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GENETIC DIVERSITY, PRESERVATION AND USE OF GENETIC RESOURCES OF MEDITERRANEAN LEGUMES: ALFALFA AND MEDICS

J.M. Prosperi[1], M. Angevain[2], I. Bonnin[2], E. Chaulet[2], G. Genier[2], E. Jenczewski[2], I. Olivieri[2] and J. Ronfort[1]

ABSTRACT

The aim of the INRA (French National Institute of Agricultural Research) Montpellier - genetic resources and lucerne plant breeding laboratory is to select useful cultivars for rangelands improvement, reseeding perennial pastures or introducing forage crops in cultivated lands (grazing vineyards or rotations with cereals) in the dry and cold Mediterranean regions. These new cultivars must provide an excellent adaptation to the natural environment and farming system. Local genetic resources and especially wild ecotypes are the basis of the variability usable for these plant breeding programs. Priority is given to the genus *Medicago,* especially because the Mediterranean basin is the center of diversification of this genus. Using agronomical and biomolecular tools (electrophoresis, RAPD, RFLP...), the researches focus on the study of the biodiversity of these species (distribution, ecology, ecotypic adaptation, population biology), the evolution and relationship between species or between wild and cultivated origins within *Medicago sativa*, relationship between the main characteristics of dry matter production, rhythms of growth, seed production and perennially.

Key words: *Medicago*, annual medics, *Medicago sativa*, genetic diversity, molecular markers, natural populations, genetic resources, biogeography

1. INTRODUCTION

Rearing in marginal lands is an old component of agricultural systems in mediterranean areas, and represents nowadays the main way to valorize and to maintain such lands in good condition, favourable to other activities such as spare time activities. What is needed to maintain rearing, and particularly sheep-farming, in the mediterranean zones, is fodder types or varieties which are able to produce high-quality feeding all over seasons associated with other forage resources such as rangelands. Improvement programs are thus based on the research of persistent forage plants, perennial or annual self-reseeding types, adapted to grazing and showing a good cultivation flexibility and quick establishment ability. To help breeders to achieve such improvement programs, our aim is to supply and to introduce wild genetic resources of fodder plants in breeding schemes.

In this context, a great part of our work is concerned with the collection and the evaluation of wild ecotypes, the estimation of their own value and the study of how particular environments are associated with particular ecotypes. This leads to precise the main factors of environmental adaptation (Prosperi *et al.,* 1989). Such investigations should not only contribute to increase the genetic variability used in breeding programs (Prosperi *et al.*, 1987; Prosperi, 1989; Mansat, 1989), but should also help to preserve the existing genetic resources from diverse mediterranean areas.

1.1. The different types of genetic resources in fodder species

Genetic resources in fodder species can be classified in three main groups (Prosperi, 1987).

(1) cultivated populations, either traditional (i.e. local populations maintained by farmers) or derived from breeding programs (cultivars or lines). These populations are maintained according to

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[1] Laboratoire de Ressources Génétiques et d'Amélioration des Luzernes Méditerranéennes, INRA- Domaine de Melgueil, 34130 Mauguio FRANCE. Tel: 33/ 67 29 06 04. Fax: 33/ 67 29 39 90.

[2] Laboratoire de Génétique et Environnement, Institut des Sciences de l'Evolution URA 327, Université Montpellier II, CC 065, 34095 Montpellier Cedex. FRANCE Tel: 33/ 67 14 49 52. Fax: 33/ 67 04 20 32.

well-defined and strict rules by seed producers so that phenotypic and genotypic characteristics are expected to be preserved.

(2) populations originating from natural meadows or partially controlled environments (fallows, or roadsides). Human influence on these plants may vary, several [cycles of] multiplications occurring classically in natural conditions. In most cases, these populations are composed of several origins so that their natural preservation remains hazardous.

(3) Wild, spontaneous populations, generally not to much abundant and met in un-controlled areas. Their *in situ* conservation is highly dependent on the maintenance of their habitat.

What must be pointed out is that, for these different genetic resources, the available diversity, but also the management as well as the conservation strategies are not the same.

1.2. The *Medicago* genus: taxonomy and geographic distribution

It is well known that the center of origin of the *Medicago* genus is the Fertile Crescent. One thousand and three hundred years B.C., alfalfa was already cultivated in Turkey but its common use in Europe started with the Roman colonization (Genier *et al.*, 1992) in spite of its former introduction in West Mediterranean Basin by Phenicians. The species was cultivated all around the world at the end of the XVIII century (Michaud *et al.*, 1988) and has been selected later in many countries.

Beside the perennial tetraploid allogamous cultivated alfalfa (*M. sativa*), many other species of this genus which could be of great interest are known:

- 20 perennial, either di or tetra -ploid, with more or less outcrossing pollination,

- 34 annual species (called medics), all autogamous and diploid except *M. scutellata* and *M. rugosa* which are tetraploid selfers (Lesins and Lesins, 1979).

In the XIX century numerous descriptions were proposed leading to a great number of homonymies and confusions between species, sub-species, and botanical varieties. The more recent and complete taxonomic investigations are those of Negre (1956), Heyn (1963) and Lesins and Lesins (1979).

Based on biogeographical characteristics, three different types of species have been distinguished among the annual species of this genus (Olivieri *et al.*, 1991):

(1) Colonizer and polyvalent species such as *M. truncatula, M. orbicularis* and especially *M. polymorpha*. These three species are among those accidentally introduced in Australia. Their spiny pods may be one reason for their successful invasion ability; such pods are easily hooked to the sheep wool (Cocks *et al.*, 1980).

(2) Species associated with particular environments, such as *M. arabica*, common in Corsica, Sardinia and Greece, but rare in Spain and North-Africa and *M. Aculeata* frequent in Spain and North-Africa but rather rare in other mediterranean countries.

(3) Rare species such as *M. scutellata* formally described as frequent in old floras. The generalization of weed-killers and deep ploughing may have lead to its rarefaction.

Among the perennials, even *M. Sativa*, is not frequently found as natural populations in the western Mediterranean region. Only sub-spontaneous morphs (plants escaped from cultivated fields) can be found in abandoned fields and on roadsides, except in Spain, where important populations of a wild ecotype of *M. sativa* referred to as the '*Mielga*' type have been reported. This ecotype is characterized by a prostrate growth habit and the ability to develop rhizomes (Delgado, 1989; Prosperi *et al.*, 1989a). In other mediterranean countries, *Medicago varia* - a spontaneous and fertile hybrid between *M. sativa* and *M. falcata* - is widely dominant. At the opposite, *M. falcata* is a nearly wild species, commonly found from the north European steppes to the north border of the Mediterranean sea (Bulgaria, Greece) due to its good adaptation to cold winters and warm summers.

1.3. An example of use of genetic resources in plant breeding program

In Australia, where the climate is typically Mediterranean with a severe summer drought and annual rainfall between 250 and 800 mm, the agriculture management is supported by sheep production and cereal crops. Taking advantage of the capacity of annual medics and sub-clovers to produce hard seeds, the Australian farmers have developed a special farming system based on the rotation of cereal and self-regenerating pasture legumes. This farming system, called ‘Ley-farming’, has replaced the traditional and unproductive fallows of Mediterranean countries (Puckridge and French, 1983).

After a first sorting among the wild ecotypes, which began in 1930, the first selected types were available in 1970. After 1950, numerous natural ecotypes were collected in most mediterranean countries by the Australian researchers. In 1990, the genetic variability was mainly issued from Australia, with 13 cultivars of subclovers among 15 existing at that time originating from australian naturalized ecotypes. Concerning annual medics, 42% of the cultivars were issued from Australia, but these varieties constitute nowadays the most widely cultivated forms.

In such a farming system, in which the forage production is mainly influenced by a good environmental adaptation (climate, agricultural systems, ...), the first step of a plant breeding program must be based on the study of the locally adapted ecotypes. Hence, a clear understanding of how genetic variability is distributed among areas is essential in such a breeding program.

1.4. Our research activities (INRA -Montpellier)

In the context of this general ‘genetic resources’ topic, the research interests of our laboratory are organized around the following complementary activities:

- the collection of a large genetic variability through an exhaustive collection of *Medicago* species, especially from mediterranean areas.

- biogeographical studies of several *Medicago* species; one of the aim being to identify the species and environmental characteristics responsible for this distribution.

- the agronomic and genetic evaluation of these genetic resources: the study of agronomic characteristics (yield, growth habit, dormancy, seasonal behaviour, different resistance) on one hand, the use of neutral molecular markers on the other hand.

- the conservation of this genetic material and breeders or research institutes supplies of this genetic diversity.

- the improvement of this material (lines, pools ...) for further use in breeding or research programs.

- theoretical approaches are also involved in order to understand the evolution of the genetic diversity, one of the aim of such studies being to identify the mechanisms responsible for both the occurrence and the maintenance of genetic variability in natural populations. Such mechanisms if reproducible, could represent interesting tools for breeding or management strategies.

2. COLLECTIONS, ACCESSIONS AND GENE BANK

Since 1985, our collection drives have been conducted in France (see below) but also in Spain and Portugal (1985, 1986, 1987), Algeria (1987, 1988) and Greece (1990) in collaboration with local research institutes: Institut National Agronomique (INA, El-Harrah -Algeria), Institut Technique des Grandes Cultures (ITGC, Sidi-Bel-Abbes, Algeria), Servicio de Investigacions Agrarias (SIA, Saragoza -Spain), Estaçao Nacional de Melhoramento de Plantas (ENMP, Elvas, Portugal). In France, the prospected areas are Corsica (1985, 1987) and the South-East of the country, i.e. the Languedoc-Roussillon and the Provence-Alpes-Côte d’Azur regions (1990-1992).

Beside the cultivated material composed of more than 500 origins (populations, lines under breeding, pools ...), the whole accession collection is composed of:

- 192 wild populations of perennial alfalfa: mainly ecotypes of *M. sativa* from Spain (the *Mielga* type, 104) and France (70);

- 2.095 accessions belonging to 25 different species of medics (among the 34 reported species) collected in France (780), Algeria (484), Greece (390) and Spain or Portugal (441), and divided as followed: *M. aculeata* Wild (113), *M. arabica* Herds (122), *M. minima* Bart (335), *M. polymorpha* L. (402), *M. rigidula* ALL (215), *M. truncatula* Gaernt (280), and others (250 accessions representing 18 species).

An accession refers to a sample of seeds collected on a given site (of one hundred square meters up to several hectares). In most cases, this accession corresponds to a wild population: a bulk of seeds originating from several mother plants - at least fifty - for allogamous perennial species, and a set of different lines for autogamous medics.

For each prospected site, a filling-card is available providing eighty descriptors of the site: localisation in terms of longitude, latitude, and altitude, passport data, vegetation and soil characteristics, mother-rock identification, intensity of human interventions and other pedo-climatic traits. All these site descriptions as well as agronomic data concerning the collected accessions are gathered in a data bank. This allows further analyses on the species distribution, and on how ecological factors are related to species differentiation (Gensollen, 1988; Prosperi, 1988; Olivieri *et al.*, 1989; Prosperi *et al.*, 1989b; Boumard, 1989; Prosperi *et al.*, 1991; Manicaci *et al.*, 1992, Chaulet et Prosperi, 1994).

Based on all the realised prospectings, an index has been edited (Prosperi, 1990-1991) and, a data bank which provide the original site characteristics and some agronomic descriptors is available. All the collected accessions are gathered at the: Station GEVES - Le Magneraud - 17700 SURGERES France, which is in charge of the management of seed stocks, the germination control, the publication of index seminum, as well as the sending of accession samples.

3. GENETIC VARIABILITY IN NATURAL POPULATIONS

It is well known that the study of the distribution of a given species and the identification of the main characteristics responsible for this distribution are the basis of the understanding of the evolution of biological diversity. Because morphological traits only represent a part of the genetic diversity, and because the distribution of this diversity is highly determined by natural selection, the identification of the mechanisms involved within populations or species (gene flow, breeding characteristics, ...) requires some studies on neutral genetic variability (i.e. not submitted to natural selection). In this context, several genetic studies based on neutral markers such as enzymes electrophoresis or DNA analysis have been undertaken and should help to precise the genetic structure of spontaneous populations and the level of gene flow between contiguous populations (Manicacci, 1989; Laganier, 1991; Olivieri, 1991; Chaulet, 1995; Bonnin, 1995).

At the present time, investigations on natural populations of both annual (medics) and perennial (the *Mielga* ecotype) are in progress and are described below.

3.1. Biogeography and population structure in *Medicago truncatula*

a/ Species distribution and diversity at a regional scale.

Starting from one hundred and two wild populations collected in 1987-1988 in Algeria, E. Chaulet (1994; 1995) is studying the diversity and a biogeographical picture of an annual diploid and selfer species *M. truncatula* Gaernt. The genetic diversity is analysed through different ways: the geographic distribution of the species, an agronomic evaluation of the populations, and isoenzyme analyses.

According to Heyn (1963), the populations of *M. truncatula* were split in three different groups according to their pods characteristics. The first two groups are linked to the subspecies *longispina* and *tricycla*. The third one is composed of populations showing intermediate pods characteristics.

Species versus subspecies distribution

For each collected site, several data are available: the data collection sheet, a soil analysis and climatic characteristics. To determine the more discriminant variables explaining the presence of the species or subspecies, we used Stepwise Discriminant Analysis. Variance analysis were used to find significant differences between sites related to the classes 'presence' and 'absence' of the species or subspecies.

M. truncatula has a large distribution all over the Mediterranean countries (Prosperi *et al.*, 1991) and is a very invasive species (Olivieri *et al.*, 1991). It was found at a frequency of 80% in Algeria, mainly found at medium altitudes (average elevation of 730 meters) and in cold situations. The subspecies *longispina* is the most frequent (55% of the populations belong to this group), following by the intermediate group (more than 30% of the populations collected). The presence of the subspecies *longispina* appears to be related to mild lowlands sites, with high annual rainfall, while the subspecies *tricycla* preferentially occurs in dry areas (less than 400 mm of annual rainfall) with sandy soils. Populations of intermediate characteristics were found on high elevation (average 1047 meters) and cold sites (mean of the minimum temperatures of the coldest month 0.7°C).

Agronomic evaluation

Fifty seven populations were used for field experimentation and scored for establishment, frost tolerance, diseases such as leaf fungi, vigour, flowering date, vegetative and seed productions. Significant differences were observed between natural populations but also exist between the three groups of populations (Table 1). The subspecies *longispina* is the most productive and the highest while *tricycla* appears as the latest, the less productive and the smallest. The intermediate group of populations has medium characteristics but shows numerous significant differences with the two other subspecies.

Table 1. Means of the different subspecies characteristics. Means followed by the same letter are not significantly different ($P \leq 0.05$) using Student-Newman and Keuls (SNK) test. (experimental design: plot of two rows, 200 seeds by linear meter, 4 replications)

Subspecies	*longispina*	Intermediate	*tricycla*
Height in winter	7.7a	5.8b	2.8c
Height April	37.5a	37.2a	18.0b
Sowing-flowering interval	201b	199b	211a
Pod production by plot (in g.)	905.6a	750.3b	523.9c
Seed production by plot (in g.)	212.5a	175.1a	80b
Seeds by pod	7.5a	3.6b	2.2a

Enzymatic variability

Four enzymatic systems (ACP, PGI, PGM, SDH) were retained among the twelve systems tested, because they were readable and show polymorphic allelic patterns. The subspecies *longispina* and *tricycla* respectively show the smallest (0.98%) and the highest (3.36%) proportion of heterozygous plants. Two loci (ACP and PGI1) show a low level of polymorphism with one common allele. For loci PGM and PGI2, we observe two common alleles, but with very few differences between the subspecies. Finally, Sdh appears as the only locus allowing to distinguish the subspecies *longispina* and *tricycla*. For this locus, the intermediate group shows the same patterns as the subspecies *longispina* (Table 2).

Table 2. Mean allelic frequencies for the Sdh system per group or subspecies

Subspecies		*longispina*	Intermediate	*tricycla*
Number of analysed plants		510	720	327
% of heterozygous plants		0.98	1.38	3.36
Sdh allel	a	3.8	11.8	0
	b	95.6	87	1.5
	c	0.6	1.1	87.1
	d	-	0.1	11
	e	-	-	0.3

This study has shown that a large genetic diversity exist in the species *M. truncatula* collected in Algeria. There is a strong relationship between the presence of a given subspecies and the ecological environment of the prospecting site. Three groups can be distinguished in terms of pods morphology, phenology, vegetative and seed productions. This suggests that the three subspecies (or groups of populations) have used different strategies to answer the selective constraints associated with the contrasted environments (Redjimi-Chaulet, 1991).

b/ Population structure at a more restricted scale: the population level.

Concurrently to the previously described biogeographic study, Bonnin (1994, 1995) is studying genetic variability in *M. truncatula* but at a lower geographical scale, the aim being to determine the relative effects of gene flow, stochastic and selective processes. To do this, the within and between population structure of 24 quantitative characters and of 21 dominant genetic markers (RAPD) have been compared. RAPD markers were chosen due to the high level of polymorphism they classically show.

Experimental procedure

Four natural populations located at a maximum of 200 km apart, were chosen and subdivided in three sub-populations. In each sub-population, one plant was collected each meter along a thirty meters transact and transplanted into a greenhouse. Each of these plants were characterized by: (1) its genotype for neutral markers using 21 RAPD markers and one morphological marker (the pod coiling direction, a monogenic dominant character), and (2) its genetic characteristics for quantitative characters, using, five offsprings of each plant, derived from spontaneous selfing (and constituting a family), sown and grown under greenhouse conditions. Studied characters included seedling traits (emergence date of cotyledons, leaf size, ..), growth traits (growth of the main stem, of secondary stems, ..) and reproductive traits (date of the first flower bud occurrence, weight of dried stems, weight of pods, seed production, ..).

Population structure based on RAPD markers

In agreement with what is expected in a selfing species, we found a strong differentiation among populations. Based on multilocus genotypes, two populations (Hérault and Corsica populations) were composed of a few, predominant lines. While the two others (Var and Aude populations) were much more polymorphic but also more structured. The Aude population showed the highest level of polymorphism and differentiation: on average, only one multilocus genotype was found common to two sub-populations. On the basis of these results, the two most structured populations were chosen for studies on quantitative characters.

Neutral versus quantitative variation and structure

Through hierarchical analyses of variance (population, sub-population and genotypic components), it has been shown that:

(1) The populations are more differentiated for quantitative characters than for neutral markers;

(2) The contribution of both within and among subpopulations components of variance to the overall genetic variance is reduced for the quantitative characters as compared to the neutral markers.

(3) At the population level, within population structure is slightly larger for markers than for quantitative traits.

Under the hypothesis that RAPD are neutral, such comparisons may be used to make hypothesis about the strength and the heterogeneity of natural selection in the face of genetic drift and gene flow. In particular, the present results suggest that for the studied populations, quantitative characters are under strong divergent selection among populations, and that gene flow is restricted among populations as well as among sub-populations.

3.2. Diversity, population structure and gene flow in *Mielga* (the wild *M. sativa* L. ecotypes from Spain)

The wild spanish ecotype of *M. sativa* is called *Mielga* in contrast with *Alfalfa*. One hundred and four wild populations have been collected in Spain, a sample accounting for interesting situations in regards of wild/cultivated interactions:

(1) in the north-west of Spain, we can fairly hypothezise that Mielgas populations have been introgressed by the cultivated alfalfa form, the plants exhibiting halfway morphological features between the two gene pools.

(2) on the mediterranean sea border, the populations exhibit traces of introgressions by *M. falcata* as can be deduced from the pattern of variegated flowers (pure *M. sativa* plants have purple flowers whereas *M. falcata* exhibit yellow ones).

(3) in the south and the center of Spain, no obvious trace of introgression, can be observed. Alfalfas are then cultivated on fertile and irrigated plots whilst *Mielga* plants settle adjacent dry areas.

A number of key issues concerning the preservation of the wild type (*Mielga*) integrity arise from these situations. Theoretically, whereas the cultivated bed integrity is artificially maintained by men, gene flow between cultivated and wild populations may greatly modify the wild plants' traits. On the other hand, the deployment of flexible reproductive barriers decreasing gene flow may further their stability. If so, what sort of reproductive barriers are at stake ? Otherwise, what other factor limit the appearance of halfway morphological plants ? Could any natural selection against some introgressed traits be involved for drought tolerance for instance ?

As said above, the Iberian Peninsula constitute an unusual zone where important wild and cultivated *M. sativa* morphotypes coexist. Because interactions between wild and cultivated compartments *in natural* could be highly informative for further breeding programs, a thesis (E. Jenczewski) has been started to elucidate gene flow patterns in these populations.

Agronomic variability

Some agro-morphological data concerning all the 104 populations have already been collected. They principally correspond to measurements of growth habit, perenniality, earlyness, variegated flowers and/or rhizoma ratio. A Principal Component Analysis confirms that wild populations from the North of Spain (supposed to be introgressed by cultivated alfalfas) can effectively be easily distinguished from the other Mielgas, and behave as cultivated types. Significant variability has been shown in 'Mielga' (Tables 3 and 4). This material is characterized by a good spring growth, slightly lower than the average of control cultivars, and by a poor level of regrowth, mainly in autumn. Nevertheless, a remarkable variability in the growth rhythm exists within all populations of 'Mielga' as the first cut in second year accounts for 39% to 75% of the total annual dry matter production. This material is more perennial than the common alfalfa cultivars, as the first cut yield of the control cultivars do not show any significant difference compared with wild populations at the fourth year. These populations also show an acceptable level of seed production: only 30% lower than the control cultivars, but with a lower 1000-seeds weight. As a result, this gives a quite equal production in number of seeds. On the other hand, these 'Mielga' populations have a prostrated habit and a great capacity of soil colonization.

'Mielga' genetic resources are particularly attractive for our topics, because they have different favourable characteristics such as prostrated habit, perenniality and drought tolerance, with a supplementary good spring yield for not selected wild populations. Nevertheless, an effort must be done to obtain higher (1) level of regrowth in autumn and at the end of winter, and (2) of resistance to diseases. Furthermore, we will now confront such a structuration with the eco-geographical data we have collected for each single site; if there is a strong correlation between the morphological and the environmental characteristics, this would suggest that the spatial differentiation is related to natural selection.

Table 3. Comparative seed and dry matter production of the wild and the cultivated form in *M. sativa*. Values for *M. falcata* are proposed as reference values. Dry matter is expressed in kg, seeds production in g

		Mielga (Min-Max)	Sativa (Min-Max)	Falcata
First cut A1	HS	1.67 (1.26-2.24)	1.87 51.21-2.11)	1.63
First cut A3	NS	1.64 (0.91-2.34)	1.73 (1.35-2.26)	1.80
Total dry matter A1	HS	2.82 (1.95-4.57)	4.56 (3.61-4.92)	2.67
% first cut A1	HS	60.7 (38.8-75.0)	41.0 (33.5-49.9)	61.1
Seed harvest A2	HS	109.7 (33-211)	136.2 (94-192)	-
1000 seeds weight	HS	1.574 (1.37-2.02)	1.968 (1.81-2.09)	1.519
Average habit	HS	6.06 (3.58-7.50)	3.80 (2.92-5.33)	5.33
Colonization	HS	5.80 (2.08-9.75)	2.38 (1.13-4.17)	3.00

HS Highly significant $P < 0.001$, NS No significant $P > 0.05$.

First cut A1: Dry matter of first cut (02/06/88) in the second year. First cut A3: Dry matter of first cut (16/05/90) in the fourth year. Total dry matter A1: Total dry matter production of the second year in 4 cuts (2nd June, 11th July, 23th August and 9th November 1988. % first cut A1: Ratio between first cut and annual dry matter production. Seeds harvest A2: Seeds produced on first cycle in third year. Seed production has not been measured on *Medicago falcata.* because of dehiscent pods. 1000-seed weight: Weight of 1000 seeds. Average habit: Score of habit (1 erect to 9 very prostrate). Colonization: Score of soil surface colonization (1 very low to 9 very important). Plants with a score of 5 to 9 are often rhizomatous.

Table 4. Relative agronomic characteristics of wild populations (Mielga type) of *M. sativa* as compared to 4 alfalfa cultivars

	ES.005	ES.096	ES.129	Europe	Magali	Aragon	Ampurdan
First cut A1	1.34	2.24	1.67	2.11	1.86	1.21	1.84
First cut A3	2.06	1.80	1.37	1.71	1.64	1.35	2.05
Total dry matter A1	1.95	3.74	2.97	4.92	4.67	3.61	4.62
% first cut A1	68.6	59.9	56.3	42.9	39.9	33.5	40.1
Seeds harvest A2	115.4	110.1	131.3	144.1	150.2	104.3	189.2
1000 seed weight	1.541	1.451	1.588	1.992	2.086	1.943	1.919
Average habit	7.08	6.00	6.00	2.92	3.33	3.92	4.17
Colonization	6.00	8.75	5.75	2.46	2.00	1.25	2.25

Origin of populations: ES.005 Zaragoza, ES.096 Granada, ES.129 Leon.

Population structure

A more accurate structuration is to be established with neutral molecular markers (RAPD, Isozymes, ...). Fifteen populations have been chosen in order to be representative of the range of situations we have observed between wild and cultivated plants, and to be regularly distributed all over the collection area. Three cultivated landraces from Spain, a 'flemish-type' alfalfa cultivar and a wild population of *M. falcata* are also analysed.

Gene flow between spontaneous and cultivated alfalfa

The population structure pattern established on the basis of the previously defined markers will not be sufficient to estimate levels of gene flow between populations and will not give any information on the causes of restricted gene flow. This is why we plan to study a restricted set of parameters, that are known to fairly participate in gene flow: pollen production and viability, pollen tube growth rate, viability of the resulting zygotes. Furthermore, in order to detect a preferential direction of gene flow (cultivated towards wild or wild towards cultivated) and to determine the parental origin of the intermediate forms, the cytoplasmic molecular variability will be helpful. We will thus develop the analysis of cytoplasmic variability through molecular markers like RFLP.

4. BREEDING PROGRAMS

For the moment, a third of the collected material has been evaluated, (Prosperi *et al.*, 1991; Volaire *et al.*, 1992) the evaluation being based on 2 steps (Prosperi, 1989):

1) A morphological description, for the taxonomic confirmation of the reported species or subspecies, and preliminary agronomic evaluation during the first multiplication step. In order to prevent the loss of material (possible susceptibility to cold conditions, hydric stress or diseases) this first step is generally carried out in controlled environments.

2) Agronomical tests performed in different places and following different modalities are realized to obtain data about phenology, relative allocation to vegetative and reproductive metabolisms, seed production, fodder quality and susceptibility to different stresses.

4.1. Medic programs

Specific differences and local adaptations for the medics

Important differences are observed between species. For example, *M. rigidula* and *M. orbicularis* are more tolerant to cold conditions than *M. truncatula* or *M. polymorpha.* This trait is one of the main adapting factors for use in high mediterranean rangelands. This tolerance is associated with a rather low winter growth; but the large variation observed between ecotypes for many species makes this parameter increasable (Prosperi, 1989). Furthermore, it seems that *M. rigidula* needs more vernalization for flowering induction than the other species.

Concerning variability all along natural distribution, we have to note a decrease of earlyness from south mediterranean areas toward north ones. So, ecotypes from France are on average later (mean note of 5.0; on a scale varying from 1: the latest to 9: the earliest) than those from Corsica (6.0), Algeria (6.5) or Spain (6.6), and Australian cutivars are the earliest (7.0). In our conditions, lateness is often associated with a decrease of pod or seed production. But the large variability met for each species for the main agronomical traits (growth habit, vegetative production, tolerance to diseases...) at the same earlyness level make the breeder optimist in his program effect.

Breeding results and potential use

So far, 4 **annual lines** (1 *M. polymorpha,* 1 *M. truncatula*, 2 *M. rigidula*) which are well adapted to cold mediterranean areas are available and ready for official registration tests. Furthermore, a work is carried out - for *M. truncatula* and *M. polymorpha* - in order to obtain recombinant lines from 6 intra-specific crosses: according to the Single-Seed-Descent method, F_6 populations of about 200-300 recombinant lines from each F_1 plant are expected in 1996.

For all the mediterranean regions, the potential use of annual self-reseeding legumes such as medics varies. Ley-farming could be the most important use of medics in North Africa or in Middle East, but in european countries their potential use is more diverse.

In France, the possibility of a ley-farming system is now limited because most farmers prefer perennial and known forage crops, mainly used for hay. But in the near future, overproduction of cereals and increase of 'set-aside' lands in rotation with cereals with EEC subsidies could provide a shift toward fallow land conversion to medic-dominated pasture.

The best ways to use medics in France are by improving winter rangeland production and oversowing degraded natural pastures under dry conditions. Several experiments have been conducted with subclovers or medics to introduce them into marginal lands. The main part of selection activity on medics of our laboratory is assigned to produce cultivars adapted to these conditions (Table 5).

Table 5. Relative selection criteria according to the management system: ley farming or improvement of rangelands.

	Ley farming for Australia	Rangelands for France
Selection for environmental conditions		
Frost tolerance		
Leaves	+	+++
Plants	+	+++
Winter growth	+	+++
Growing season	4-6 months	8-9 months
Flowering date	Early	Late
Maturation period	Medium to long	Neutral
Resistance to		
Fungal diseases	+	+++
Insect pests	+++	+
Selection for farming systems		
Seedling vigor	+	+++
Competition with weeds	+	+++
Seed production	Important	Neutral
% of hard seeds	> 80%	40 - 60%
Break of hardseededness	After 1 year	Neutral

Important (+) to very important (+++)

Medics and subclovers also can be interseeded in vineyards to provide grazeable forage and particularly to limit soil erosion on sloping land. This second utilization should be applicable to non-Mediterranean regions such as the west of France where winter temperatures are not too cold and would permit an annual legume growth period in the fall and spring.

The last possibility is to use them as spring annual forage legume crops throughout France, in place of *Vicia* or *Pisum*. In this case, we need some high-producing, erect species, such as *Medicago* *scutellata*, to produce hay. In mediterranean countries, this utilization also is feasible as a forage mixture with grasses or cereals, as a substitute for traditional *Vicia* or *Avena* production.

A very significant use for medic will be in combination with perennial forage legumes, which have their own specific role in these north mediterranean zones.

4.2. Genetic, phenotypic, and environmental correlations in *M. lupulina* L.

Genetic correlations arise from pleiotropy, or linkage disequilibrium, or both. As they determine how traits will change in relation to each other, an understanding of genetic correlation among life history traits is crucial for an understanding of coordinate evolution through correlated responses to natural selection (Falconer, 1981). In the context of plant breeding, a certain comprehension of genetic correlations is particularly useful to perform indirect artificial selection on characters that show low heritability and/or are difficult to measure (Gallais, 1990).

Experimental procedure

In the present study we have investigated the relationship between phenotypic and genotypic correlations among a large number or quantitative traits (36) in three different environments in order to determine their degree of disparity. Twenty accessions (full-sib families) of autogamous *M. lupulina* were grown in three environments. In two of these three types of environment, stresses were generated by harvesting plants at flowering and by growing plants in competition with barley, respectively. A third environment, with no treatment, was used as control.

Results

Average values of pod and shoot weight indicate that competition induces the highest level of stress. The genetic and phenotypic correlations among the 36 traits were compared. Significant phenotypic correlations were obtained easily, while there was no genetic variation for 1 or the 2 characters being correlated. The large positive correlation between the genetic and phenotypic correlation matrices indicated a good proportionality between genetic and phenotypic correlations matrices but not their similarity. In a given environment, when only those traits with a significant genetic variance were taken into account, there were still differences between genetic and phenotypic correlations, even when levels of significance for phenotypic correlations were lowered. Consequently, it is dangerous to substitute phenotypic correlations for genetic correlations. The number of traits that showed genetic variability increased with increasing environmental stress, so that the number of significant genetic correlations also increased with increasing environmental stress (Figure 1a). In contrast, the number of significant phenotypic correlations was not influenced by the environment (Figure 1b). The structures of both phenotypic and genetic matrices, however, depended on the environment, and not in the same way for both matrices.

4.3. Growth rhythms variability in Alfalfa and effects of contrasted environments

a/ Growth rhythm and habit in lucerne: the genetic study of erect X prostrate hybrids

Due to the large diversification of use in lucerne, variation in growth rhythm and habit have to be investigated. Growth rhythm of a given genotype here defines the ability of the genotype to grow at various periods of the year. In this context, 140 F1 hybrids (2480 plants) originating from hand hybridization between erect types (eight mainly non dormant populations) and prostrate or semi-prostrate types (eight dormant or semi-dormant populations) have been product and studied under spaced plants conditions (Table 6). During three years, fourteen variables were obtained referring to seasonal (re)growth, spring and summer height and diameter, frost tolerance, growth habit, number and size of stems.

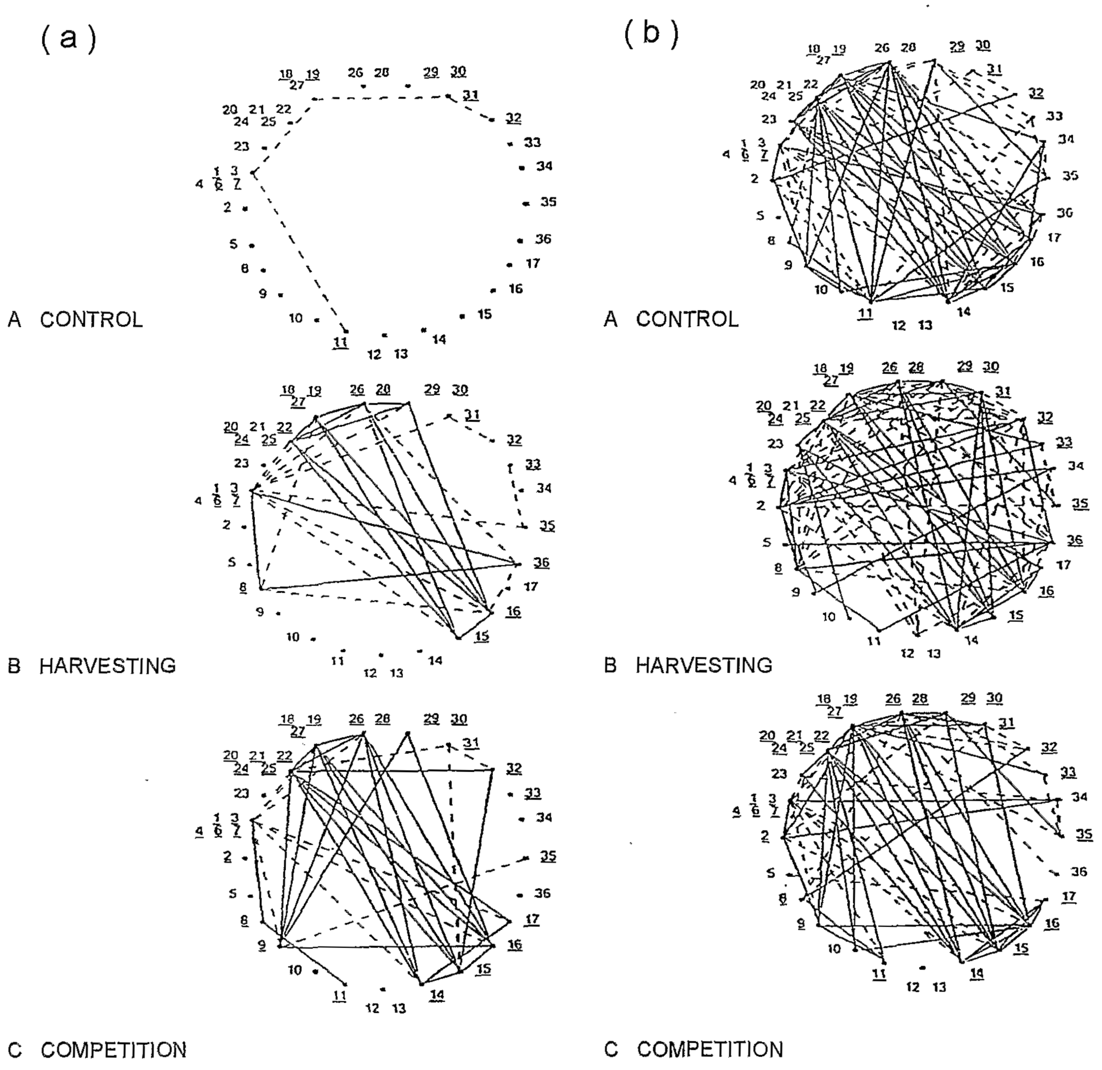

Figure 1. (a): Genetic correlations and (b): Phenotypic correlations among all the 36 traits in control (A), harvesting (B) and competition (C) environments. In both figures, the correlations among traits which were significantly different from zero at P<0.05, P<0.01 and P<0.001 are represented by *lines* connecting the traits. *Solid lines* indicate positive correlations; *dashed lines*, negative correlations. The traits which are underlined show significant genetic variability.

Table 6. Dormance type of 16 different parental genotypes used for the study of the genetic component of growth habit. In italic: ecotypes or breeder lines

Dormant	Semi-dormant	Non-dormant
Coussouls (Semi-prostrate) *Crau T2* (Semi-prostrate) *Crau T5* (Prostrate) Europe (Erect) Luzelle (Semi-prostrate) Pancrudo (Prostrate)	Ida aridae (Semi-prostrate) Magali (Erect) *Mielga 2.84* (Prostrate) *Zaragoza 4a* (Semi-prostrate)	Alep (Erect) Chypre (Erect) *Colin 2.14* (Erect) Cuf 101 (Erect) Demnate (Erect) Guilboa (Erect)

Parents characteristics: As shown on Table 7, parental populations differ clearly by their growth habit and behaviour in autumn and winter. No significant differences between types of growth appear either for spring or summer biomass, or for diameter and height. This could be probably explained by the "explosive" growth of dormant types in spring which balances the delay of start of growth observed in the beginning of the spring in comparison with non dormant types. Comparison of spring "volume" between the two successive years (Spv1/Spv2) shows a similar behaviour for dormant plants, the correlation (r=0.67; *p<0.01*) is highly significant. At the opposite, the behaviour of non dormant plants is strongly dependent on winter conditions *(r=0.23; p<0.05)*. A correlation also exists in autumn between growth habit and biomass: the more prostrate the habit is, the weaker the biomasses are.

Table 7. Mean values for different agronomic characters for the parental genotypes according to their growth type and results of variance analysis

	Dormant	Semi-dormant	Non dormant	Significance
Growth habit	**6.5a**	**4.8 b**	**4.0 b**	***
Spring Volume year 1 (SpV1)	4.6	5.6	6.3	NS
Spring Volume year 2 (SpV2)	3.2	4.0	3.4	NS
Correlation SpV1/SpV2	**0.67**	**0.40**	**0.23**	
Summer Volume	4.9	5.8	6.1	NS
Autumn Volume	**3.8 b**	**5.1 b**	**6.0 a**	*
Beginning Winter Volume	**1.8 c**	**3.4 b**	**6.3 a**	***
End Winter Volume	**2.2 v**	**4.0 b**	**5.8 a**	***
Spring Diameter	33	32	30	NS
Spring Height	18	26	26	NS
Summer Diameter	67	64	63	NS
Summer Height	37	46	45	NS
Stem size	4.3	4.6	4.9	NS

The "volume" is a score of aerial biomass from (1): small to (9) large. Growth habit varies from (1) erect to (9) prostrate. Score of stem size from (1) fine to (9) thick.

F1 hybrids characteristics: It seems possible in F1 hybrids to break down a part of the linkage we observed (during the autumn) between growth habit and biomass in the parental populations. As for the parents, a strong negative correlation is observed between frost tolerance and winter growth. Prostrate plants show a greater number of stems than the erect ones. Taking into account the cross direction, a strong maternal effect could be observed: F1 behaviour is always nearer the female parent than the male parent, especially for growth habit and winter "volume" (Table 8).

Table 8. Comparison between parental and hybrid values for growth habit and "volume" at the end of winter according to the cross direction

	Growth habit			Winter volume		
Genotype	Parental value	Hybrid value		Parental value	Hybrid value	
		Female effect	Male effect		Female effect	Male effect
Coussouls	6.02	5.16	4.59	2.66	3.44	3.59
Crau T2	6.12	5.75	5.01	2.90	2.46	2.68
Crau T5	5.41	5.24	5.14	2.45	3.01	3.14
Luzelle	6.04	5.54	5.19	2.00	2.42	3.00
Pancrudo	8.90	6.18	4.78	1.02	1.58	3.55
Dormant	**6.49**	**5.57**	**4.94**	**2.20**	**2.58**	**3.19**
Alep	4.18	4.56	5.10	5.18	4.00	3.18
Chypre	3.90	5.11	5.16	6.94	4.83	3.24
Colin 2.14	4.04	4.58	5.16	4.46	3.01	2.70
Cuf 101	3.94	4.68	5.11	6.25	4.40	2.88
Demnate	3.75	4.99	5.59	5.97	3.36	2.12
Non dormant	**3.96**	**4.78**	**5.22**	**5.76**	**3.92**	**2.82**

The mean values of the 14 synthetic variables for each F1 hybrids were analysed by a Principal Component Analysis. The first axis represents 30% of the total variance and is highly correlated to the autumn growth and generally to all scores of "volumes", while the second axis, with 18% of the total variance, is related to frost tolerance, number of stems and growth habit (Figure 2). A clear opposition appears between the frost tolerant hybrids with a low capability of winter growth (female parent populations are dormant or semi-dormant) and hybrids showing an important winter growth and a less frost tolerant (originating generally from a non-dormant mother population). This strong maternal effect is also evident on this first plan.

To answer the new applications of alfafa, it seems possible to diversify growth rhythms and habit in Lucerne through a large breeding program including non-dormant and dormant populations. The improvement of the cultivars will concern the lengthening of the vegetative growth in autumn-winter and, the break of the relationship between the habit of the plant and the capability to growth at low temperature. In most cases, the level of dormancy will have to be chosen according to the required level of frost tolerance.

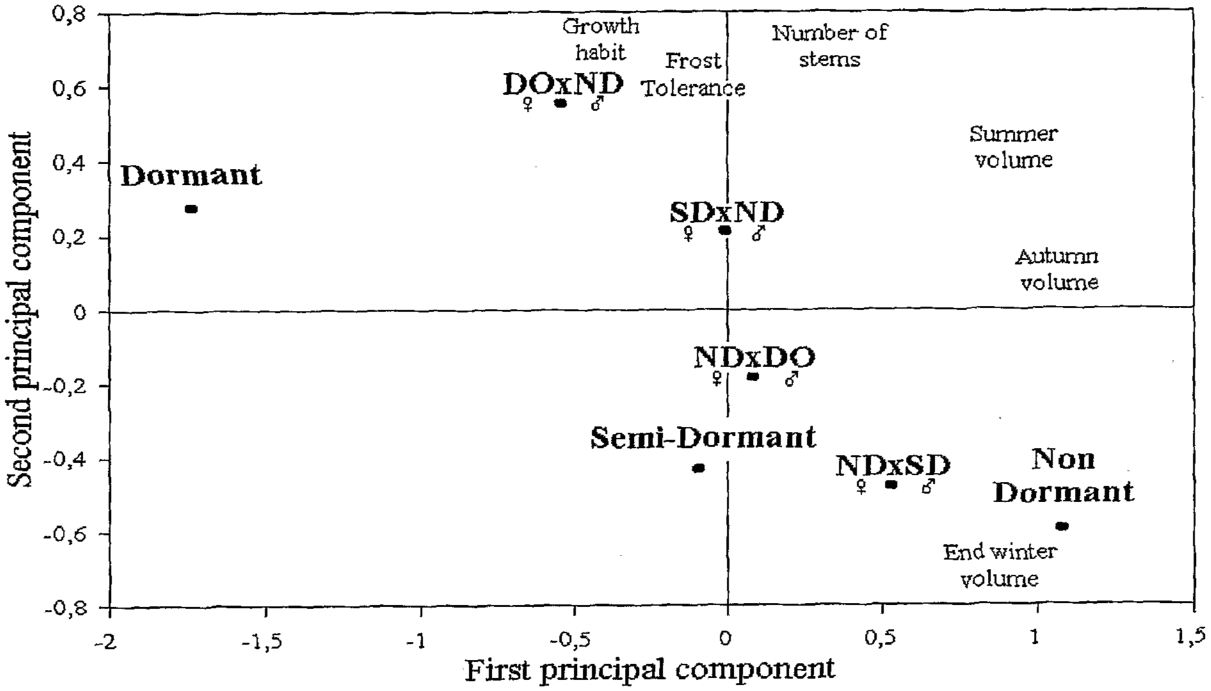

Figure 2. First plan of the Principal Component Analysis, performed on the 14 synthetic variables measured on F1 hybrid plants (Axe 1 and Axe 2 represent respectively 30 and 18% of the total variance).

b/ Genetic variation for growth rhythms in lucerne, in two contrasted and cold mediterranean environments

Experimental design

In order to define lucerne accessions adapted to Mediterranean environments, eighty-two genotypes showing different growth rhythms were studied in two climatic situations from 1986 to 1990.

The "**Mauguio**" site located in a littoral plain (altitude of 10 meters) is characterized by mild temperatures and two months of summer drought. The experiment was irrigated during the first three years (1986-88) and not irrigated in 1989 and 1990. Plots were harvested five times a year, at early flowering of the control cultivar "Magali".

The "**Saint Gely"** site belongs to the garrigue zone (altitude of 105 meters). It is wetter and cooler than the "Mauguio" site, with a longer summer drought. Experiment was not irrigated and plots were grazed four times a year by sheep.

Results

Sixty-three measures were collected during five years. They were derived from visual estimations and concerned: aerial biomass or "volume", growth habit, blooming date and intensity of flowering, diseases tolerance, size of plants, number and size of stems, etc... Eighteen synthetic variables (Table 9), 9 of each site were derived from the primary measures and were analysed through a Principal Component Analysis. The three first axis explain more than 90% of the total variability. The first component, with 45% of the total variance, opposes winter growth to cold tolerance. The second component explains 24% of the total variance. On this axis, the genotypes can be distinguished according to their growth rate after the winter and during the spring: It could be interpreted as a "vigour" axis. The third plan clearly opposed the two experimental sites, the difference between the two sites being maximum in spring and in summer. An ascendant hierarchical clustering method was performed based on Euclidean distances between genotypes from the first three principal components (Figure 3). In this case, it is easy to divide the tree in eight clusters ranging from strong winter dormancy, high spring productivity and adaptation to grazing to continuous winter growth, very little cold tolerance and short persistency.

Table 9. Number of primary variables used to obtain the synthetic variables

Variables	Saint-Gely		Mauguio	
Establishment	GVI	1	MVI	1
Winter Hardiness	GWH	1	MWH	1
Winter Growth	GWH	3	MWG	5
Spring Growth	GSG	2	MSG	2
March "volume"	GMV	3	MMV	3
June "volume"	GJV	3	MJV	1
Summer "volume"	GSV	4	MSV	2
Autumn "volume"	GVA	4	MVA	3
Persistency	GDP	1	MDP	3
Total	**9**	**22**	**9**	**21**

5. A SPECIFIC STUDY: A GENETIC MAP FOR *MEDICAGO TRUNCATULA*

In France, breeding of annual medics (especially *M. truncatula, M. rigidula, M. polymorpha)* began at INRA Montpellier 10 years ago, in order to introduce it in extensive, mediterranean managements. Because of its genetic structure (diploid and selfer species), and because it has been chosen as a model plant in studies of plant/rhizobium symbiosis, *M. truncatula* is particularly adapted to studies of mapping inside the *Medicago genus.*

Starting from 2 crosses, the present analysis of F2 progenies using molecular, symbiotic, and morphological markers would allow the 'INRA-CNRS laboratoire de biologie moléculaire des relations plantes-microorganismes' in Toulouse (T.Huguet[3]), in relation with our lab, to map the *M. truncatula* genome in next few years. More than a model of genetic technology, this approach is of great agronomical interest: several characters should be interesting for breeding improvement of cultivated alfalfas. For example, maps realised on diploid *Medicago* species could be used for tetraploid ones. In this way, synteny analysis have been planned.

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[3] Laboratoire de Biologie Moléculaire des Relations Plantes Microorganismes. Chemin de Borderouge. B.P. 27. 31326 Castanet Tolosan Cedex, France. Tel: 33/ 61 28 50 28. Fax: 33/ 61 28 50 61.

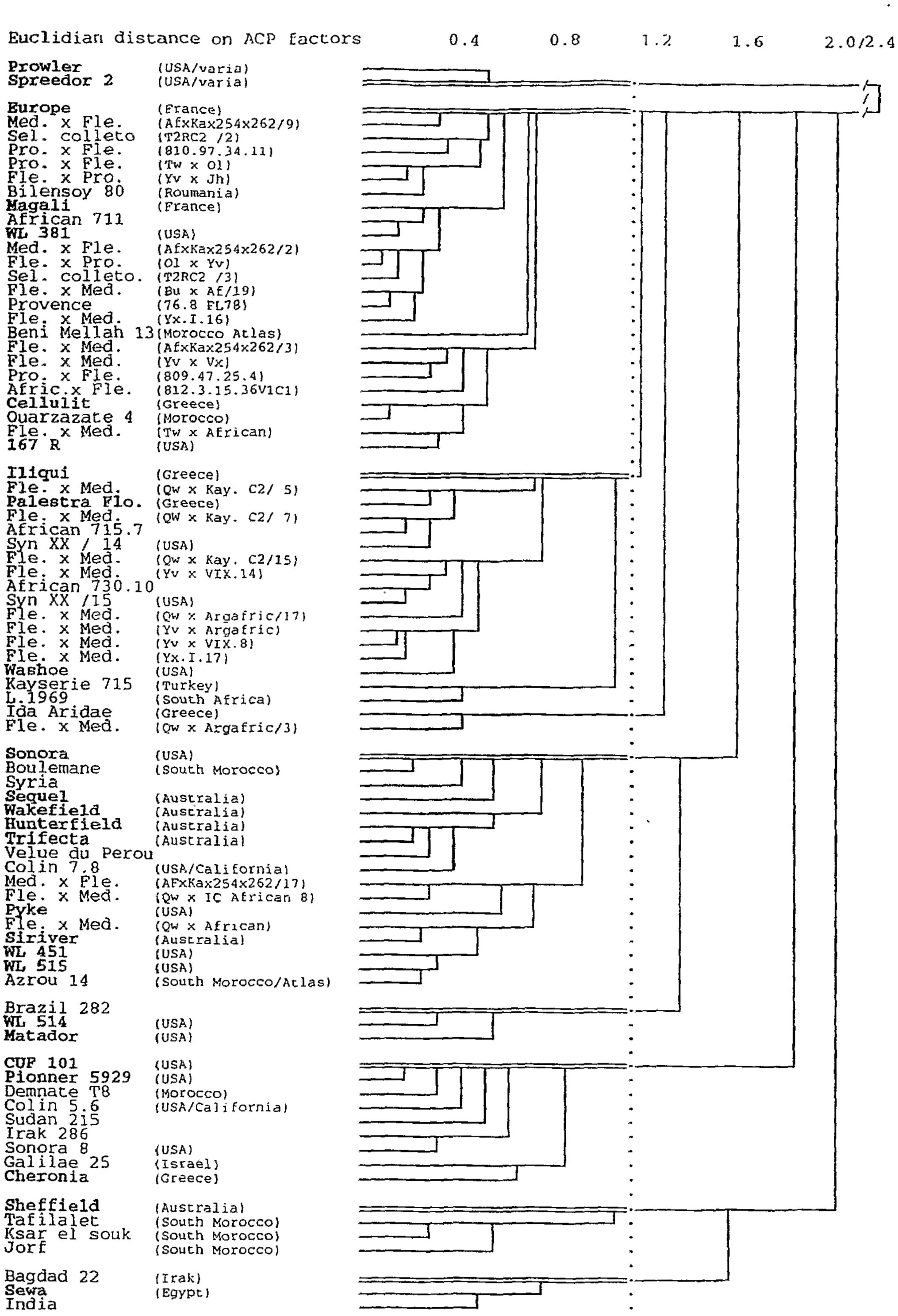

Figure 3. Hierarchical classification tree of the 82 genotypes and partition into 8 clusters. In bold, registred cultivars. Fle. = Flemish, Pro. = Provence, Med. = Mediterranean.

6. FORESEEN PROGRAMS

These programs will continue for several years, some of them will be intensified especially:

* Collection and multiplication of temperate and mediterranean accessions in order to develop a bank of Genetic Resources at european level.

* Further studies referring to biogeography of different *Medicago* species with extension analysis to the whole primary distribution area of these species including the east part of mediterranean basin.

* Carrying on and generalization of populations genetic studies based on correlations between description of original sites and evaluation characteristics in order to precise the influence of natural selection on parameters such as phenology or cold tolerance.

* Expanding of molecular marking technics on these species, annual and perennial (use of cytoplasmic molecular analysis such as RFLP).

* Extension of phylogenic studies of the genus Medicago using particularly molecular markers (DNA sequence analysis on neutral genomic fragments but also on genes involved in symbiotic interactions) in order to confirm some hypothesis about the biogeography of these species and the evolution of this genus.

* All this work do open into cultivars creation in relation with private breeding companies.

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Mediterranean lucerne cultivars:
1971: Magali
1971: Mireille (List B)
1988: Medalfa (co-obtention ACVF)
1994: Meldor
***Sainfoin*:**
1967: Fakir

REGISTRATION REQUESTS (1995):
Medics:
M. rigidula: 2 lines
M. truncatula: 1 line
M. polymorpha: 1 line

Mediterranean Lucerne (Alfalfa):
'Coussouls' (synthetic cultivar): for grazing
'ND87' non-dormant type for hay production