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GENETIC VARIATION IN THE *MEDICAGO SATIVA* COMPLEX

B. Julier, Ch. Huyghe, P. Guy and M.L. Crochemore[1]

ABSTRACT

Historically, theoretical and experimental studies were developed on quantitative genetic of tetraploids, based on lucerne. Genetic variation for agronomic, morphological, biochemical and molecular traits is under study. Results are presented on the variation within the *Medicago sativa* complex for such traits. Using agronomic and morphological traits with Principal Component Analysis, two groups of populations were separated, the first one with all the tetraploid, cultivated populations from the subspecies *sativa*, the second one with all the wild populations, diploid or tetraploid, from both subspecies *sativa* and *falcata*. The content in medicagenic acid, part of the main anti-nutritional saponin, is much lower in the pure *sativa* populations than in the *falcata* populations, and the *sativa* populations hybridised with *falcata* types had intermediate level. The lab is now focused on nutritive value of lucerne. It appears that stem digestibility, relatively to dry matter yield, is higher in wild than in cultivated populations. Molecular markers such as RAPD were used to study the relationships between populations. The approach developed here was based on population specific bands, so each population was represented by the mixed DNA of 30 individuals. Genetic distances were calculated, and trees drawn. The distinction between the populations from the two subspecies *sativa* and *falcata* was clear. However, the classification within the *sativa* subspecies was not related to the genetic origins or the agronomic characters of the populations. However, this study shows that RAPD markers could be used in variety distinction, as specific bands were found.

Key words: *Medicago*, genetic diversity, *falcata-sativa* complex, forage quality, molecular markers.

INTRODUCTION

The "lucerne" lab of INRA Station d'Amélioration des Plantes Fourragères of Lusignan (France) is now focused on genetic variability of the perennial lucernes from the *Medicago sativa* complex, and on forage quality. However, the lab had previously studied plant growth in the canopy, genetic variability and quantitative genetics of tetraploids. Subsequently, the past works on genetics will be described. Then, results obtained on a wide range of populations with morphological, agronomic and chemical characters will be presented. At the moment, perennial and tetraploid lucerne populations are under study, using agronomic, molecular and physico-chemical traits, and this work will be described.

I. THE HISTORY OF THE RESEARCH

Tetraploid genetic

Polyploidy is frequent in cultivated species, especially in forage crops. Lucerne is a good plant model for studies of genetics. Several works in USA and above all in Europe (Demarly, Gallais, Guy at Lusignan in France, Rotili, Zannone at Lodi in Italy) were carried out to develop methods for tetraploid crop breeding. These studies are now old, and can seem difficult to understand.

A good variety is a good association of genes and functions in a heterozygous structure. In lucerne, the heterozygosity is responsible for vigour and seed production of the cultivars (Guy, 1987). A good variety model allows, starting with a population of genitors, to build the most possible

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heterozygous variety. Research at Lodi and Lusignan was complementary and gave tools to the breeders.

We studied theoretically and experimentally the proportion of vigour and yield due to heterozygosity, in order to determine the level of inbreeding of the parents, and the optimal number of parents in a synthetic variety. At Lusignan, we worked on the notion of structure (Guy, 1966) that is the partition between the different genotypic states, generalised to the whole genome. The expected value of a quantitative trait *Y* results from a linear equation between the structure and the weighted value of the different states (m_1, m_2, m_3, m_4, m_5):

$$Y = m_1 y_1 + m_2 y_2 + m_3 y_3 + m_4 y_4 + m_5 y_5$$

with the parameters y_1-y_5 defining the structure.

Accepting a small lost of information, they can be substituted by synthetical parameters: *F* is the inbreeding coefficient by Kempthorne, *P* is the isoaction coefficient (Guy, 1972) and *N* is the mean number of alleles per locus and per plant (Guy, 1972).

In the 60's and 70's, we mainly worked on inbreeding, parentage, that is on identity. The molecular markers now allows to approach more to the genome, and to work on isoaction. Probably, the matrix of relationships between identity and isoaction (Gillois, 1966; Guy, 1972) and every similar approach might be useful to geneticist and to breeder. Let us remind that identity, isoaction and allele richness(Ψ) are linked by:

$$1 - P = (1 - \Psi)(1 - F)$$

For a long time, the inbreeding coefficient *F* of Kempthorne was a reference, but the parameters that allow to evaluate the number of alleles per locus and its evolution in the various breeding systems (inbreeding, crosses, random mating) should be developed (Guy, 1966, 1968, Rotili et Guy, 1991). Experimentally, the number of alleles per locus better takes into account the differences in vigour, dry matter yield between the variety models than *F*.

The lucerne canopy

The understanding of plant canopy functioning requires the description of the structure of the canopy in its 3 components, stems, leaves and roots. The assimilate partitioning between roots and aerial parts depends on genotype and climate (temperature, photoperiod). These environmental factors also determine the growth rate of the canopy, for one regrowth cycle, for one year and for successive years. Even if the growth analysis, by regrowth cycle, was extensively studied, with the research of invariant relationships and of genetic variability, a global analysis is necessary to take into account the cutting regime and the interaction between cuttings.

French research was focused on 2 variety types, Flamande (Flemish) and Provence, originating from north and south of France, respectively. In the 80's, we progressively widen the genetic variability in 3 directions: the cultivated forms (cultivars and landraces), types adapted to pasture with a prostrate growth, the *Medicago sativa* complex with wild forms from the sub-species *sativa*, *falcata*, *tunetana* and *glomerata*. The genetic variability available in all these populations was described. We found a large variation for growth, and a relationship between the regrowth, the plant morphology and the winter dormancy. Indeed, 3 types of populations were identified. The type 'Flamand' had a slow regrowth after a cutting followed by a growth rate rather high, an early entrance in dormancy in autumn, and a « horizontal » morphology (a small height for a leaf area index of 1). Cv Europe and Julus belong to this group. The Mediterranean type adapted to mild winter had no winter dormancy, was cold susceptible and had a « vertical » morphology (tall plants for a leaf area index of 1). Cv Totana, Demnat and Gabès are of this type. The Mediterranean type adapted to cold winter, as cv Lodi, Equipe, Aragon, Victoria, had an important regrowth in summer and autumn, a late winter dormancy and a rather good winter resistance.

Forage quality

The various possible uses of lucerne forage are direct animal feeding, and also industrial treatments: dehydration and now protein extraction, perhaps fiber extraction tomorrow. Regarding these different uses, it is better to refer to characters rather than to quality. For polygastric animals, the main characters were protein content and are now digestibility (% of metabolisable energy in the forage), and intake. The improvement of these characters are necessary for the feeding of highly productive animals. For monogastric animals, we need to add to the former characters the low medicagenic acid content, as this component of some saponins is an anti-nutritional factor.

We improved the protein content by breeding. The cv Lutèce, Sitel and Alizé have 1% more protein than the control Europe. We improved the cutting regime for dehydration. A breeding program is in progress to release a Flamande variety with low medicagenic acid content.

We studied the intake with sheep, and looked for genotype effect through stem thickness. A genetic variability was observed for intake, without relationship with stem thickness. The cv Lutèce and the experimental genotype 63-28P were the most interesting. The feeding of dairy cows with Europe or 63-28P proved than 63-28P allowed a milk production improved by 1 litre per day and per cow (Emile *et al.*, 1993). A strong relationship was found between dry matter production and digestibility (Guy *et al.*, 1971; Lemaire et Allirand, 1993). The genetic variation for this relationship is under study. It clearly appears that the improvement of digestibility has to be done with a control of dry matter yield.

2. Genetic variability for agronomic and quality traits in diploid and tetraploid perennial lucerne population of M. sativa complex

The *M. sativa* complex shows a large genetic variability due to both natural and human selection under various climates and locations. Its main 2 sub-species, *sativa* and *falcata* show very different morphological traits. The ssp *sativa* has purple flowers, a tap root, an erect growth habit, coiled pods and no winter dormancy. The ssp *falcata* has yellow flowers, fasciculate roots, a prostrate growth habit, sickle-shape pods, strong winter hardiness and winter dormancy (Quiros & Bauchan, 1988). In both sub-species, diploid and tetraploid forms exist, and cultivated or wild populations were observed. For ages, and at the same ploidy level, hybridisation have occurred between *sativa* and *falcata* (Lesins & Lesins, 1979). The combination of an erect growth habit and winter hardiness is necessary for cultivation in regions with cold winters.

The study concerned the genetic variability observed among diploid or tetraploid, wild or cultivated populations from *M. sativa* complex. We recorded morphological and agronomic traits, and measured saponin content and digestibility. More data on the same populations are presented in Julier *et al.* (1995 a, b).

Material and methods

Twenty-five populations were studied. In the ssp *sativa*, 5 French landraces and varieties (Flamande, Europe, Marais de Luçon, Provence, Magali), 3 populations from Morocco (Dem3, Pool5, D15), 2 landraces from Tunisia (Gabès, Maktar), 4 Spanish landraces (Ampurdan, Aragon, Tierra de Campos, Mediterraneo) and 3 Spanish wild Mielga (Pancrudo, Villanueva de Jara, Monte-Oscuro), and one diploid *coerulea* population were chosen. In the ssp *falcata*, a Canadian diploid variety (Anik), 2 French wild tetraploid populations (Malzeville, Maron), an Ukrainian tetraploid population (Krasnokutskaya), and 2 Russian diploid populations (*romanica*, *quasifalcata*) were studied.

The agronomic evaluation was made at Lusignan. In 1993, 21 populations were planted in nursery, and, in 1993 and 1994, observations on growth habit, stem size, leaf area, inflorescence shape, regrowth scores, diameter of the plants, plant height, % of rhizomatous plants, flower colour and winter survival were recorded. Flowering date, mean seed weight, pod set and number of seeds per pod were evaluated. The 25 populations were sown in 1993 in a 2-row plots design with 4 randomised blocks. Two cutting regimes were applied, one with normal cutting dates, the other with

early cutting dates. Dry matter yield and stand height under the two cutting regimes were recorded. All these characters showed a population effect in a variance analysis, and were used in Principal Component Analysis (PCA) to investigate the relationships between the populations and to structure the variability in groups.

The populations were analysed for saponin content and medicagenic acid content (responsible for the anti-nutritional effect of lucerne on monogastric animals), and stem digestibility was evaluated. Leaves and stem samples, originating from the 2-row plot trial, were used for saponin titration, and digestibility, respectively. Digestibility was compared to yield production, as the 2 characters are negatively correlated.

Results

In the 1993 nursery, the PCA gave 2 components accounting for 72.5 and 11.7% of variation respectively. The first component was explained by regrowth scores, growth habit, stem size, inflorescence shape, plant heights, number of seeds per pod and mean seed weight. The second component was explained by winter survival (Fig. 1). Two groups were isolated, one composed of *sativa* populations, the other of wild populations, *sativa* or *falcata*. The Ukrainian *falcata* population was intermediate between the 2 groups, and *glomerata* had lower regrowth scores and plant heights than the other wild populations. On average, populations from the south of the Mediterranean sea had lower frost resistance than French landraces and varieties. Among the wild populations, *sativa* Mielga populations could not be distinguished from the others, except by flower colour (data not shown) that were purple for the Mielga and yellow for the *falcata*.

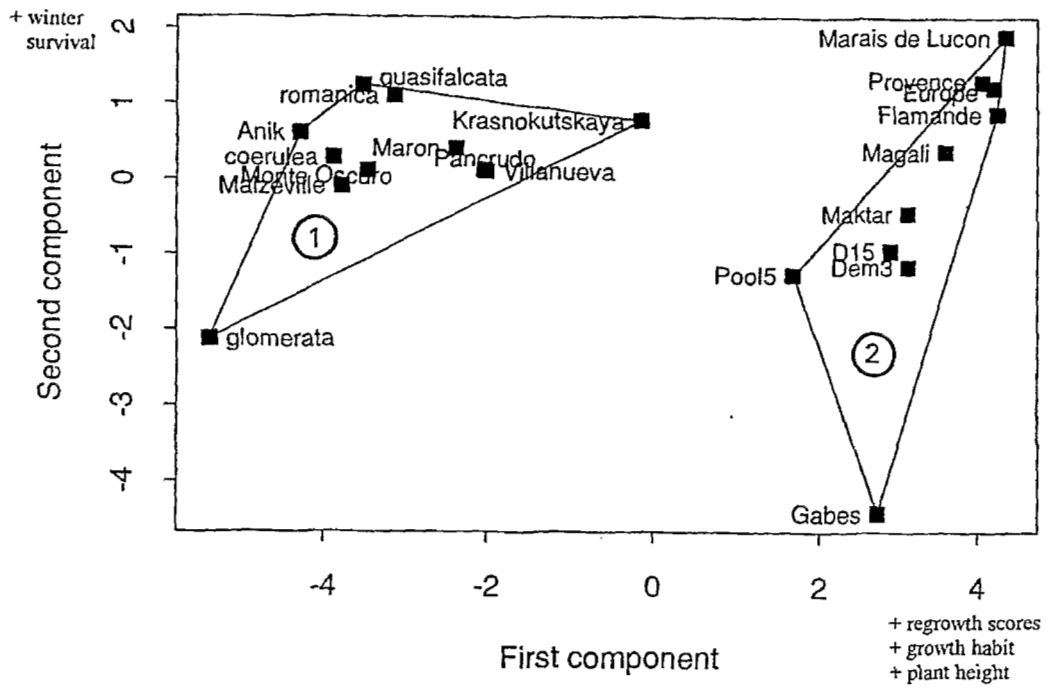

Figure 1. PCA diagram on the 1-2 plane of 21 populations studied in 1993 and 1994 in the 1993 nursery. 1 and 2 are the 2 groups shown by partition from Euclidian distances on the components of PCA.

In the 2-row plot trial, the first 2 components of the PCA made on yield and height measurements explained 81.1 and 14.2% of the variation respectively. All height measurements and yield per cutting except on the first cutting contributed to the first component. The second component was positively correlated to yield of the first cutting in both regimes, and negatively to yield of autumn cutting. Two main groups appeared on the PCA diagram (Fig. 2), one with the cultivated *sativa*

populations that were high yielding and tall, the other with the wild populations that were lower yielding and shorter. Within the cultivated populations, the French populations adapted to a cool climate were separated from the Mediterranean populations because of their higher yield in the first cutting and lower relative yield in autumn.

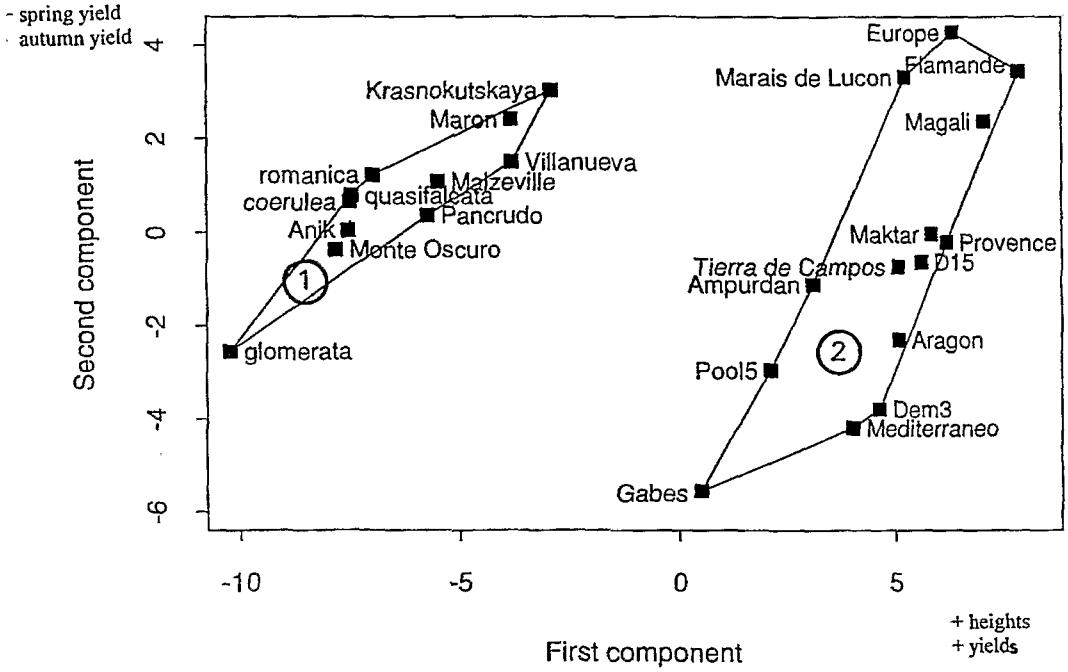

Figure 2. PCA diagram on the 1-2 plane of 25 populations studied in 1994 in the 1993 2-rows plot design. 1 and 2 are the 2 groups shown by partition from Euclidian distances on the components of PCA.

The saponin content varied from 3.79 for Gabès to 5.60% for Dem3 (Table 1), but the population effect was not significant. However, the medicagenic acid content showed a population effect. In average, the *falcata* populations had more medicagenic acid than all the *sativa* populations (wild or cultivated) (Table 1). Among the *sativa*, the populations from the south of the Mediterranean Sea and the wild Mielga populations from Spain had lower medicagenic acid contents than the cultivated populations from France and Spain.

Stem digestibility was negatively correlated to dry matter yield (Fig. 3). However, the wild populations (*falcata* and *sativa*) had higher digestibilities than expected according to their yield, and the cultivated *sativa* populations were less digestible.

Discussion

Whatever the morphological and agronomic characters used to describe the populations, 2 main groups of populations were separated. The first group contained the French, Spanish and North-African varieties and landraces, all of them from the ssp *sativa*. They are characterised by high yield, tall plants, erect growth habit and quick regrowth after cutting. Under the climate of Lusignan, the French populations yielded more than the Mediterranean ones, and had purple flowers variegated with yellow instead of pure purple. The Mediterranean populations produced a larger proportion of their annual yield in autumn than the other cultivated populations. The second group contained wild *falcata* and wild *sativa* populations, including the Canadian *falcata* population Anik, and were characterised by reduced growth, low forage yield especially in autumn, and prostrate growth habit. The *glomerata* population was close to this group. The *falcata* Krasnokutskaya tended to be intermediate between the 2 groups, because of better regrowth scores and higher yield than the remaining *falcata* populations. The Mielga populations could not be distinguished morphologically and agronomically from the *falcata*, except by the flower colour.

Table 1. Content in total saponins and in medicagenic acid, in % of leaf dry matter, for 25 lucerne populations

	Ssp, use, ploidy	Total saponin	Medicagenic acid
Flamande	*sativa*, cultivated, 4x	4.39	0.109
Europe	"	5.29	0.173
Provence	"	4.58	0.147
Marais de Luçon	"	4.75	0.305
Magali	"	4.60	0.145
D15	"	4.53	0.060
Dem3	"	5.60	0.033
Pool5	"	4.87	0.030
Gabès	"	3.79	0.119
Maktar	"	4.37	0.088
Aragon	"	5.11	0.244
Mediterraneo	"	4.88	0.140
Tierra de Campos	"	4.96	0.225
Ampurdan	"	4.11	0.227
Villanueva	*sativa*, wild, 4x	3.84	0.093
Monte Oscuro	"	4.44	0.043
Pancrudo	"	4.23	0.094
coerulea	*sativa*, wild, 2x	4.76	0.097
Maron	*falcata*, wild, 4x	4.96	0.402
Malzeville	"	5.12	0.277
Krasnokutskaya	"	5.05	0.373
Anik	*falcata*, cultivated, 2x	5.13	0.333
romanica	*falcata*, wild, 2x	3.94	0.417
quasifalcata	"	4.55	0.444
glomerata	*glomerata*, wild, 2x	4.62	0.134
Mean *sativa*		4.65	0.134
Mean *falcata*		4.80	0.350
Subspecies effect		NS	***
Standard Error		0.84	0.090

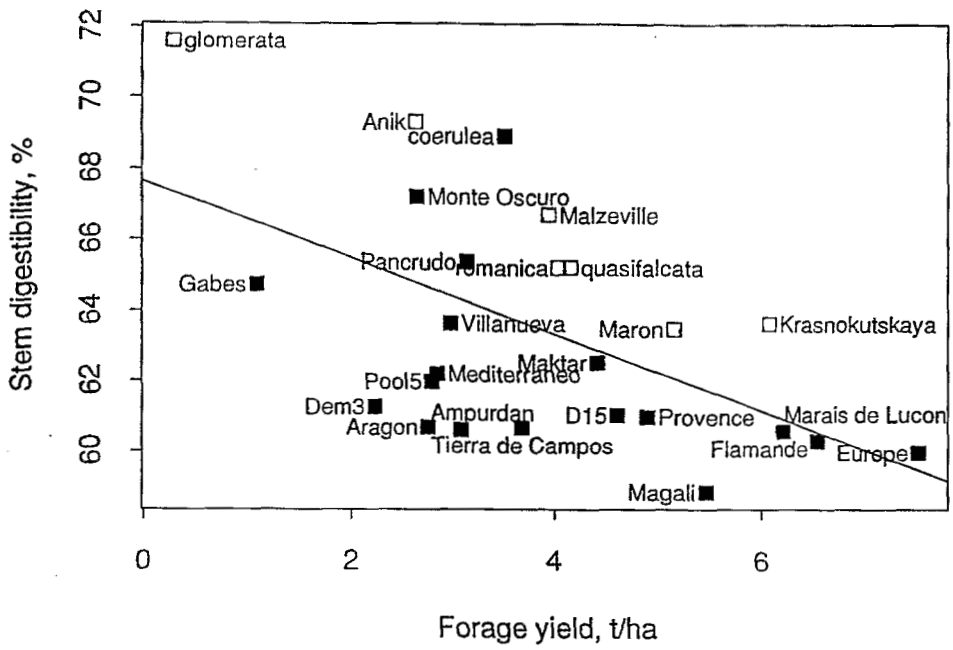

Figure 3. Relationship between stem digestibility and forage yield for 25 populations studied in 2-rows plot design.

The total saponin content was independent of the genetic origin of the populations. At the opposite, for the medicagenic acid content, there was a range from the low content of the *sativa* populations from Maghreb and wild Spain populations, to the high content of the *falcata* populations, with the intermediate of the French and Spanish cultivated populations.

The *falcata* and wild *sativa* populations were more digestible relatively to dry matter yield than cultivated *sativa* populations. This feature could be interesting in breeding programs to improve forage digestibility, and nutritive value of the lucerne.

The variegated flower colour of the French populations, and their intermediate content in medicagenic acid provides evidence for the genetic introgression of the *falcata* germplasm in these *sativa* populations. The crosses could have occurred in Northern and Eastern France and Europe where both sub-species coexist. The frost resistance of the *falcata* was useful for adaptation of lucerne to northern climates of Europe, and the erect growth habit, the high productivity and the low medicagenic acid content of the *sativa* was useful for cultivation and use. The large variation for medicagenic acid offers prospect to reduce the levels of this anti-nutritional factors in cultivars.

3. GENETIC VARIABILITY IN PERENNIAL TETRAPLOID LUCERNE POPULATIONS STUDIED WITH MORPHOLOGICAL, BIOCHEMICAL AND MOLECULAR CHARACTERS

A study is presently carried out on the genetic variability available in the complex *sativa* - *falcata*, and focuses on the tetraploid level. This study uses three types of characters.

The morphological characters aim to describe the structure and the growth of the plants during the first two growing cycles during two consecutive years, the year of planting and the next one. This part of the study involved 47 populations (Table 2) covering the widest possible range available in the complex at the tetraploïd level. The experimental design included three replications. In each replication, 30 spaced plants were analyzed for each population. The very preliminary analysis carried on the data showed that the wild populations involved in the study were behaving very differently from the rest of the material showing prostrate growth habit, long winter dormancy and slow regrowth after the cuts. But, it was difficult to separate the wild *falcata* from the wild *sativa* (Mielga). In the cultivated populations, the classical gradient of winter dormancy was found. These analyses will be carried on further in the next months.

In the previous experimental design, one stem per plant was sampled at each cut. The 30 stems per replication were gathered. After grinding, the powder will be analyzed by NIRS (Near Infra-Red Spectrophotometry). A whole spectrum of 350 wavelengths will be collected per population, per replicate and per cut. A prediction equation has been calibrated for predicting the leaf / stem ratio and this character will be taken into account in the first approach. The 350 values of reflectance obtained for the 350 wavelengths will be analyzed as different characters. The first step in the analysis will be to summarize the information available in the 350 characters through the research of the principal components, the number of principal components being the ones witch allow to represent 95% of the variation available in the original matrix of data. The second step will consist in the analysis of these components as independent characters through a factorial discriminant analysis, the grouping factor being the populations, and the individuals of the analysis being the replicates x cuts.

The third approach of this study is the research of Random Amplified Polymorphic DNA markers. This approach was carried on a subsample of 24 populations chosen among the original 47 populations (Table 2). Two strategies were compared. In a first strategy, called Individual Based Analysis (IBA), 30 plants per population were analysed. The DNA was extracted from each plant and the PCR (Polymerase Chain Reaction) was performed on each plant with 4 primers which were known to be polymorphic in the *Medicago sativa - falcata* complex. Out of a series of more than 100 polymorphic bands, 29 were selected to be reproducible and used for the subsequent analyses. The frequencies of occurrence of the bands for each population were calculated and a multiple correspondence factorial analysis was performed on the frequencies. The dendrogram obtained from a clustering analysis performed after this factorial analysis did not produced any pattern which could have been expected from the type of plant material involved in the study. The reason for this is the importance of the within-population variability compared to the between-population variability. The

molecular data will be reanalysed with the calculation of mean within-population diversity and mean between-population diversity as carried out on other species with a wide within-population genetic variability (Baruffi *et al.*, 1995).

Table 2. Origin of the 47 populations, landraces or cultivars studied with morphological and biochemical characters. (*) indicates the 24 populations studied with RAPD markers

Continent	Country		Population name	Subspecies	Type
Europe	Chypre		Chypre	*sativa*	landrace
	France	*	Flamande	*sativa*	landrace
			Provence	*sativa*	landrace
		*	Marais de Luçon	*sativa*	landrace
		*	Maron	*falcata*	wild
		*	Malzeville	*falcata*	wild
		*	Magali	*sativa*	variety
		*	Europe	*sativa*	variety
			Medalfa	*sativa*	variety
		*	Rival	*sativa*	variety
			Résis	*sativa*	variety
			Maya	*sativa*	variety
			Allegro	*sativa*	variety
		*	Orca	*sativa*	variety
		*	63-28P	*sativa*	experimental cv
		*	Luzelle	*sativa*	variety
		*	Coussouls	*sativa*	variety
			27-48	*sativa*	experimental cv
	Germany		Altfranken-Schmidt-Steinbach	*sativa*	variety
	Greece		Lamia	*sativa*	variety
	Italy	*	Lodi	*sativa*	variety
	Russia		Radouga	*sativa*	variety
	Spain		Villa Nueva	*sativa*	wild
		*	Monte Oscuro	*sativa*	wild
			Aragon	*sativa*	variety
			Ampurdan	*sativa*	variety
			Tierra de Campos	*sativa*	variety
		*	Mediterraneo	*sativa*	variety
			Totana	*sativa*	variety
	Sweden		Julus	*sativa*	variety
	Turkey		Kayserie	*sativa*	variety
	Ukrain		Krasnokutskaya	*falcata*	wild
Africa	Egypt	*	Sewa	*sativa*	variety
	Morocco	*	Pool3	*sativa*	landrace
		*	Pool5	*sativa*	pool
	Soudan	*	Higazi	*sativa*	landrace
	Tunisia	*	Gabès	*sativa*	landrace
America	Brasil		Crioula	*sativa*	variety
	Canada	*	Sabre	*sativa*	variety
			Victory	*sativa*	variety
			Rhizoma	*sativa*	variety
	U.S.A.	*	CUF101	*sativa*	variety
			Alfagraze	*sativa*	variety
		*	WL514	*sativa*	variety
Asia	China		"falcata"	*falcata*	wild
	India		2929	*sativa*	landrace
	Japan	*	Natsuwakaba	*sativa*	variety

An other strategy was developed which was looking for Population Specific Bands (PSB). For this purpose, aliquote DNA samples for each of the 30 plants were gathered to produce an unique DNA sample per population. This bulk of DNA was analysed with 60 primers. Thirty-three primers out of the 60 proved to provide polymorphic and reproducible patterns of bands. A screening of the bands was performed to select the highly reproducible polymorphism and this yielded in 103 markers. The comparison between both strategies (IBA and PSB) showed that as soon as a reproducible marker was present in more than 20% of the plants, it was shown in the bulked DNA. Distance matrices were calculated on the data. Two types of distances were calculated, the Jaccard distance and the Sokal and Michener distance or its transformation into a Nei's distance. Both yielded very similar results, the difference between these two measurements being the fact to take (Sokal and Michener) or not (Jaccard) into account the case when a band is absent in both populations as a common band. Figure 4 shows the dendrogram obtained by the Unweighted Pair Grouped Method Average of aggregation (UPGMA) on the Sokal and Michener distance. This shows that the wild *falcata* populations (Malzeville and Maron) and the Mielga population (Monte Oscuro) involved in that study are very different and from the rest of the populations. Within the rest of the tree, some associations are found as expected, such as Flamande and Europe, Julus and Luzelle, Sewa and Higazi. Some associations were not expected such as Lucon, a French landrace and Pool 5, a Moroccan landrace, Lodi and CUF101 or Orca, an old French variety and Sabre, a Canadian variety. To test the stability of the phenogram, a bootstrapping technique was used (Felsenstein, 1985) and yielded in a majority rule consensus tree (Fig. 5). This tree obtained with 30 bootstraps showed the stability of the position of the *falcata* populations and the relative stability of that of the Mielga as well as the relative stability of the associations refereed to above. This shows that the associations are found in about one third of the bootstraps which means that a few specific markers exist for each population and for each of the associations while a lot of markers are available to separate the sub-species. Such a feature was already observed in other plant and animal species.

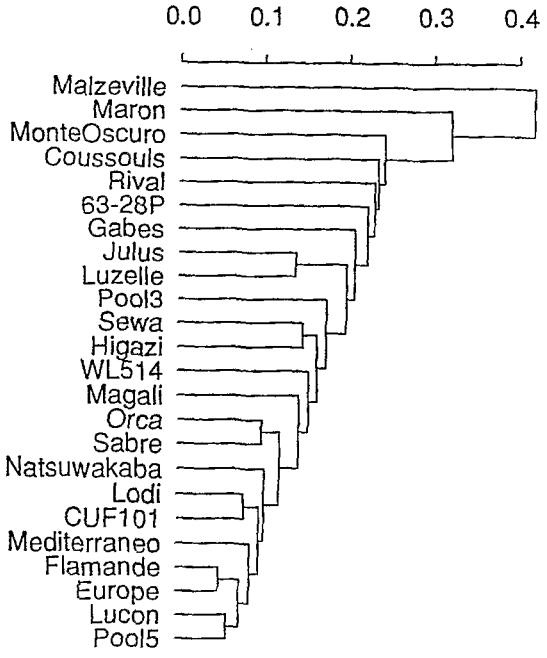

Figure 4. Tree obtained with Sokal et Michener distance, and UPGMA aggregation.

An other method of aggregation, the Neighbour Joining tree method developed by Saitou and Nei (1987) was used. This method allows to draw phylogenetic trees and to identify likely progenitors of populations. Interestingly, the *falcata* populations appears to be associated (Fig. 6) with the only populations where plants with yellow flowers have been identified, namely Julus and Luzelle. The Mielga population appeared somehow associated with Magali, a Provence-type variety. Orca which was previously associated with Sabre is now completely separated from the rest of the material which would suggest that this variety belongs to a different genetic group. The analyses on the three types of markers will be carried on and the relationships between the different distance matrices obtained from the different types of markers will be studied.

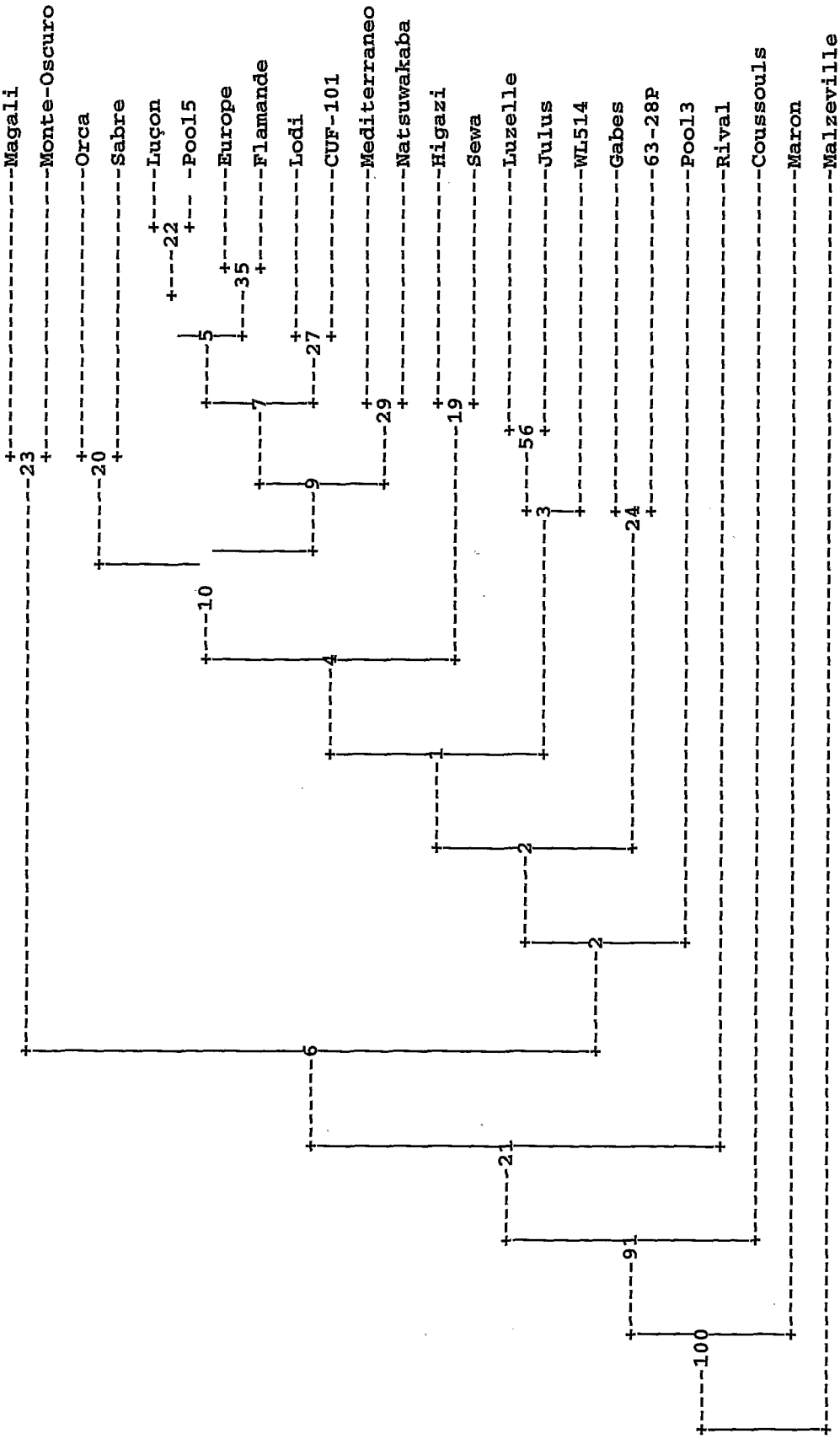

Figure 5. Majority consensus tree, obtained with 100 bootstraps, based on the Sokal and Michener distances, with UPGMA aggregation. The number of forks indicate the number of times consisting of the populations which are to the right of that fork occurred among the trees.

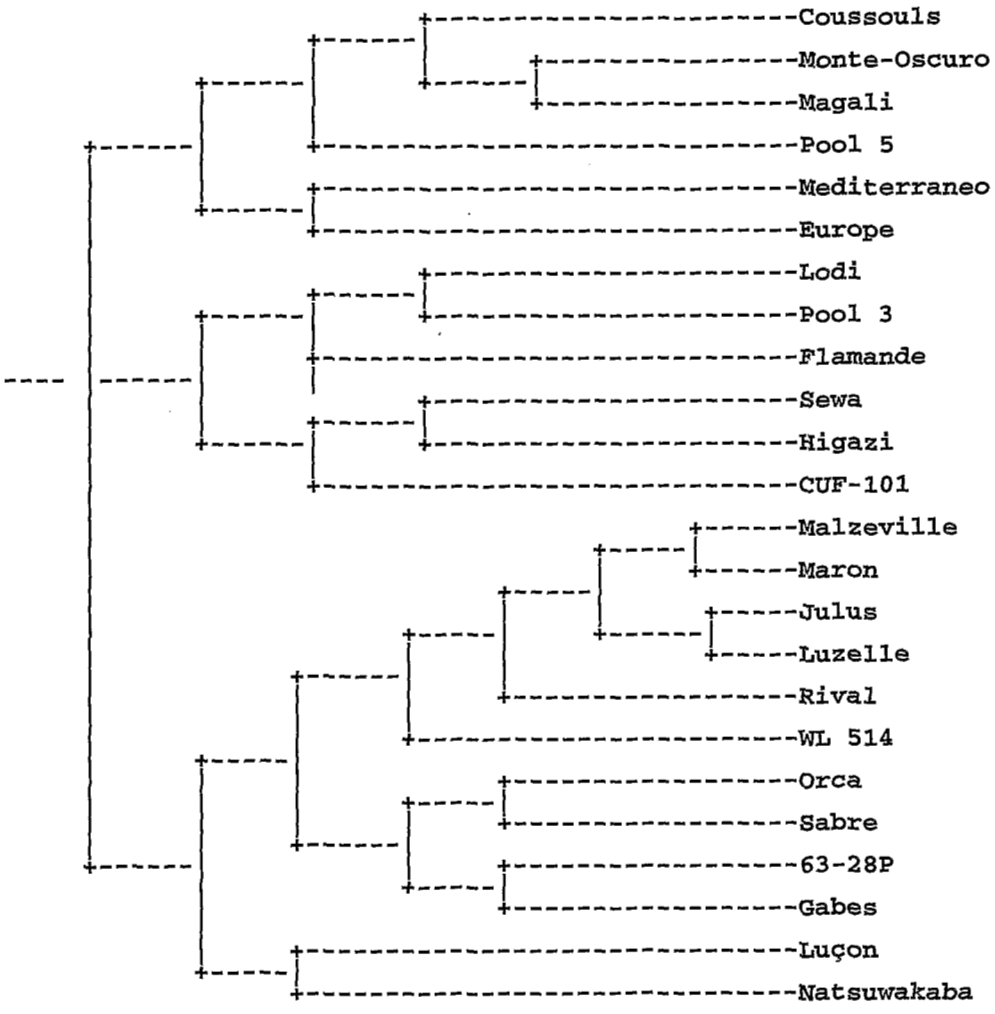

Figure 6. Tree obtained with Sokal et Michener distances, and Neighbor-Joining aggregation.

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