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Characterization of *Vitis vinifera* L. subspecies *sylvestris* (Gmelin) Hegi in the Ebro river Basin (Spain)

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Summary

A wild grapevine population of 76 vines of *Vitis vinifera* L. subspecies *sylvestris* (Gmelin) Hegi was found along the Iregua river valley (Northeastern Spain), located in the Ibérica mountain-range. The characterization of this phylogenetic resource was based on an ampelographic description of the male and female individuals and an evaluation of their sanitary state. This shows that the imported North American downy and powdery mildews are the main pathogens, but no symptoms caused by phylloxera on roots were found. Different degrees of infestation caused by Eryophid mites, *Colomerus vitis* and *Calipitrimerus vitis*, were also registered on leaves. On the other hand, the red wines obtained show an alcoholic degree situated between 8.5 and 10.8, pH around 3.5 and a high intensity of colour, between 10.2 and 11.3. The vinegar had only 3.5° of acetic acid. Its phenolic composition is similar to those reported from red and Sherry vinegars produced from cultivars. The genetic analysis based on 18 samples, using 16 nuclear microsatellites, shows a low genetic diversity ($He = 0.45$). This might be due to inbreeding caused by mating among siblings in this isolated population. The genetic comparison with the allowed cultivars of this Guarantee of Origin showed two distinct gene pools. So it indicated that there is no genetic contribution of these native vines to current instead of actual local varieties. It is necessary to preserve this phylogenetic resource to be used in breeding programs and to restore its destructed habitats by human different impacts.

Key words: ecology, genetic study, La Rioja (Spain), parasites, vinegar, wine.

Introduction

The Eurasian wild grapevine belongs to the taxon *Vitis vinifera* L. subspecies *sylvestris* (Gmelin) Hegi. This is a dioecious relative of cultivated varieties, which are mostly hermaphrodites (OLMO 1995, ZOHARY and HOPF 1994). The anthropical action is destroying wild grapevine habitats. So, this subspecies appears as an endangered plant in the red list published by the International Union for Conserva-

tion of Nature (IUCN 1997). According to studies conducted with chloroplastic microsatellites, the presence of certain haplotypes in western varieties which are not present in Transcaucasian wild grapevines indicate the possible existence of other secondary outbreaks of domestication (ARROYO-GARCIA *et al.* 2006). The aim of this paper is a holistic characterization of a wild grapevine population found in the riparian forest of the Iregua river, a tributary of the Ebro river, called *Iberus flumen* by Romans, which gave the name to the Iberian Peninsula. This population extends along the road N-111, parallel to Iregua river, since the village of Castañares de las Cuevas (km 311) (N 42° 18' 26.7"; W 2° 33' 24.4") until the km 305 (N 42° 16' 21.3"; W 2° 35' 39") on fluvisols, like the majority of the locations carried out in Southern Europe (ARNOLD *et al.* 1999, OCETE *et al.* 2002), with an average altitude around 600-700 m. A part of the population is concentrated along the Fuente del Perro creek (Nestares), between N 42° 16' 4"; W 2° 35' 2" N and 42° 16' 42.8"; W 2° 35' 16.2". Genetic data were compared with those belonging to allowed cultivars in La Rioja wine producing area. However wine and vinegar were produced from wild grapevines in forests from the antiquity to present time (OCETE *et al.* 2007), an evaluation of the qualities of both were included.

Material and Methods

A prospection of the Iregua river valley was carried out during the flowering stage of the wild grapevines (2nd-4th week of June). Individuals with clear ampelographic characteristics of the wild subspecies were georeferenced using GPS.

Ampelographic description: The ampelographic characterization of the female and male individuals, along their phenological development, was carried out according to the criteria exposed by IPGRI, UPOV and OIV (1997). Seeds were collected from a group of 30 mature berries in the second week of October from 4 vines, with available harvest, in a small area under the same microclimate conditions. When the seeds had been extracted, 50 were chosen at random and their length (L) and width (A) were measured. The coefficient A/L (STUMMER 1911) was calculated for each wild seed and 50 cultivated seeds from a no clonal Tempranillo cultivar (90 years old). Average values of coefficients obtained were analyzed by ONE

WAY ANOVA test ($p \leq 0.05$) and were compared according to RENFREW (1973).

Parasites: The identification of phytophagous arthropods and pathogens was carried out on roots and the aerial organs of the vines in summer time, from 2004 to 2008. Roots were discovered up to a maximum depth of 40 cm. The observation of symptoms on shoots, sarmets, leaves and bunches were made to a maximum height of 3.5 m. Elisa test to determine the presence of Grapevine fanleaf virus (GFLV) was made on 3 leaves of each plant, according to the procedure of GUGERLI *et al.* (1984).

Wine study: Harvest took place in 2004, at the second week of October. Wines were obtained with indigenous yeasts, with a maceration of 10 d without an addition of potassium metabisulphite, at 20 °C. Analytical data were obtained from microvinifications of 4 isolated vines and from a sample of wine elaborated with must obtained from 20 female vines. The methods used for oenological analysis are shown in Tab. 1, according to procedures of MINISTERIO DE LA PRESIDENCIA (1977) and EEC (1990).

Vinegar study: Vinegar was obtained by traditional acetification process from the mixture wine. Dry extract and acidity were determined according to Spanish Official Methods (MINISTERIO DE LA PRESIDENCIA 1977). The total phenols index (TPI) was determined by the Folin-Ciocalteu micro-method (WATERHOUSE 2001). The total monomeric anthocyanins (TA) were estimated by a pH differential method (GIUSTI and WRÖLSTAD 2001). Ethyl acetate, acetaldehyde, methanol, ethanol and propanol were quantified by GC-FID using the method proposed by MORALES *et al.* (2001). Minor volatile compounds were determined by Headspace Sorptive Extraction GC-MS Analysis (HSSE-GC-MS) (CALLEJÓN *et al.* 2008). HPLC analysis of phenols was performed using the rapid method proposed by IBERN-GÓMEZ *et al.* (2002).

Descriptive sensory test was carried out by an expert panel in wine vinegar sensory analysis composed of seven tasters (five females and two males). All members were trained according to international protocols. 15 ml of vinegar sample was presented in dark glass covered with plastic dish. The wine vinegar sensory profile was built with previously established descriptors (TESFAYE *et al.* 2008). The selected attributes were compiled in a tasting-card and panellists were asked to rank each descriptor on a 10 cm unstructured scale (from not noticeable to very strong).

Genetic study: DNA extraction and PCR amplification: Total genomic DNA was ex-

tracted from young leaves, using the DNeasy™ Plant Mini Kit (Qiagen). Extracted DNA was quantified and used as a working DNA solution of 10 ng·µl⁻¹. A set of 16 microsatellite loci well scattered on the genome: VVMD5; VVMD7, VVMD21, VVMD24, VVMD25, VVMD28, VVMD32 (BOWER *et al.* 1997, 1999); VVIn16, VVIv67, VVIv37, VVIq52, VVIp60, VVIh54, VVIb01, VVIp31 (MERDINOGLU *et al.* 2005); VVS2 (THOMAS and SCOTT 1993). Six of these markers belong to the core set chosen by the international grape community: this allows comparison of our data with the local varieties from La Rioja.

Amplification reactions were performed in a total volume of 20 µl with 30 ng of DNA template, 0.25 to 0.5 µM of forward primer labelled either with 6-FAM, HEX, NED or PET fluorophore, 0.5 µM of non-labelled reverse primer, 150 µM of each dNTP (Boehringer, Mannheim, Germany), 2.5 mM MgCl₂, 1X buffer AmpliTaq and 0.8 units AmpliTaq polymerase (PE/Applied Biosystems, Foster City, CA). The PCR was carried out using a GeneAmp PCR system 9700 thermocycler (PE/Applied biosystems). The cycling program consisted of the following steps: 10 min 94 °C followed by 35 cycles of 45 s at 92 °C, 1 min at (5,257 °C) according literature and 1 min 30 s at 72 °C and a final extension step of 5 min at 72 °C. Labelled amplification products were resolved onto an automated 310 ABI PRISM DNA sequencer (PE/Applied Biosystems), using a HD400-ROX as an internal size standard. Allelic data were cored using GENEMAPPER 3.0 software and the genotype of each sample was determined.

Genetic diversity values were measured for nuclear microsatellites by estimating the average gene diversity or expected heterozygosity (*He*). These genetic parameters were calculated using GENALEX. This program was also used to analysed Principal Component Analysis in order to represent genetic relationship among individuals.

Results and Discussion

In the counting conducted in 2004 there were 76 individuals (44 male and 32 female). The cleaning of the riverbanks during 2005 winter reduced the number of vines to only 53, 25 females and 28 males.

Ampelographical description: Main ampelographical data are similar to those shown in Ocete *et al.* (2007). Each vine exhibits a wide range of leaf morphologies. Individuals of a greater size, some with more

Table 1

Analysis of microvinifications

Parameters	vine 1	vine 2	vine 3	vine 4	Mixture	Methods
Alcoholic degree	10.30	10.80	10.10	8.50	10.47 %	Near Infrared (NIR)
pH	3.53	3.48	3.45	3.40	3.48	Automatic Potentiometry
Total Acidity	8.85	8.20	8.22	9.50	5.94 g/l	Automatic Potenciometry
Volátil acidity	0.76	0.65	0.73	0.70	0.83 g/l	Autoanalyser FCSA
Tartaric acid	6.20	6.28	6.19	7.05	0.83 g/l	Autoanalyser FCSA
Malic acid	3.98	4.10	4.00	4.75	6.33 g/l	Autoanalyser FCSA
Intensity of color	10.2	11.4	10.3	11.5	3.70 g/l	O.I.V.

than 20 m height, are located along the Iregua riverbanks.

The number of flower bunches on male plants by shoot is usually 4. In the case of the female vines, usually 2-3. Male bunches have more flowers and are longer than the female ones. The number of berries per bunch is highly variable, being higher in those female grapevines located next to the male individuals. All female exemplars exhibited red berries, with a small size, with a maximum diameter around 1 cm. The scarce pulp is not colored. The harvest ranges between 152 g and 2.548 g·vine⁻¹.

This population (Average coefficient: 0.691) must be considered as wild, according to RENFREW (1973), and differs significantly from that belonging to the 'Tempranillo' cultivar (Average coefficient: 0.582) (ANOVA test: 75.81; p ≤ 0.05). Fig. 1 shows the frequencies of the Stummer's index over the whole wild population and the cultivar 'Tempranillo'. The higher values belong to wild seeds.

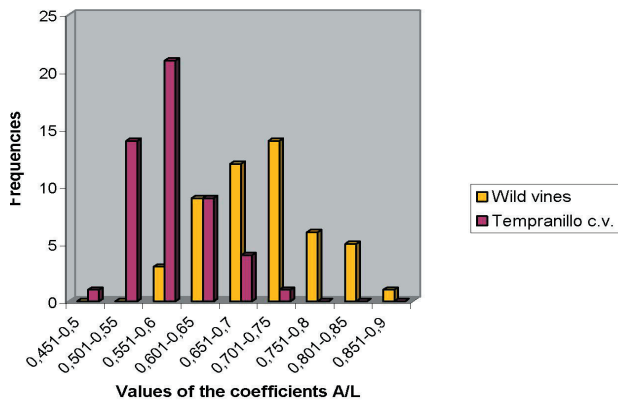


Fig. 1: Frequencies of the Stummer's coefficients.

Parasites: From 2004 to 2008 a list of symptoms caused by parasitic species was recorded (Tab. 2). No symptoms caused by the subterranean cycle of phylloxera, *Daktulosphaira vitifoliae* (Fitch) (Hemiptera, Phylloxeridae) and by Fanleaf (GVLV) virus were found in any sample. However, wild grapevines are sensible to phylloxera, as it was proven by OCETE and LARA (1994). It might be due to the high level of moisture of the soils, caused by

flooding, which prevents the presence of the insect. On leaves, the symptoms caused by the Erineum strain mite, *Colomerus vitis* (Pagenstecher) (Acari, Eryophiidae), can be found in all the vines. This mite is present in all European and North African populations observed (Ocete *et al.* 2007), so this mite could be a typical monophagous species on wild exemplars which along the development of the viticulture was imported to vineyards. About 50 % of the vines exhibited symptoms caused by another obligate parasitic mite, *Calepitrimerus vitis* (Nalepa) (Acari, Eryophiidae). These begin to appear during the phenological state E, when crisp leaves with slow growth and discoloured points, observable against the light, can be detected. Both mites are cosmopolitan eriophyoid pests on vineyards from different vineyards of all over the world including Australia (BERNARD *et al.* 2005).

During 2004, 2007 and 2008 summers, heavy symptoms were caused by downy mildew, *Plasmopara viticola* Berl. & De Toni, on leaves and bunches induced by rainy weather and average temperatures about 21 °C. Also, symptoms caused by powdery mildew, *Uncinula necator* (Schw.) were detected.

Grapevines show quite different level of damages caused by pests and pathogens. This fact, together with the wide variation in ampelographic characters are the result of its biodiversity. It is an inherent characteristic of wild grapevines populations, where exists sexual reproduction. The absence of GFLV virus was confirmed by Elisa test results.

Wine study: The volume of must obtained is very small, only about 16 % of the berry weight. So more complete determinations, like total polyphenols, anthocyanins and tannins, etc was not possible. Tab. 1 shows the analysis of wines obtained from 4 vines elaborated separately and a sample made from 20 individuals. Ethanol concentration is low, not exceeding 11 % Vol. It could be due to microclimatology of the habitat which causes ripening differences in berries belonging to a single cluster. In Southern Spain, under a warmer climate, wines from wild vines reach 13.5 °C (OCETE *et al.* 2007). A remarkable fact is the high intensity of colour, due to the small proportion of must obtained from each berry and the high proportion of pigmented skin. The intensity of colour was 113, meanwhile in the Guarantee of Origin Rioja, a wine is considered red from a value of 3.5 before malolactic fermentation (Consejo Regulador de la Denominación de Origen Calificada Rioja 2001).

The total acidity shows a high value mainly due to the content of malic acid before the malolactic fermentation. In the majority of the Spanish vineyards, red cultivars make problems due to lower the total acidity of their musts. It is necessary to remark that total acidity and high tonality of colour may be of interest to transfer to traditional cultivars under warmy climatologies.

Vinegar study (Tabs 3 and 4): The vinegar presents TPI values slightly lower to those found in red wine vinegars from cultivars of *Vitis vinifera* L *sativa* (DC.) Hegi and higher TA (CEREZO *et al.* 2008). A total of seven phenolic compounds were identified. Caftaric acid was the major phenolic, followed by gallic acid and tyrosol. Regarding the

Table 2

Incidence of parasites from 2004 to 2008

	2004	2005	2006	2007	2008
<i>Colomerus vitis</i> (on leaves)	76	53	53	53	53
<i>Calipitrimerus vitis</i> (on leaves)	29	24	27	21	18
<i>Uncinula necator</i> (on leaves)	61	45	44	22	27
<i>Uncinula necator</i> (on bunches)	13	4	19	5	3
<i>Plasmopara viticola</i> (on leaves)	41	34	27	43	49
<i>Plasmopara viticola</i> (on bunches)	13	9	7	22	31

Table 3

Physicochemical parameters of vinegar	
	Mean \pm SD
Acetic degree (g per 100 mL)	3.5 \pm 0.0
Dry extract	1.8 \pm 0.01
Total Polyphenol Index ^a	1178 \pm 25
Total Monomeric Anthocyanins ^b	0.75 \pm 0.02
Phenolic Compounds (mg·L ⁻¹)	
Gallic acid	40.54 \pm 0.06
Protocatechuic acid	4.61 \pm 0.00
Tyrosol	12.4 \pm 0.1
Vanillic acid	3.31 \pm 0.01
(+)-Catechin	2.74 \pm 0.03
Cafaric acid	117.0 \pm 0.2
Caffeic acid	4.22 \pm 0.00

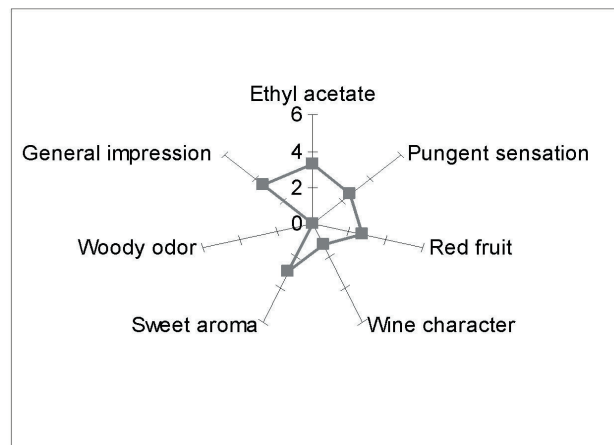


Fig. 2: Aroma profile of vinegar.

aromatic profile 47 compounds were determined. The main aromatic compound was ethyl acetate. The content of acetoin is within the range of concentration of this compound in traditional vinegars (TRONCOSO and GUZMAN 1987)

The results of a sensory descriptive test are shown in Fig. 2. As it can be seen in this figure, the sample presented low pungent sensation according to the low acetic degree. The value of ethyl acetate descriptor was in agreement with

the concentration of this ester in the sample. The woody aroma is due to the presence of isomers of oak lactones. These compounds were not detected by HSSE-GC-MS in the food seasoning so the panel did not detect the corresponding aroma in the sample. Hence the value of woody aroma descriptor was 0.

Genetic study (Tabs 5 and 6): The analysis of 18 wild individuals from Rioja using 16 nuclear mic-

Table 4

Volatile Compounds of vinegar

Volatile compounds ($\mu\text{g}\cdot\text{L}^{-1}$)	Mean \pm SD	Volatile compounds ($\mu\text{g}\cdot\text{L}^{-1}$)	Mean \pm SD
Aldehydes		Acetic esters	
Acetaldehyde* ¹	150.2 \pm 0.4	Methyl acetate*	13.9 \pm 0.3
2-Furfuraldehyde	328 \pm 32	Ethyl acetate* ¹	2619 \pm 15
Benzaldehyde	126.2 \pm 0.2	Propyl acetate	1530 \pm 30
Total aldehydes*	150.5	Isobutyl acetate	2264 \pm 27
Acetal		Butyl acetate	94 \pm 8
Acetaldehyde diethylacetal*	397 \pm 11	Isoamyl acetate*	6.2 \pm 0.1
Ethyl esters		Hexyl acetate	164 \pm 8
Ethyl propanoate	666 \pm 14	2-Phenylethyl acetate	729 \pm 4
Ethyl isobutyrate	321 \pm 8	Total acetic esters*	2644
Ethyl butyrate	64.1 \pm 2.5	Alcohols	
Ethyl 2-methylbutyrate	26.9 \pm 0.1	Metanol* ¹	71 \pm 4
Ethyl isovalerate	380 \pm 6	Etanol* ¹	5135 \pm 124
Ethyl hexanoate	155 \pm 8	1-Propanol* ¹	12.7 \pm 0.4
Ethyl lactate*	10.8 \pm 0.5	Isobutanol*	12.5 \pm 1.5
Ethyl octanoate	143 \pm 11	2-Methyl-1-butanol*	7.1 \pm 0.7
Ethyl furoate	34.7 \pm 0.2	3-Methyl-1-butanol*	53.4 \pm 1.4
Ethyl phenylacetate	81.2 \pm 0.3	1-Hexanol	1229 \pm 13
Diethyl succinate	1232 \pm 17	Cis-3-hexen-1-ol	84.3 \pm 0.3
Total ethylic esters*	13.9	Benzyl alcohol	188 \pm 6
Ketones		Furfuryl alcohol	1445 \pm 184
Acetoin*	364 \pm 34	2-Phenylethanol*	13.2 \pm 0.1
Lactones		Total alcohols*	5372
γ -Butyrolactone	3064 \pm 416	Acids	
Phenols		Isovaleric acid*	12.2 \pm 0.3
Guaiacol	14.5 \pm 0.9	Hexanoic acid	767 \pm 27
4-Ethylphenol	60.2 \pm 3.0	Octanoic acid	374 \pm 26
Total phenols	74.7	Decanoic acid	20.4 \pm 0.2
		Total acids*	

*: concentration in mg·L⁻¹; ¹ GC-FID; ² LLE-GC-MS

Table 5
Genotype of wild individuals. (RJ1-19)

RJ1	VVMD28	VVIVB01	VVMD5	VVMD27	VVMD25	VVS2	VVIV37	VVIQ52	VVIP67	VVIP60	VVIP31	VVIN73	VVIN16	VIH54	VVMD21	VVMD24
234	261	289	222	184	248	150	152	80	364	311	188	263	161	165	245	208
RJ2	234	289	222	184	248	150	152	80	364	311	188	263	161	165	245	208
RJ4	234	287	222	176	246	148	150	78	364	313	182	263	153	167	245	210
RJ5	234	287	226	176	246	148	150	78	364	303	188	263	153	167	245	208
RJ6	234	287	222	184	254	148	150	84	364	311	188	263	153	167	245	210
RJ7	234	287	224	184	246	156	164	80	364	303	182	263	153	167	245	208
RJ8	234	287	228	186	246	150	164	80	364	303	182	263	153	167	245	208
RJ9	234	287	228	186	246	150	164	80	364	303	188	263	153	167	245	208
RJ10	234	287	222	186	252	150	152	78	364	313	182	263	nd	149	245	210
RJ11	234	287	222	184	252	150	152	78	364	313	182	263	153	167	245	208
RJ12	234	287	228	184	246	156	152	78	364	313	182	263	161	165	245	208
RJ13	234	287	228	186	246	152	154	80	364	313	182	263	153	165	245	204
RJ14	234	287	228	184	246	152	154	78	364	311	nd	263	153	165	245	210
RJ15	226	261	287	184	246	154	152	78	364	311	182	263	153	165	245	204
RJ16	226	261	287	184	246	150	152	78	362	364	176	263	153	165	245	208
RJ17	226	234	287	184	246	150	152	78	362	364	176	263	153	165	245	214
RJ18	226	234	287	186	246	150	152	78	364	313	176	263	153	165	245	210
RJ19	226	234	287	186	246	150	152	78	364	313	176	263	153	165	245	208
cvrj1	261	261	289	184	240	142	144	84	368	327	180	256	151	163	245	208
cvrj2	246	246	287	194	240	135	144	88	358	365	176	184	153	163	241	216
cvrj3	250	261	289	182	240	142	144	82	362	374	176	178	151	165	247	214
cvrj4	246	261	289	180	262	137	150	88	358	365	180	191	151	165	247	214

Table 6

Genotypes of the wild accessions and cultivated grapevine from Rioja. Cvrj1; Tempranillo; cvrj2; Garnacha Tinta; cvrj3; Mazuelo; cvrj4; Graciano. Nd; no determined

Loci	Na	He
VVMD28	3,000	0,471
VVIVB01	3,000	0,452
VVMD5	5,000	0,569
VVMD27	4,000	0,625
VVMD25	5,000	0,708
VVS2	5,000	0,681
VVIV37	4,000	0,562
VVIQ52	4,000	0,576
VVIP67	3,000	0,290
VVIP60	5,000	0,548
VVIP31	4,000	0,701
VVIN73	2,000	0,327
VVIN16	3,000	0,439
VIH54	3,000	0,426
VVMD21	3,000	0,554
VVMD24	6,000	0,730
Mean	3,875	0,541

rosatellites showed a level of genetic diversity $He = 0.45$. This level is lower than the genetic diversity analysed in cultivated grapevine (THIS *et al.* 2006). This might be due to inbreeding caused by mating among siblings in small isolated populations, as revealed in the analyses. The genotype data can be obtained by the author on request.

A genetic comparison between wild grapes from the Iregua river and the local varieties 'Tempranillo', 'Garnacha tinta', 'Mazuelo', 'Graciano', 'Malvasia' showed two distinct gene pools. Principal Component Analysis as used to investigate the genetic dataset without detecting intermediate individuals (Fig. 3). Our results showed that wild grapevine samples and the varieties that are cultivated at the present time in La Rioja DOC clearly belong to two distinct genetic pools. It means there is not a genetic contribution of this native grapevines to present-day cultivars authorized in this region.

In conclusion, the maintenance of genetic variability within wild grape populations has become a priority primarily due to the concurrent risks of increased human impact on flood-plain areas and the spread of new pests. Fragmentation of species habitat will reduce both the number and size of the population, and decrease the genetic variation within populations. So the existence of different genetic pools within this population is remarkable and the conservation of this germplasm becoming more interesting. This population, as the rest situated in Spain, has not a specific preservation statute. It is necessary to take into account that Spain is the country with the largest area of vineyards all over the world: 1. 200. 000 ha, a 15 % of the full area (HUEZT DE LEMPS 2009), and it is affected by a heavy process of genetic erosion (OCETE *et al.* 2007). In consequence, there is an urgent need to bring this material that could be propagated to nurseries for use in the restoration of riparian

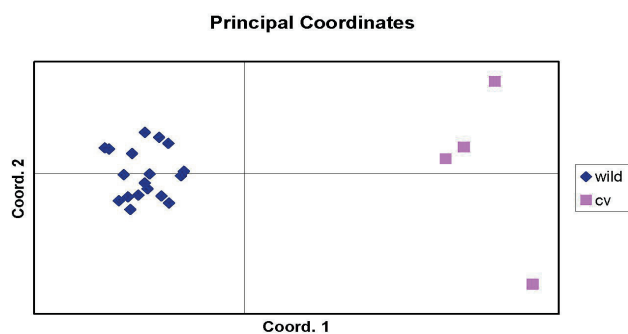


Fig. 3: PCA analysis between cultivated and wild individuals from Rioja.

forests and undertake breeding programmes of cultivars and rootstocks. Particularly, the low incidence of pests and diseases is remarkable, the high acidity of the wines and their high intensity of color total, interesting characteristics can be transferred by crossing with cultivars from Mediterranean areas. On the other hand, the immersion tolerance, absence of rot root and symptoms caused by nematodes could be interesting for obtaining new rootstocks, hybridizing with traditional rootstocks, specially when many vineyards have fertirrigation or are planted on clayey soils under a rainy climate, as it was indicated by OCETE (2010).

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