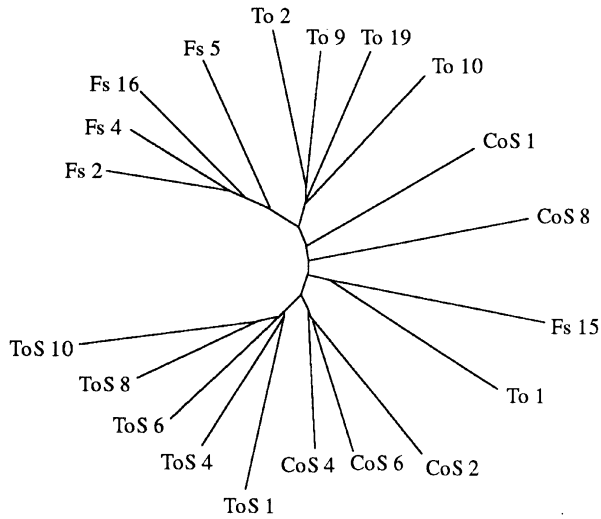

Fig. 7 Dendrogram for two populations of *Fagus sylvatica* L. and two 'tortuosa' populations of Verzy and of Süntel classified by banding pattern using 25 primers. *Fs* Common beech of Verzy, *CoS* common beech of Süntel, *To* 'tortuosa' of Verzy, *ToS* 'tortuosa' of Süntel (populations nos. 3, 4, 9, 10)

beech of Süntel constituted a subcluster close to the 'tortuosa' cluster of the same location. The four remaining individuals (To 1, Fs 15, CoS 8 and CoS 1) were distant from all clusters and could be considered as unclustered. As already seen previously, the heterogeneity of the Süntel common beech population hindered clear clustering. In spite of this uncompleted clustering, two main groups could be distinguished: they corresponded to geographical locations (common beech and 'tortuosa' of Verzy on the one hand, and 'tortuosa' and common beech of Süntel on the other hand), which were more easily discriminated than the varieties.

This study emphasizes the very tight relationship between *Fagus sylvatica* L. and its 'tortuosa' variety within each site and confirms previous data obtained by Démesure et al. (1995): using isozyme analysis, they found the same allozymic fragments in common beech and 'tortuosa' and different ones in other species. Our results also indicate a genetic difference between a given variety within both sites: the 'tortuosa' population of Verzy clusters separately from the population of Süntel. A similar observation was made with the common beech populations. Démesure et al. (1995) were also able to discriminate between the populations of Verzy and Süntel on the basis of their location and observed clustering of the common beech and the 'tortuosa' populations of each site.

Analysis of genetic relationships may suggest hypotheses on the origin and the appearance of the 'tortuosa' variety. An important mingling exists among the common beech and the 'tortuosa' populations within given geographical sites. While keeping more or less their genomic features since they could be easily differentiated by pairwise analysis, common and 'tortuosa' varieties share a significant part of their genome. This similarity, more important than similarities among a given variety taken from both geographical sites, invalidates the hypothesis of a recent tree transfer from Verzy to Süntel, as has been suggested (Laplace and Masson 1979; Metz 1989). Actually, in the hypothesis of a transport of a few 'tortuosa' specimens during the Middle Ages, and given the high longevity of these trees, a few generations only would have existed since the transport. We would then expect to see a greater similarity between both populations of 'tortuosa' and a greater difference between the local common beech population and this introduced 'tortuosa' population. Another proposed hypothesis suggests that identical spontaneous mutations in at least two different sites would have occurred, leading to the appearance of the 'tortuosa' variety (Démesure et al. 1995). Such an event is statistically highly improbable. In the Verzy forest, the 'tortuosa' phenotype has been described in two other species, *Castanea sativa* and *Quercus petraea*. Furthermore, 3 'tortuosa' specimens out of the 800 'tortuosa' specimens of Verzy are in fact chimeras with a trunk of 'tortuosa' phenotype and a main branch featuring stable common beech traits. These observations suggest that we are probably not in the presence of a spontaneous mutation. We hypothesize that an infectious agent (bacteria, virus...) could have stably infected these trees, thus leading to the 'tortuosa' character.

Population discrimination through RAPD with a small number of selected primers will be useful for establishing the genotype of *in vitro*-regenerated plants (Fontaine 1995; Druelle 1996), as another program of *in vitro* propagation of the 'tortuosa' variety has been undertaken in our lab.

In conclusion, we have shown that while the common beech and 'tortuosa' are very closely related varieties they can be discriminated by our DNA-based technique, which indicated a genetic difference between these two distinct phenotypes. Up to now, many hypotheses on the 'tortuosa' phenotype origin have been expressed, from soil influence to virus contamination. As yet, no clear response is available, but we can now ensure that the genome is involved in the differences between common beech and 'tortuosa' varieties. Further investigations, such as the search then the identification of a specific 'tortuosa' marker, would be needed to clearly specify the gene(s) involved in the 'tortuosa' phenotype.

Acknowledgements We thank Dr. P. Devaux (Florimond Desprez) for critically reading the drafts of this article. This work was funded by the French MESR.

References

Balakrishna P (1995) Evaluation of intra-specific variability in *Avicennia marina* Forsk. using RAPD markers. Curr Sci 69: 926–929

Boureux JP (1992) Les Faux de Verzy: naissance, développement et maintien d'une légende. In: Parc Naturel Régional de la Montagne de Reims (eds) Sites et paysages. Reims, pp 184–191

Brown PTH, Lange FD, Kranz E, Lörz H (1993) Analysis of single protoplasts and regenerated plants by PCR and RAPD technology. Mol Gen Genet 237 : 311–317

Demeke T, Adams RP, Chibbar R (1992) Potential taxonomic use of random amplified polymorphic DNA (RAPD): a case study in *Brassica*. Theor Appl Genet 84 : 990–994

Démesure B (1991) Les Faux de Verzy (Marne). DEA University of Bordeaux III, France

Démesure B, Comps B, Thiébaut B (1992) Les faux de Verzy, aspects génétiques. In: Parc Naturel Régional de la Montagne de Reims (eds) Sites et paysages. Reims, pp 138–144

Démesure B, Comps B, Thiébaut B, Barrière G, Letouzey J (1995) Les hêtres tortillards en Europe occidentale. Aspects génétiques. Ann Sci For 52 : 103–115

Druelle JL (1996) Ontogenèse *in situ* des bourgeons du hêtre commun (*Fagus sylvatica* L.) et du hêtre tortillard de Verzy (*Fagus sylvatica* L. var *tortuosa* Pépin). Application à la régénération *in vitro*. PhD thesis, University of Reims Champagne-Ardenne, France

Fontaine F (1995) Micropropagation du hêtre commun (*Fagus sylvatica* L.) et du hêtre tortillard (*Fagus sylvatica* L. var 'tortuosa' Pépin). MSc thesis, University of Technology, Compiègne, France

Graham J, McNicol RJ (1995) An examination of the ability of RAPD markers to determine the relationships within and between *Rubus* species. Theor Appl Genet 90 : 1128–1132

Iqbal MJ, Paden DW, Rayburn AL (1995) Assessment of genetic relationships among rhododendron species, varieties and hybrids by RAPD analysis. Sci Hortic 63 : 215–223

Kremer A, Durel CE, Petit R, Villar M (1994) Marqueurs moléculaires et génétique des arbres forestiers. Biofutur 131 : 17–23

Kump B, Javornik B (1996) Evaluation of genetic variability among common buckwheat (*Fagopyrum esculentum* Moench) populations by RAPD markers. Plant Sci 114 : 149–158

Lange F (1974) Morphologische untersuchunegen an des Süntelbuche. Mitt Dtsch Dendrol Ges 67 : 24–44

Laplace Y, Masson M (1979) Les Faux de Verzy. CRDP, Reims, France

Liu Z, Furnier GR (1993) Comparison of allozyme, RFLP, and RAPD markers for revealing genetic variation within and between trembling aspen and bigtooth aspen. Theor Appl Genet 87 : 97–105

M'Ribu HK, Hilu KW (1994) Detection of interspecific and intraspecific variation in *Panicum* millets through random amplified polymorphic DNA. Theor Appl Genet 88 : 412–416

Metz R (1989) Des arbres pas comme les autres : les faux de Verzy. Arborescences 20 : 9–13

Moreau F (1993) Recherche de marqueurs appliquée à la différenciation moléculaire entre chêne sessile (*Quercus petraea*) et pédonculé (*Quercus robur*). PhD thesis, University of Poitiers, France

Morell MK, Peakall R, Appels R, Preston LR, Lloyd HL (1995) DNA profiling techniques for plant variety identification. Aust J Exp Agric 35 : 807–819

Nei M, Li W-H (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76 : 5269–5273

Novy RG, Kobak C, Goffreda J, Vorsa N (1994) RAPDs identify varietal misclassification and regional divergence in cranberry (*Vaccinium macrocarpon* (Ait.) Pursh). Theor Appl Genet 88 : 1004–1010

Pépin (1861) Les hêtres tortueux de Verzy. Rev Hortic Paris

Rani V, Parida A, Raina SN (1995) Random amplified polymorphic DNA (RAPD) markers for genetic analysis in micropropagated plants of *Populus deltoides* Marsh. Plant Cell Rep 14 : 459–462

Rogers SO, Bendich AJ (1988) Extraction of DNA from plant tissues. In: Gelvin SB, Schilperoort RA, Verma DS (eds) Plant molecular biology manual. Kluwer Academic Publ, Dordrecht, pp 1–10

Rol R (1955) Les Faux de Verzy. Bull Soc Bot Fr 102 : 25–29

Thiébaut B, Vernet P (1981) Biologie de la reproduction sexuée. In: INRA (eds), Le Hêtre. INRA, Paris, pp 198–205

Thiébaut B, Bujon P, Comps B, Mercier J (1992) Développement réitératif des hêtres tortillards (*Fagus sylvatica* L. var 'tortuosa' Pépin). CR Acad Sci Paris 315 ser 3 : 213–219

Thiébaut B, Bujon P, Haddad S, Comps B, Mercier J (1993) Morphologie et architecture des faux de Verzy ou hêtres tortillards (*Fagus sylvatica* L. var 'tortuosa' Pépin). Can J Bot 71 : 848–862

Tschammer J, Zyprian E (1994) Molecular characterization of grapevine cultivars of Riesling-type and closely related Burgundies. Vitis 33 : 249–250

Welsh J, McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res 18 : 7213–7218

Whitty PW, Powell W, Sprent JI (1994) Molecular separation of genera in *Cassiinae* (*Leguminosae*) and analysis of variation in the nodulating species of *Chamaecrista*. Mol Ecol 3 : 507–515

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18 : 6531–6535

Yang H, Schmidt H (1994) Selection of a mutant from adventitious shoots formed in X-ray treated cherry leaves and differenciation of standard and mutant with RAPD. Euphytica 77 : 89–92