Vitis **33**, 217–224 (1994)

A chemotaxonomic investigation on *Vitis vinifera* L. II. Comparison among ssp. *sativa* traditional cultivars and wild biotypes of ssp. *silvestris* from various Italian regions

by

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S u m m a r y : An extensive screening on regional representatives of *Vitis vinifera* sspp. *sativa* and *silvestris* was carried out to look for relationships and differences between different taxa. Total proteins in the pH range 4.0-5.5, and the enzymes AcP, ADH, EST, G-6-PDH, MDH, PGM and POD were recorded. Only patterns of storage protein subunits, AcP, EST and G-6-PDH were taxonomically informative. Dendrograms were computerized on the basis of presence/absence of individual bands; these always distinguish at different levels in homogeneous groups between ssp. *sativa* and ssp. *silvestris*. The acidic subunits showed a dichotomy from the first branching of the cladogram. These observations are a demonstration that neither hypotheses of direct or indirect origin of the Italian ssp. *sativa* from Italian ssp. *silvestris* via domestication is tenanble. The authors suggest that, the morphological, the ecological and the biochemical differences between the two taxa support the hypothesis that *V. sativa* and *V. silvestris* should be regarded as two separate taxa that have had reciprocal interactions such a long period of time that a precise location of origin is no longer possible.

K e y w o r d s : seed proteins, storage proteins, isoelectric focusing, enzyme.

Introduction

Grouping within the genus *Vitis* - a member of the Vitaceae family – is a controversial issue. Some authors (Olmo 1976; Galet 1988) sort a large number of *Vitis* species into two subgenera: *Euvitis* and *Muscadinia*, where the number of chromosomes (38 and 40 respectively) and the fiber arrangement in the secondary phloem are assumed as being the consistent and distinctive characteristics for each group.

There are two peculiar points to this genus. First, there are no species-specific restraints of cross-hybridization within *Vitis*, except in the case of *Euvitis* x *Muscadinia* crosses which do not occur very often and if they do, only occur with great difficulty (Olmo 1976). Secondly, for this reason genetic traits are able to spread across the various species. This implies a lack of specificity for any morphological characteristics, floral pattern included. A description and characterization of different taxa has often had to resort to an analysis of the transient features during the vegetative cycle, such as juvenile characteristics, bud hairyness, etc.

The authors were prompted to investigate the biochemical markers of *V. vinifera* and *V. silvestris* as a means of classification because of the taxonomic uncertainties just mentioned and also because of the economic and agronomic importance of grapevine. Representative samples of the former group were collected from all Italian districts where vine growing ranks as one of the main agricultural activities. Only traditional cultivars were chosen which had been described and diffused in the areas where it originated several centuries ago, i.e. long before phylloxera, oidium and mildew pandemics occurred. In other words, they were selected before non-autochtonous types replaced those which had been destroyed by infection and did not allow to determine either the initial diffusion area of *V. vinifera* or the possible correlations with spontaneous forms of *V. silvestris*. An effective way of identifying a given biotype should include the largest amount of data capable of describing the subject from various points of view. Thus, a panel of morphological traits has been summarised for each named cultivar. Biochemical markers are best suited as distinctive parameters when correlations are being drawn among taxonomic units, a similarity of the effectors of basic metabolic pathways being more circumstantial than are resemblance in size and shape (Kubitzki 1984).

Seed proteins are assumed to be useful reference parameters for cultivar characterization (Larkins 1981). In fact, they are usually polymorphic, either by size or by surface charge (Stegeman 1983; Cooke 1984). Moreover, a large amount of literature states that protein in seed is under genetic control with little or no influence from the environment (Nelson 1980; Boulter 1981). A previous investigation by the authors (Gianazza *et al.* 1989) described the protein content of cv. Chardonnay seed extracts to investigate the variability between individual self-pollinated grape seeds by scoring the electrophoretic banding pattern of the major storage protein (Gianazza *et al.* in preparation) and of a number of enzymes with high specific activity. By studying Chardonnay, phenotypically homogeneous, and Sangiovese, which shows great variability, no relationship was observed between the degree of morphological and of biochemical variability (Tedesco *et al.* 1991).

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With *V. vinifera* L. the distinction of two subspecies - *sativa* and *silvestris* (GALET 1988) – is based on the floral morpho-physiology (monoicous hermaphoditic versus dioicous plants), ecology (cultivated versus spontaneous forms) and the preference to different environments. However, when the environment is modified by human activities, the latter rule fails (GALET 1988). It is still not clear whether *V. vinifera* ssp. *silvestris* should be regarded as a taxon on its own deriving from either the autochtonous forms or from mixtures of autochtonous plants crossed with the domesticated forms of *V. vinifera* ssp. *sativa*. Variability among ssp. *silvestris* individuals, as entailed by these assumptions - would in the former case - be adaptive to different environments (such as modified by farming). A third, but less probable hypothesis states that ssp. *silvestris* is a feral cultivated variety. Under experimental conditions, neither sex expression nor pest resistance patterns similar to ssp. *silvestris* were ever reported for ssp. *sativa*. In Italy, ssp. *silvestris* plants from the northern and southern part of the country can be readily differentiated. In Central Italy where most of *silvestris* specimen are reported, however, most of the morphological traits seem to lie between both of the above types and overlap with the cultivated varieties (FAILLA 1992).

Materials and methods

M a t e r i a l s : Seeds were obtained from ripe berries of self-pollinated *V. vinifera* ssp. *sativa* from the collection of Istituto Agrario, San Michele all'Adige, TN (Tab. 1a, Fig. 1). *V. vinifera* ssp. *silvestris* was sampled both in the wild (by Dr. O. FAILLA) and in the collection of Azienda Agricola 'Villa Banfi', Montalcino, SI (Tab. 1b, Fig. 1).

P r o t e i n e x t r a c t i o n was performed with a 0.2 M isoelectric solution of glycine (GIANAZZA *et al.* 1989) on the endosperm of 35 randomly selected seeds from various samples of self-pollinated *V. vinifera* using a tissue to buffer ratio of 1:10 (TEDESCO *et al.* 1991). Tissues were crushed in a ice-chilled mortar, the slurry suspended in the extraction medium, stirred for 1 h at 4 °C and centrifuged twice 20 min at 12,000 rpm.

T a b l e 1

List of samples under investigation of (a) *V. vinifera* ssp. *sativa* and (b) *V. vinifera* ssp. *silvestris*

(a)

Vitis vinifera* ssp. *sativa

Neyret	***Val d'Aosta***	1
Vien de nus		
Barbera 1		
Barbera 2		
Nebbiolo		
Freisa	***Piemonte***	2
Bonarda		
Lambrusca di Alessandria		
Favorita		
Malvasia di Casorzo		
Pignola		
Croà rosso		
Croatina		
Durella		
Moradella		
Moretto	***Lombardia***	3
Timoraccio		
Uva d'oro		
Schiava lombarda		
Trebbiano di Lugana		
Trebbiano Valtenesi		
Lagrein		
Lambrusco foglia frastagliata		
Marzemino		
Schiava gentile		
Schiava grossa	***Trentino***	4
Schiava grossa 1		
Schiava grigia		
Trollinger		
Teroldego		
Cabrusina		
Corvina cl. 7		
Corvina cl. 47		
Dindarella		
Rondinella	***Veneto***	5
Rondinella cl. 77		
Rossetta di montagna		
Torbiana di Soave		
Trebbiano di Soave		
Malvasia di Asolo		
Cividino		
Fumat		
Picolit	***Friuli***	6
Refoscone		
Ribolla n.		
Ribolla spizade		
Coda di volpe di Lavico		
Coda di volpe di Vermentino		
Pigato	***Liguria***	7
Vermentino di Finale		
Lambrusco grasparossa		
Lambrusco Maestri		
Lambrusco oliva		
Lambrusco salamino		
Lambrusco di Sorbara		
Ancellotta	***Emilia Romagna***	8
Cagnina		
Canina		
Trebbiano romagnolo		
Malvasia di Candia		
Brunello		
Colorino		
Mammolo		
Prugnolo	***Toscana***	9
Sangiovese		
Malvasia lunga Chianti		
Trebbiano toscano		
Vernaccia di S. Gimíniano		
Malvasia bianca	***Lazio***	10
Malvasia di Candia non ar.ca:		
Aglianico	***Campania***	11
Greco bianco		
Grechetto bianco	***Umbria***	12
Vermentino spoletino		
Malvasia di Lecce	***Puglia***	13
Verdicchio	***Marche***	14
Cannonau		
Vernaccia	***Sardegna***	15
Carignano		

(b)

Vitis vinifera* ssp. *silvestris

Canalone 2			
Canalone 4	*Villa*		
Madonnina 8	*Banfi*		
Canalone nuova			
Chiesetta			
Iterzi 1			
Iterzi 2	*Poggio*		
Reppi 1	*del*		
Reppi 2	*Sasso*	*Ombrone*	
Reppi 3			
Pioppeto 3			*Toscana* **A**
Ombrone nuovo			
Pioppeto 3 bis	Strada		
Ponte 5			
Badia Ardenga 1			
Badia Ardenga 2	*Badia Ardenga*		
Castelgiocondo			
Guinzone	Guinzone		
Rigonsano 1			
Rigonsano 3	*Rigonsano*		
Rigonsano 5			
Adelio			
Adelio 2			
Ch 1			
Sinni 1			
Sinni 3			
Sinni 5a			
Sinni 5b	*Basilicata* **B**		
Sinni 5c			
Sinni 6			
Sinni 7			
Martignano Lago			
Martignano Strada			
Bolsena	*Lazio* **L**		
Ponte S.Pietro 1			
Ponte S.Pietro 2			
Ponte S.Pietro 3			
Vialta	*Veneto* **V**		
Vallarom	*Trentino* **T**		
Biferno	*Molise* **M**		

Isoelectric focusing was run on immobilized pH gradients (IPG) (Bjellqvist *et al.* 1982) with a T4, C4 polyacrylamide matrix (T = % total monomer concentration in a polyacrylamide gel, C = relative % of the cross-linker). An exponential gradient course with a range pH 4 - 10 (Gianazza *et al.* 1985) was chosen in order to resolve the native proteins and the isozymes of esterase (EST), phosphoglucomutase (PGM) and alcohol dehydrogenase (ADH). Acid phosphatase (AcP) and peroxidase (POD) were resolved on pH 4.5-7, gluconate-6-phosphate dehydro-genase (G-6-PDH) on 4-6 and storage protein subunits on 4.0-5.5 linear gradients (Gianazza *et al.* 1984). The slabs were polymerized (Righetti *et al.* 1990), dried and reswollen in 0.5 % w/v carrier ampholytes (CA) of the relevant pH range (for the non linear 4-10 range, the following mixture was used: 0.14 % Pharmalyte 3-10, 0.1 % 4-6.5, 0.14 % Ampholine 3.5-10, 0.12 % 4-7, 0.1 % 4-6, from Pharmacia LKB). For the analysis of native total proteins, CA concentration was increased to 2 % w/v. For protein analysis under reducing and/or dissociating conditions, the protein extracts were diluted 1:1 with 8 M urea, with or without 2 % 2-mercaptoethanol, and applied to a slab which was reswollen in 8 M urea. Samples were loaded near the cathode; the separation was run overnight at 10 °C (15 °C in presence of urea) at 50 V/cm, then 1 h at 150 V/cm. The protein stain was carried out with Coomassie Blue (Righetti and Drysdale 1974).

Zymograms: The enzyme activity was revealed by incorporating the relevant chromogenic substrates and cofactors into 1 % agarose (high electroendoosmosis - low gelling temperature; from Merck) which was poured on GelBond foils (FMC Corp.). The sandwiches of IPG slab and agarose overlay were then incubated in a 37 °C oven for about 2 h. The staining mixtures were as follows:

G-6-PDH (Stuber and Goodman 1980) and *PGM* (Spencer *et al.* 1964): gluconate-6-phosphate in pH 7 Tris buffer, or glucose-1-phosphate and glucose-6-phosphate dehydrogenase in pH 8 Tris buffer, respectively, NADP, phenazine methosulfate (PMS), methyl thiazolyl blue (MTT).

ADH (Smith *et al.* 1971) and *malic dehydrogenase* (Davidson and Cortner 1967): ethanol, or malic acid, in pH 8 Tris buffer, NAD, MTT and PMS.

AcP: α-naphthyl-phosphate in pH 4.5 citrate (Swallow and Harris 1972).

EST: α-naphthyl-acetate in pH 6.5 phosphate buffer (Coates *et al.* 1975); color development with Fast Blue RR.

Peroxidase (POD): The IPG slab was shaken in the dark at room temperature in a pH 7.0 phosphate buffer containing hydrogen peroxide, phenol, NADH and nitro blue tetrazolium (NBT) (Taketa 1987).

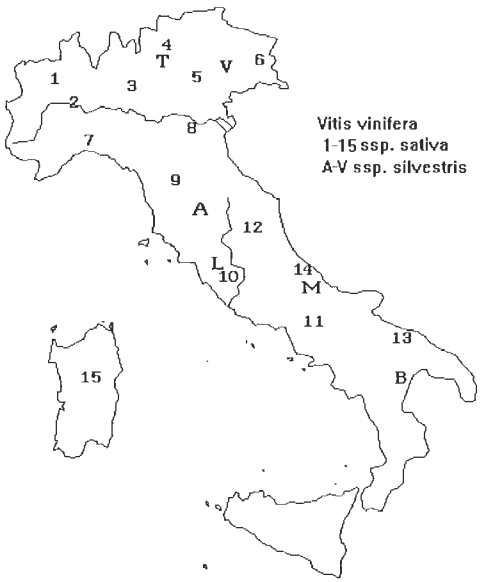

Fig.1: Geographic distribution of samples from Tab. 1.

Statistical analysis of the results was carried out using the NTSYS-pc 1.3 programme (Rohlf 1987) on an Olivetti M250 microcomputer. For each sample, a binary chart was compiled to include distinctive bands on the basis of their presence (1) or absence (0). Similar sample matrices were computed from these data using the Jaccard coefficient. Dendrograms relating the different samples were then created via the UPGMA clustering method (Unweighted Pair-Group Method using Arithmetic Averages). The evaluation of the cophenetic correlation coefficient was the criterion used to determine the relationships observed among its Operational Taxonomic Units (OTUs) (Sneath and Sokal 1973; Orloci 1975; Sokal 1986; Sneath 1989). This was then verified using the recommandations of several researchers (Burgmann and Sokal 1989; Sneath 1989).

Results

Total proteins under native and denaturing conditions: Total protein extracts from *V. vinifera* sspp. *sativa* and *silvestris* seeds were analyzed both under native conditions (on 4-10 IPGs reswollen in 2 % CA in order to improve globulin solubility at and near pI) and in the presence of 8 M urea. The latter treatment (i.e. exposure to a dissociating but not a reducing medium) splits the quaternary assembly of the major storage protein, ampelin, to 60 kDa subunits, which focus neatly above pH 6 (Gianazza *et al.* 1989; Gianazza *et al.* in preparation). In the former case 33 protein bands were resolved while in the latter, 42. With native proteins the inter-sample variability was negligible, and it was only marginal with protein subunits; hence, these parameters are of little use for any taxonomic analysis (see Discussion).

Storage protein constituent peptides: Releasing ampelin building blocks (-S-S-bridged acidic 40 kDa and basic 20 kDa peptides) with a reducing agent (Gianazza *et al.* 1989; Gianazza *et al.* in preparation) disclosed great banding variability, mostly due to the acidic peptides. Samples were therefore screened on a 4-5.5 gradient to optimize the resolution. In Fig. 2 are shown (as example) the patterns of some *V. vinifera* ssp. *silvestris* plants and a sketch of all the common and variable bands

of *V. vinifera* sspp. *sativa* and *silvestris* together with their numbering. This score results are given in Tab. 2 (a 0-1 array for absence-presence of distinctive bands in individual samples).

The dendrogram of Fig. 3 which includes all 104 (65 + 39) specimens under investigation, shows a clear-cut discrimination between ssp. *sativa* and ssp. *silvestris*. In ssp. *sativa* extracts, overall 47 components were resolved; only 3 bands (# 21, 24 and 53) are common to all biotypes investigation while bands 14, 18, 25, 32, 36 and 47, observed in *V. silvestris,* were missing in all. The statistical analysis of the uncommon band distribution of the dendrogram of Fig. 3 (*sativa*) showed dichotomies at various levels; for certain samples the protein pattern were similar at similar geographical locations. For ssp. *silvestris* the banding repertoire includes 51 components, 6 of which are present in all protein extracts (10, 12, 17, 22, 24 and 46) while 2, 7 and 16 did not occur. Cluster analysis of Fig. 3 (*silvestris*), did not show differentiation of biotypes originating from Southern and Central Italy. When the two sets of data were merged together, a total of 53 individual components could be listed (Tab. 2 as example). Just one of these bands, #24, was common to all members of both groups while bands #21 and 53, were distinctive of the ssp.*sativa* in comparison to bands #10, 12, 16, 22, 46 for ssp. *silvestris*

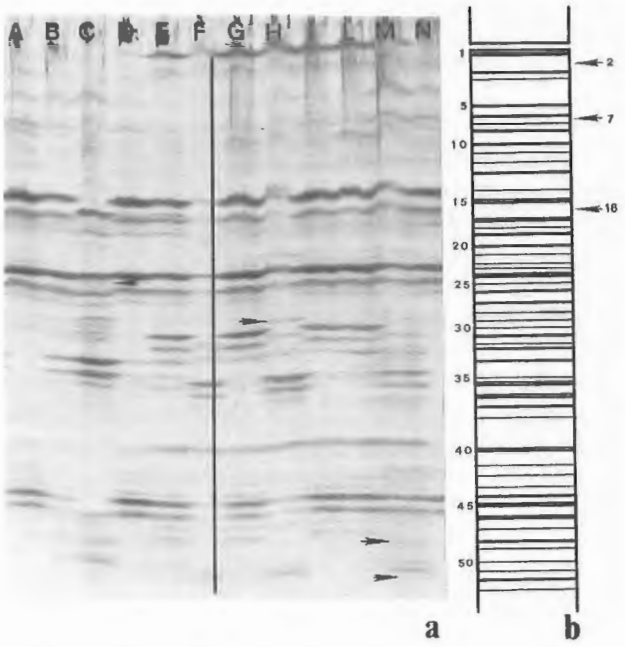

Fig. 2: Isoelectric focusing of some *V. vinifera* ssp. *silvestris* samples under investigation in the pH range 4-5.5, in presence of 8 M urea and 2 % 2-mercaptoethanol. The sketch shows the peptide bands identified in the samples, and their relative number. Bands 2, 7, 16 indicated by arrows are absent by this samples. **A** Martignano lago, **B** Sinni 5c, **C** Sinni 5b, **D** Sinni 7, **E** Sinni 5a, **F** Sinni 6, **G** Sinni 3, **H** Sinni 1, **I** 322/2, **L** Rigonsano 5, **M** Rigonsano 3, **N** Rigonsano 1.

Details on enzymes: Details of the results, the 0/1 matrices describing absence/presence of individual bands, had been arranged in 5 plates for referee's convenience; this data will be made available to collegues upon request.

A c P : Acid phosphatase isozymes were evenly resolved over a 4.5-7 IPG (Fig. 4). Sixteen different elements (Fig. 4 d) were identified for ssp. *sativa* banding patterns (Fig. 4 a and b); none of these showed distinctive subspecies markers, each component was represented in both ssp. *sativa* and ssp. *silvestris* samples (compare with Fig. 4 c). An attempt to group into clusters gave no correlation to geographical distribution nor to morphological characters (such as berry color). *V. vinifera* ssp. *silvestris* specimens gave a total of 12 active bands, 3 of them common to all members of the group. The dendrogram in Fig. 5 compares all the samples under investigation. The two subspecies did not segregate but the homogeneous groups, instead, showed branching at different levels to include either *sativa* or *silvestris* specimen.

A D H : The specific activity of alcohol dehydrogenase was highly variable. It was near zero for all ssp. *silvestris* seed extracts and for 30 of the 66 cultivars of ssp. *sativa*. However, whenever present, 10 bands were observed focusing in the pH range 5.8 - 7.5. These data which rely on quantitative instead of qualitative variation were not processed further (see Discussion).

E S T : Esterase isozymes showed a very complex focusing pattern, including 40 bands spread over the entire 4-10 pH range. In all samples of both sspp. *sativa* and *silvestris* 28 components were constant. The varying elements were clustered in the neutral to slightly alkaline pI region. An attempt to compare *sativa* cultivars to *silvestris*

Table 2

0/1 entries for absence/presence of individual peptide bands in some *V. vinifera* ssp. *silvestris*

	01	05	10	15	20	25	30	35	40	45	50
A Martignano lago	1 0 0 1 1 0 0 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 0 1 0 1 0 0 0 0 1 1 0 1 1 1 1 1 1 1 1 0 1 1 1 0 0 0
B Sinni 5c	0 0 0 1 1 0 0 1 1 1 0 1 1 1 1 0 1 1 1 1 1 1 1 1 1 0 1 1 0 1 0 1 0 0 1 1 1 1 1 0 1 1 1 1 1 1 1 0 1 1 1 0 0
C Sinni 5b	0 0 0 1 1 0 0 0 1 1 0 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 0 0 1 1 0 1 1 1 1 1 1 1 1
D Sinni 7	1 0 0 1 1 0 0 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 0 1 0 1 0 1 0 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 0 0 1 1 1 1 1
E Sinni 5a	1 0 1 1 1 0 0 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 0 1 0 1 0 1 1 1 1 0 1 0 1 1 1 1 0 1 1 1 0 1 1 1 1 1 1 1 1 0 1 1 1 1
F Sinni 6	1 0 1 0 1 1 0 0 1 1 1 1 0 0 1 0 1 0 1 1 1 1 0 1 0 1 1 1 1 0 1 1 0 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 0 1 1 0
G Sinni 3	1 0 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 0 1 0 1 1 1 1 0 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 0 0
H Sinni 1	1 0 1 1 1 1 1 0 1 0 1 1 1 1 1 1 1 0 1 0 1 0 1 1 1 1 1 1 0 1 1 1 0 1 1 1 1 1 0 1 0 1 1 1 1 1 1 1 1 1 1 0 0 1 1 1 0
I 322/2	1 0 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 0 1 1 1 1 0 1 0 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 0 1 1 1 0
L Rigonsano 5	1 0 1 1 1 1 1 0 1 1 1 1 1 1 1 0 1 0 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 0 1 1 1 1 0 1 1 1 0 1 1 1 1 1 1 1 1 1 0 1 1 1 0
M Rigonsano 3	1 0 1 1 1 0 0 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1
N Rigonsano 1	1 0 1 0 1 1 0 1 1 1 1 1 1 0 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 1 0 0 1 1 1 0 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1

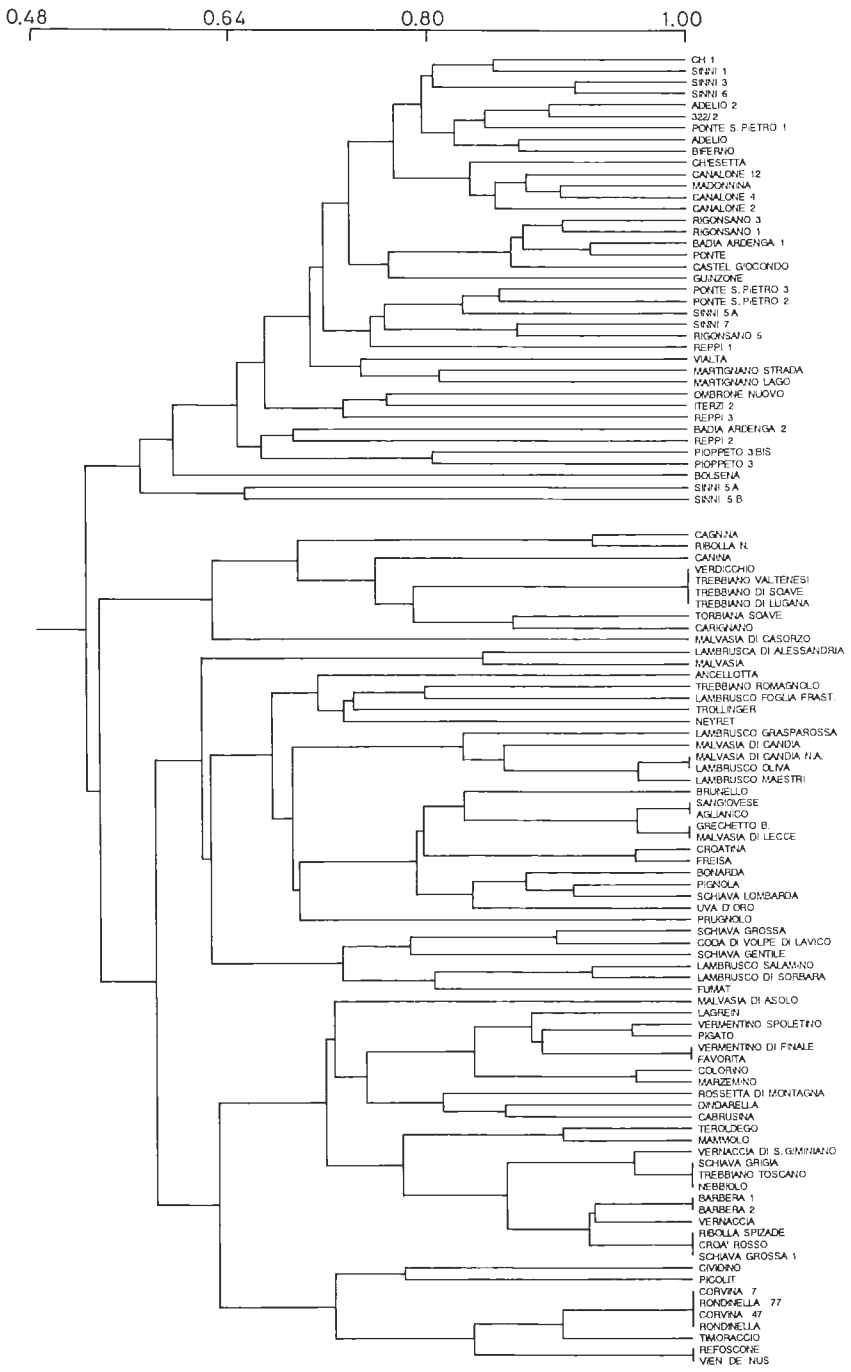

Fig. 3: Comparative dendrogram of *V. vinifera* sspp. *sativa* and *silvestris* computed from data on peptides.

based on geographical distribution or morphological affinity was inconclusive.

G - 6 - P D H : Ten zones in the pH range 4-6 were reactive to gluconate-6-phosphate dehydrogenase *V. vinifera* ssp. *sativa* samples correlated to each another whereas for *silvestris* specimens only 3 typical banding patterns were obtained. Once again, no correlation to environment, morphology, or geographical origin was found.

M D H : All ssp. *sativa* samples exhibited the same quali- and quantitative distribution, with 35 bands lying in the pH 4-10 range. The dendrogram was therefore dispensed of for ssp. *silvestris* seed extracts.

P G M : The focusing pattern of PGM in the pH range 5 - 6.5 showed that specific activity of this enzyme was usually low, and hardly any bands were detectable for ssp. *silvestris* samples. 11 bands were resolved in ssp. *sativa*, and variability was scarce; these data were not processed further.

P O D : Whenever present, peroxidase was always resolved in 8 distinct isobands.

Discussion

The wealth of data obtained in this study was assembled in the dendrogram (not shown) which includes data from 4 biochemical markers (storage protein constituent peptides, AcP, EST, G6PDH). The scoring procedure was

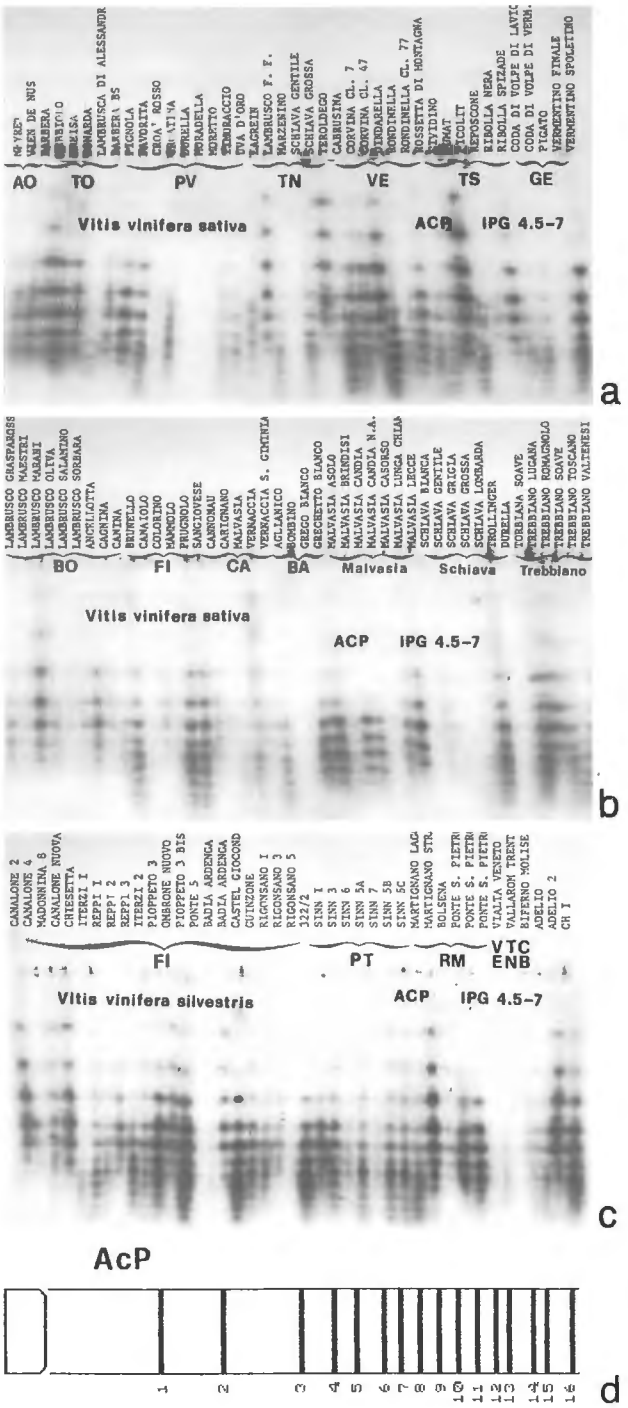

Fig. 4: Banding pattern for acid phosphatase (AcP) after isoelectric focusing on a 4.5-7 IPG. *V. vinifera* ssp. *sativa* (panels a and b) and ssp. *silvestris* (panel c) samples are listed according to their geographical origin. d: schematic drawing of the enzyme bands identified in the samples under analysis.

based on a qualitative evaluation, that is, the presence/absence of individual bands. This approach however, was unable to fully exploit the information provided by, for example, the zymograms of ADH and PGM. In these cases, the contrast in terms of specific enzyme activity in *V. vinifera.* ssp. *sativa* versus ssp. *silvestris* samples was striking, but no scoring value was associated to lack of a detectable signal. A similar conclusion can be made for data on the total native protein distribution: even if the number of resolved bands was constant throughout, the relative intensity of the different components was found to be highly variable; this finding was not entered into a discrimination grid. Any analysis which includes and evaluates quantitative differences should require the use of both a complex image analysis system (a few of them are currently available on the market) but also a different and more careful processing of the samples. In fact, to avoid underestimation or missing of minor isozymes, identical loading for functional stains should be based on enzyme activity instead of standard sample extraction or protein quantitation. Statistical analysis as well should be approached from a different standpoint (SOKAL 1986). The above results have been evaluated along two guidelines: *V. vinifera* biotypes characterization and ssp. *sativa* versus ssp. *silvestris.*

Vitis vinifera biotype characterization: The dendrogram in Fig. 3 is difficult to interpret. Certain clusters are significantly valid in that they can be associated to well-known characteristics while others seems to show no apparent explanation. One can observe that by using protein subunits the Verdicchio, Trebbiano di Soave, Trebbiano di Lugana and Trebbiano di Valtenesi seem to be similar genetic entities. A more important clustering is that made up of Vermentino spoletino, Pigato, Vermentino di Finale and Favorita which are vines from a single biotype population (Vermentino-Rolle-Valentine). On the other hand, Bonarda, Pignola, Schiava lombarda and Uva d'oro; Rossetta di montagna, Dindarella, Cabrusina; Cividino, Picolit; Corvina, Rondinella show yet another clustering pattern and even if they show great morphological variability, they all come from the same geographical region. The 1st group of these vines is autochtonous of the Lombard-Piemontese plain; the 2nd and 4th group of vines originate in the Adige valley and Verona's hillsides and the vines of the 3rd group come from the Friuli area.

In comparison to conventional ampelographic methods, a procedure which relies on the electrophoretic analysis of biochemical markers has the disadvantage of being more complex and labour intensive. The advantages, on the other hand, are manifold. Biochemical markers - mainly seed protein make-up - have been shown to express a plant's genotype. In ampelography, the comparison lies between given organs of different individuals; in chemotaxonomy, instead, it lies between parametrical entries. The identification of the different biotypes through the 0/1 arrays as in Tab. 2, may be added over time, and can be easily stored in a data base and retrieved for future comparison. It is mandatory in this respect that band identification in a given sample be unambiguous. Besides the general strategy of including a reference along with test samples, the technology of immobilized pH gradients (IPG) for protein isoelectric fractionation used in this investigation offers both reproducibility and high resolution, mainly because the width of the pH ranges may be tailored for the optimal fractionation of individual components. The outcome of the proposed procedure may thus be the positive identification of each ssp. *sativa* cultivar.

V. vinifera ssp. *sativa* versus ssp. *silvestris:* The distribution of the various biochemical

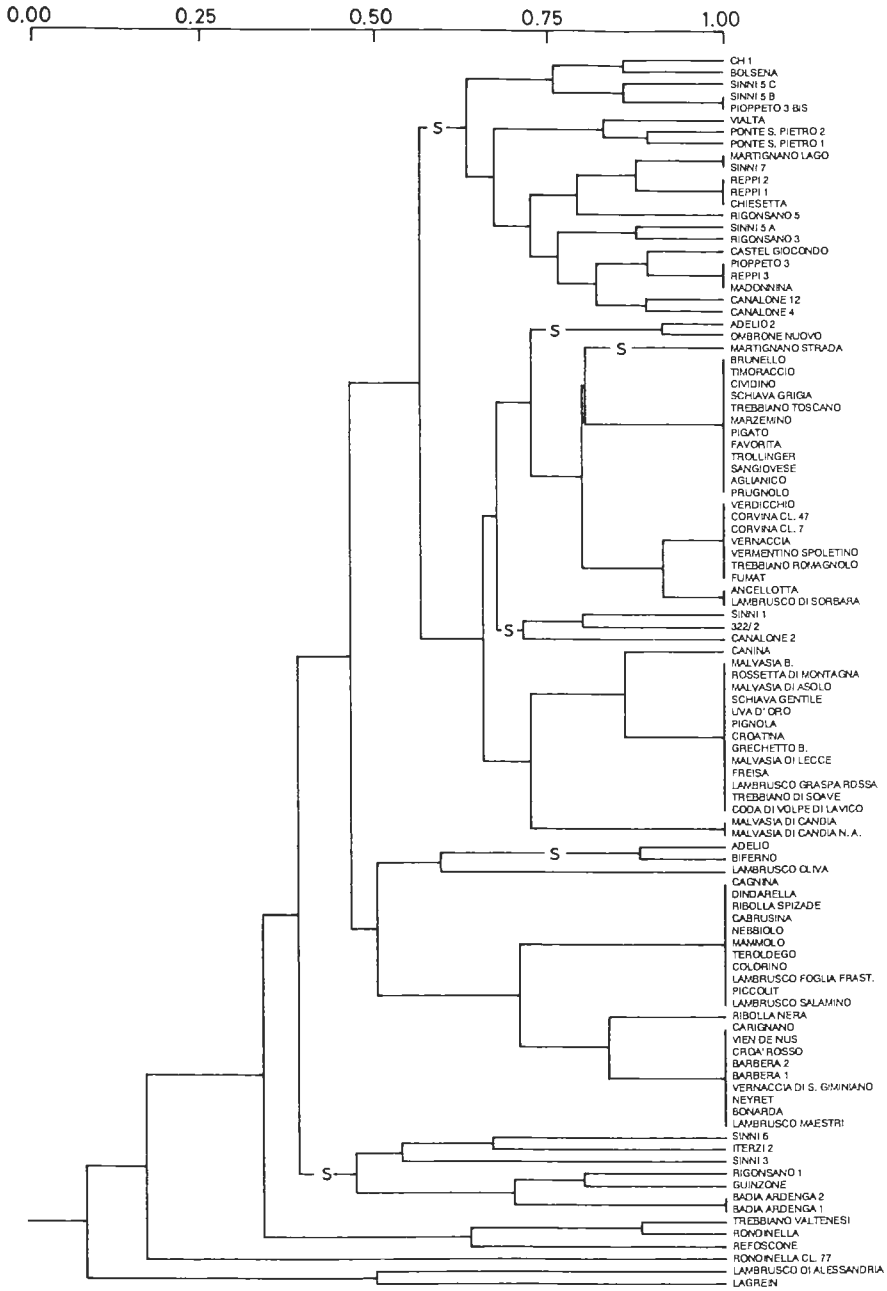

Fig. 5: Comparative dendrogram for AcP of all samples investigated (from data in Fig. 4). **S** = *V. vinifera* ssp. *silvestris*.

markers between sspp. *sativa* and *silvestris* biotypes is not unequivocal, hence the hierarchic arrangements defined by the clustering procedure vary for each different parameter (compare the dendrograms based on storage protein peptides - Fig. 3 - and on AcP - Fig. 5). From the lowest branching level, for no enzyme are all the specimen from the two subspecies grouped together. On the contrary, one notes identical banding patterns and no disjunction with each enzyme for panels of sspp. *sativa* and *silvestris* biotypes. Similarly, the complete homogeneity in the banding pattern of enzymes MDH and POD is non-informative with regard to variability, hence its ability to characterize and discriminate the different individuals. This feature, however, could also correspond to a genus, or species-specific invariant trait.

Conflicting evidence derives from other sets of data. As mentioned at the beginning of this section, the specific activity for PGM, and often for ADH, is always much higher in ssp. *sativa* than in ssp. *silvestris*. This could be interpreted as a genetic difference, not at the level of the structural gene but involving some regulatory element(s). In any case, the two subspecies should be recognized as different at the phenotype level for the character being investigated. Within the limits of the scoring system detailed above however, an effective discrimination between ssp. *sativa* and ssp. *silvestris* is observed only when storage protein peptides are the reference parameters (Fig. 3): in this case the two sets of samples segregate from the lowest branching level of the dendrogram.

In contrary to other arrangements, for which no correlation could be detected to any other parameter (see Results), some of the groups in Fig. 3 (*sativa*) correspond to either the presence of peculiar morphological characteristics or close geographical origin or similar habitats. This

correlation however could not be generalized to include all biotypes. Such a limitation could be due to an insufficient number of samples analyzed in the present investigation (only 65) in comparison to the ca.1,000 and over cultivars grown across Italy. One might speculate that only a continuum of elements could give an adequate historical representation of the spread of the different varieties, while in a few cases, identical names are given to different vines or, on the contrary, different local names would eventually specify the same biotype.

The discrimination between sspp. *sativa* and *silvestris* is maintained when all data are merged to give the comprehensive dendrogram (not shown): the divergence between their storage protein patterns overcomes some uniformity of enzyme isoforms. Heterodispersion is, in most cases, more extensive for storage proteins than for enzymes. The former are usually encoded by multiple gene copies, whose codominant expression speeds up the accumulation of aleurone grains in the seed endosperm; divergence as a result of mutation may easily arise within such gene families. Selective pressure, on the other hand, is likely to occur randomly. The function of storage proteins is just the accumulation of aminoacids to be metabolized upon germination and an almost infinite number of structures might carry out this role. The sole requirements seem to be that the storage granules be efficiently packed - which entails some type of balance between their hydrophilic and hydrophobic regions - and that protease target sequences are preserved. Naturally, structural constraints have to be much more stringent in order to preserve enzyme activity. From this study one can conclude that the enzymes selected for screening - although distinct for each subspecies - cannot differentiate *V. vinifera* ssp. *sativa* from ssp. *silvestris* as the assortment of their isoforms are similar. Only the characteristic storage protein bands and the small amount of intermixing with regards to most enzyme banding patterns presented in this paper provides evidence of a clear-cut separation between *sativa* and *silvestris* specimens and for this reasons one can rule out the hypothesis of a possible derivation of one biotype from the other with time.

Acknowledgements

This research was funded by the Ministerio Italiano dell'Agricultura in the framework of the project "Resistenze genetiche delle piante agrarie agli stress biotici e abiotici" and in part by a grant from Ministero della Pubblica Istruzione, Roma (Fondi 40 %).

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Received March 9, 1994