

# ***Juglans regia* L., phenotypic selection and assessment of genetic variation within a simulated seed orchard <sup>§</sup>**

Fulvio Ducci<sup>1</sup>\*, Anna De Rogatis<sup>1</sup>, Roberta Proietti<sup>1</sup>

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**Abstract** – Noble hardwoods are very important for the Italian furniture industry. Since 1985, approximately 170,000 ha have been planted in Italy with noble hardwoods. Among them, about 50% of species are represented by walnuts. Walnut (*Juglans regia* L.), not native in Italy, has been the focus of a substantial research effort for breeding and improvement programmes. The priority has been to preserve the *in situ* genetic resource still existing after intensive felling. Phenotypes suitable for timber production showing important traits such as straight stem, nice branch architecture, dominance and adaptation (phenology) have needed to be developed and selected. In order to reach this goals, selection of valuable progenies and the evaluation of the interaction *genotype x environment*, methods based essentially on a multi-trait Selection Index, were developed. Studies have been undertaken also to measure the variation of phenological traits, more correlated to traits valuable for architecture; in addition, neutral markers were used to assess genetic variation among different intensities of the adopted selections. The individual genetic component was found to be higher than at the inter-population level. Results showed that a hypothetical seed orchard made with progenies selected by morphology, phenology and genetic traits could provide material with a good performance and supply a variability similar to larger populations as the total plantation or the pseudo-natural system chosen for comparison.

**Key words:** *Juglans regia L.*, *walnut*, *phenotypic selection*, *half-sib progenies*, *genetic variation*, *seed orchard*.

**Riassunto** – *Juglans regia* L., selezione fenotipica e stima della variabilità genetica in un arboreto da seme simulato di progenie *half-sib*. *Juglans regia* è originaria dell'Asia centrale ed è stata introdotta, diffusa e ampiamente coltivata in Europa per la produzione sia di frutti che di legno pregiato. Gli studi effettuati da diversi autori hanno evidenziato che il noce comune è caratterizzato da debole strutturazione genetica sia in Italia che in Europa, probabilmente a causa del ridotto *pool* genico introdotto e della lunga selezione antropica subita nel corso di vari secoli. La manipolazione, orientata soprattutto verso la produzione di frutti, è evidente osservando le caratteristiche fenotipiche delle risorse di *J. regia* sul nostro territorio, ad esempio la ridotta dominanza apicale, la ramificazione consistente, una marcata adattabilità a contesti ambientali diversi, soprattutto per la fenologia. Obiettivo di questo lavoro è valutare quanta variabilità è possibile ottenere dal *pool* genico attuale per costituire arboreti da seme destinati a produrre materiali di riproduzione per l'arboricoltura da legno. A questo scopo si studia un piantagione, parte di un test bi-stazionale di progenie *half-sib*, ottenute da fenotipi selezionati in Italia centro-settentrionale e settentrionale (Toscana, Emilia-Romagna e Trentino-Alto Adige). Dieci progenie *half-sib*, allevate in due siti diversi per suolo e per clima: Ponteburiano (Ar) e Ficulle (Tr), sono state poste a confronto tra loro e, localmente, anche con altre 10 non presenti in entrambe le località. Per la selezione fenotipica sono stati impiegati caratteri considerati ad alta ereditabilità materna relativi ad accrescimento (altezza e diametro), architettura della pianta (forma del fusto, dominanza apicale, dimensione e numero dei rami, angolo di inserzione dei rami), sensibilità a fattori abiotici (gelate tardive) e fenologia (epoca di apertura delle gemme). I caratteri relativi all'architettura della pianta e alla fenologia sono stati valutati tramite punteggi e sono stati sintetizzati in un Indice di Selezione multi-carattere (IS). Questo indice è stato usato per selezionare le progenie migliori e, all'interno di queste, i fenotipi migliori. Sono state selezionate solo le progenie e gli individui con IS superiore almeno del 10% rispetto alla media del sito considerato. Le analisi genetiche sono state effettuate su tutti gli individui presenti nella parcella di Ponteburiano (Ar) e su una popolazione italiana di riferimento, impiegando marcatori genetici di tipo biochimico (8 sistemi isoenzimatici che hanno consentito di individuare 19 *loci* e 40 alleli). I risultati relativi ai caratteri fenotipici e, soprattutto, quelli ad essi fortemente correlati riguardanti la fenologia, suggeriscono che l'epoca di apertura delle gemme è il carattere con maggior valore discriminante delle progenie. L'epoca di inizio della vegetazione condiziona non solo l'accrescimento ma, soprattutto, l'architettura della pianta. Le progenie migliori sono infatti quelle più efficienti nello sfruttare la stagione vegetativa ma soprattutto quelle che riescono ad evitare le gelate tardive che, in genere, danneggiano la gemma apicale in vegetazione. I risultati dell'analisi genetica indicano che la componente principale della variabilità genetica è quella individuale, intra-popolazione; manca infatti una strutturazione geografica della variabilità e l'eccesso di omozigosi è elevato. L'applicazione severa dell'indice IS ha consentito di selezionare solo 4 delle 10 progenie e 58 piante, risultate superiori fenotipicamente e con bassa interazione *genotipo x ambiente*. I parametri genetici di questa popolazione non hanno evidenziato diminuzione né del numero di alleli per *locus*, né della variabilità genetica rispetto alla "popolazione" totale, intesa come l'insieme delle discendenze esaminate, di Ponteburiano. Di conseguenza, è possibile convertire la parcella sperimentale in arboreto da seme eliminando le progenie con basso IS e mantenendo, tuttavia, un buon livello di diversità genetica.

**Parole chiave:** *Juglans regia L.*, *noce*, *selezione fenotipica*, *progenie di fratellastri*, *variabilità genetica*, *arboreto da seme*.

*F.D.C.: 176. 1 Juglans regia: 165. 3*

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<sup>1</sup> CRA - SEL Council for Research and Experimentation on Agriculture, Research Centre for Silviculture, Arezzo, Italy

\* Corresponding author *fulvio.ducci@entecra.it*

## Introduction

In Italy only 20% of noble hardwoods timber requirements are met from domestic markets. Noble hardwoods are very important for the Italian furniture industry, which uses about 60% of the total European import of valuable wood.

About 170,000 ha have been planted with noble hardwoods since 1992 (COLLETTI 2001) and 70% of this future stock is represented mainly by walnut and wild cherry. Of that amount about 60% has included walnut as the main species.

It is estimated that the only turnover for seed and seedling supply exceeded 50 million € during this period (DUCCI 2003).

Walnut is one of most valuable European woods. This tree seems to be not native in western Europe and it was probably introduced from the nearest Orient and spread by human migrations during different times for fruits, oil and for wood. Several investigations (FORNARI *et al.* 1999; MALVOLTI *et al.* 1995 and 1997) highlighted a very poor genetic structuration of walnut in Europe and in Italy. Very low variation rates are probably due to the very small gene pool introduced. The effect of the long time selection addressed to improve fruit crop production was also evident on the phenotype of trees: low apical dominance, low straightness, wide crowns and big branches are typical traits dominant in walnut stocks in the country. Anyway, FADY *et al.* (2003) showed higher variation in adaptive traits as bud-break phenology in the framework of a wide European experimentation. This variation was punctual by latitude and by altitude of provenance and was probably due to the selection pressure exerted by humans for their own utility.

Strategies for walnuts have been initially focused on preserving the remaining Italian resources of the species interesting their traits and to be used for high quality wood plantations. Activity focused on phenotypic selection and on the establishment of *half-sib* progeny test networks, aimed to transform experimental plots into *half-sib* seed orchards.

This work is aimed at: a) simulating different models for a seed orchard to be established after a *half-sib* progeny trial; b) assessing the variation levels within the simulated seed orchard; c) comparing the genetic parameters with those of pseudo-natural populations.

## Materials and method

A comparative two-field test network including in total 20 *half-sibs* progenies was established in 1992. Progenies were collected from different northern Italian regions (Figure 1): Tuscany, province of Arezzo (Ar), Emilia-Romagna, provinces of Ravenna (Ra), Modena (Mo) and Parma (Pr), and Trentino, province of Trento (Tn). Test sites were located at: 1) Ficulle (province of Terni, Lat. 42°49'23.80"N - Long. 12°04'19.86"E, altitude 450 m, volcano soils); 2) Ponteburiano (province of Arezzo, Lat. 41°53'23.6"N - Long. 12°04'19.6"E, 209 m, alluvial soils of the River Arno). Ten progenies were common to both those test sites, and overall 280 trees were phenotypically tested at Ponteburiano and 212 at Ficulle (Table 1).

### Basic material selection

On seedlings 8 years old (due to the need to use a smaller tree size for recording phenology data) and trees aged 12 years, according to methods and scores specified in the framework of European research net-

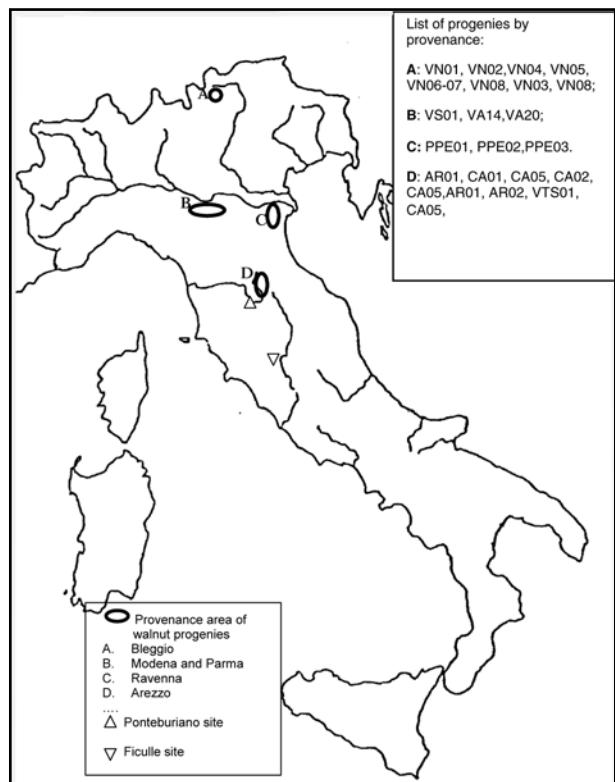


Fig. 1 - Provenance areas of the walnut progenies used in the experiment and test sites.

Provenienze delle discendenze di noce usate nei due siti e localizzazione delle aree sperimentali.

**Tab. 1** - List of progenies tested (X) in the experiments carried out in Ponteburiano (Arezzo) and Ficulle (Terni).  
*Lista delle descendenze di fratellastri in prova nei due siti presi in esame, Ponteburiano (Ar) e Ficulle (Tr).*

Code	Progeny name	Test site		Provenance
		Ficulle	Ponteburiano	
CA01	Boschi M. Grazia	X		Poppi (Ar)
CA02	Carola 1- 2	X		Poppi (Ar)
AR01	Il Mulino	X	X	Ponte alla Chiassa (Ar)
AR02	Gragnone	X		Gragnone (Ar)
VTS01	Lilli 1	X		Molin nuovo (Ar)
CA05	Fabbri 3	X	X	Pagliericcio (Ar)
PPE02	Marzocchi	X		Valle del Reno (Ra)
VS01	Palagano	X	X	Palagano (Mo)
VN01	Grazi 1	X	X	Bleggio (Tn)
VN02	Grazi 2	X	X	Bleggio (Tn)
VN03	Serafini 3	X		Bleggio (Tn)
VN04	Serafini 4	X	X	Bleggio (Tn)
VN05	Serafini 5	X	X	Bleggio (Tn)
VN06-07	Serafini 6 e 7 (mix)	X	X	Bleggio (Tn)
VN08	Serafini 8	X		Bleggio (Tn)
GAG	Gaggio	X		S. Maria Villiana (Ra)
VA14	Roffi Giovanni 1	X	X	Panigaro (Pr)
VA20	Vettola	X	X	Vettola di Valmozzola (Pr)
PPE01v	Missiroli-Vecchia		X	Castiglione di Cervia (Ra)
PPE01	Cane		X	Castiglione di Cervia (Ra)

works (DUCCI 1995; FADY *et al. op. cit.*) the following traits were examined: - bud break (score 1-7, Figure 2), surveyed during 2 years and averaged on fixed dates; - height and height increase (cm); - basal diameter (cm) and DBH (cm); - mean branch angle (scored: 0 vertical to 7 horizontal); - basal branch diameter (cm); apical dominance (scored: 1 bad to 7 very good); - total number of main branches; stem form (scored: 1 crooked to 7 straight); frost damage resistance (scored: 1 - very bad resistance, 4 - poor resistance, 7 - small damages, 10 - very good resistance) after the hard frost which occurred between 17<sup>th</sup> and 19<sup>th</sup> April 1997 when 14 hours at -7°C were recorded in both sites.

All these traits were initially analysed by ANOVA (data only partially shown, Annex 1) keeping each 2 areas separated. This was necessary due to differences within sites for soil. At Ficulle, progeny plots were planted with 6 plants in a rectangle, at 6 x 6 m spacing, whilst in Ponteburiano, seedlings were in singleline 9-tree plots, at 5.5 x 5.5 m spacing. Plots were randomly distributed within 3 blocks. Due to the absence of mortality, a one-way analysis of variance was performed within each site for complete balanced blocks in accordance with the following factorial model (1) (CAMUSSI *et al.* 1986):

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma k + \varepsilon_{ijk} \quad (1)$$

where  $Y$  is the value of a variable (examined traits) for  $i$  as a level of factor A (progenies),  $j$  as a level of factor

B (soil fertility),  $k$  as a level of blocks within sites;  $\mu$  is the population average,  $\alpha_i$  and  $\beta_j$  are the effects of different levels of factors A and B;  $(\alpha\beta)_{ij}$  represents the interaction effect among factors A and B for their different levels;  $\gamma$  the effect of  $k^{\text{th}}$  block, and finally,  $\varepsilon$  represents residuals where factors are difficult to account for and where they can influence the  $j^{\text{th}}$  experiment unit within the  $i^{\text{th}}$  treatment within the  $k^{\text{th}}$  block.

In order to summarize results and to focus on the genetic aspects treaten in this paper, the results of the ANOVA analyses are not shown here.

Multiple comparisons were carried out (Duncan's test) by area and traits with special reference to phenology (showed in this paper), the main adaptive trait correlated to a good quality of the general architecture in walnuts. Anyway, the main correlation among traits were assessed. Performance intra-site indices for each trait (ratio between the rank of each progeny and the number of progenies tested in each area) were computed and used to compare progenies across sites. Values near 1.00 express the best 'performance', lower values indicate lower performance. Several traits, especially those concerned with the plant architecture and those correlated to phenology (HANSCHE *et al.* 1972), were considered with high maternal inheritance (LANDE and KIRKPATRICK 1990, THIEDE 1998, BARRET 2002, MICHLER *et al.* 2007). The Principal Component Analysis (PCA) was used to point out the most important traits useful for material selections. The phenotypically-best progenies and the best individuals within these progenies were selected and results were used to simulate different possible seed orchard models at different intensity of selection.

The selection criterion of both progenies and trees was based on a multi-trait selection index (ZOBEL and TALBERT 1984). This parameter is generally considered a total score to be assigned to each individual. Theoretically, the selection index should include coefficients depending on heritability, correlation among traits and/or their economical importance. In this test it was decided for simplicity to attribute similar weights to the considered traits and to exclude the total height, which was the most important measured trait (2)

$$SI = \Sigma n_f \text{Perf. H99} + \text{Perf.}[(\text{Frost Res./Avoid.} + \text{Stem} + \text{Branch} + \text{Straight.} + \dots)/N_t] \quad (2)$$

In the above formula  $n$  is the number of trees within families,  $f$  the number of families,  $\text{Perf.}$  the performances for each trait,  $N_t$  the amount of the con-

Walnut half sib progenies in Ponteburiano (Arezzo)		19/4/96	9/5/96	21/4/97	
Code and name of the progeny					
● 12 VN 06 - VN07 (Serafini6 e 7-Bleggio T)	2,05	● VN 01(Graffi1-Bleggio Tn)	6,25	● VN 01(Graffi1-Bleggio Tn)	2,26
● 11 VN 02(Graffi2-Bleggio Tn)	2,28	● VN 06 - VN07 (Serafini6 e 7-Bleggio)	6,47	● VN 06 - VN07 (Serafini6 e 7-BI)	2,33
● 2 VN 04 (Serafini4-Bleggio Tn)	2,3	● VN 04 (Serafini4-Bleggio Tn)	6,61	● VN 02(Graffi2-Bleggio Tn)	2,33
● 13 VN 05 (Serafini5-Bleggio Tn)	2,35	● VA 14 (Panigaro-Roffi - Pr)	6,64	● VN 05 (Serafini5-Bleggio Tn)	2,5
● 10 VN 01(Graffi1-Bleggio Tn)	2,52	● VA 20 (Vettola di Valmozzola - Pr)	6,67	● Fabbri 3 (Castel S. Niccolò AR)	2,64
● 9 VA 14 (Panigaro-Roffi - Pr)	2,64	● VN 05 (Serafini5-Bleggio Tn)	6,7	● VA 20 (Vettola di Valmozzola	2,69
● 8 VA 20 (Vettola di Valmozzola - Pr)	2,83	● Fabbri 3 (Castel S. Niccolò AR)	6,72	● VA 14 (Panigaro-Roffi - Pr)	2,7
● 7 Fabbri 3 (Castel S. Niccolò AR)	3	PP-E-01 (Cast. di Cervia-cane-Ra)	6,73	PP-E-01 (Cast. di Cervia-cane)	2,81
5 PP-E-01 (Cast. di Cervia-cane-Ra)	3,17	● VN 02(Graffi2-Bleggio Tn)	6,8	● AR 01 (Il Mulino AR)	2,83
6 PP-E-01 (Cast. di Cervia-vecchia-Ra)	3,58	● VS 01(Palagano MO)	6,8	● VN 04 (Serafini4-Bleggio Tn)	2,85
● 4 AR 01 (Il Mulino AR)	3,68	PP-E-01 (Cast. di Cervia-vecchia-Ra)	6,91	● VS 01(Palagano MO)	2,87
● 3 VS 01(Palagano MO)	3,88	● AR 01 (Il Mulino AR)	6,92	PP-E-01 (Cast. di Cervia-vecchia-Ra)	2,92
Average	2,84	Average	6,68	Average	2,64
<b>Ficulle</b>	<b>Name</b>	<b>19/4/96</b>	<b>9/5/96</b>	<b>21/4/97</b>	
11 VN 03 (Serafini 3-Bleggio - Tn)	2,25	VN 03 (Serafini 3-Bleggio - Tn)	4,5	VN 03 (Serafini 3-Bleggio - Tn)	3,25
● 14 VN 06 - VN 07 (Serafini 6e7-Bleggio-T)	2,44	● VN 05 (Serafini 5-Bleggio - Tn)	4,73	● VN 05 (Serafini 5-Bleggio - Tn)	3,81
● 12 VN 04 (Serafini 4-Bleggio - Tn)	2,5	● VN 04 (Serafini 4-Bleggio - Tn)	5	● VN 04 (Serafini 4-Bleggio - Tn)	4,07
● 13 VN 05 (Serafini 5-Bleggio - Tn)	2,63	VN 08 (Serafini 8-Bleggio - Tn)	5,27	VN 08 (Serafini 8-Bleggio - Tn)	4,09
15 VN 08 (Serafini 8-Bleggio - Tn)	2,73	● VN 02 (Graffi 2-Bleggio - Tn)	5,36	● VN 01 (Graffi 1-Bleggio - Tn)	4,15
● 9 VN 01 (Graffi 1-Bleggio - Tn)	2,77	● VN 01 (Graffi 1-Bleggio - Tn)	5,54	● VN 06 - VN 07 (Serafini 6e7-BI)	4,3
● 6 Fabbri 3 (Castel S. Niccolò - Ar)	3,41	● VN 06 - VN 07 (Serafini 6e7-Bleggio)	5,67	● VN 02 (Graffi 2-Bleggio - Tn)	5,125
● 10 VN 02 (Graffi 2-Bleggio - Tn)	3,45	● AR 01 (Il Mulino - Ar)	6	Gaggio (S. Maria Villiana)	5,18
● 3 AR 01 (Il Mulino - Ar)	3,61	VTS 01 (Lilli 1 - Ar)	6,5	● Fabbri 3 (Castel S. Niccolò - Ar)	5,25
4 AR 02 (Gagnone - Ar)	3,75	● Fabbri 3 (Castel S. Niccolò - Ar)	6,58	PP-E-02 (Marzocchi)	5,25
7 PP-E-02 (Marzocchi)	3,91	● VS 01 (Palagano - Mo)	6,64	CA 02 (Carola 1-2, Ponte a P.)	5,33
16 Gaggio (S. Maria Villiana)	4	● VA 20 (Vettola di Valmozzola - Pr)	6,67	Roffi Giovanni Pamigaro	5,33
● 16 VA 20 (Vettola di Valmozzola - Pr)	4,08	CA 02 (Carola 1-2, Ponte a Poppi - A)	6,75	● VA 20 (Vettola di Valmozzola -	5,33
2 CA 02 (Carola 1-2, Ponte a Poppi - Ar)	4,25	PP-E-02 (Marzocchi)	6,75	● AR 01 (Il Mulino - Ar)	5,41
● 17 Roffi Giovanni Pamigaro	4,25	● Roffi Giovanni Pamigaro	6,75	● VS 01 (Palagano - Mo)	5,45
● 8 VS 01 (Palagano - Mo)	4,27	Gaggio (S. Maria Villiana)	6,82	AR 02 (Gagnone - Ar)	5,58
1 CA 01 (Boschi M. Grazia - Casentino)	4,42	CA 01 (Boschi M. Grazia - Casentino)	6,83	CA 01 (Boschi M. Grazia - Cas	5,83
5 VTS 01 (Lilli 1 - Ar)	4,5	AR 02 (Gagnone - Ar)	6,92	VTS 01 (Lilli 1 - Ar)	6
Average	3,52	Average	6,07	Average	4,93



1 - Buds closed.  
1 - Gemme chiuse.



2 - Scales start to open.  
2 - Perule in fase di allargamento.



3 - Green scale appear.  
3 - Appaiono le perule interne verdi.



4 - Leaves appear.  
4 - Visibili le foglioline all'interno.



5 - Leaves out of the bud.  
5 - Foglie fuoriuscite dalla gemma.



6 - Leaf distension starts.  
6 - Avvio della distensione.



7 - Shoot elongation starts.  
7 - Avvio dell'allungamento del germoglio.

These three last steps represent the most sensitive phases to late frost damages.

Queste ultime tre fasi sono le più sensibili alle gelate tardive.

**Fig. 2** – Duncan's test results concerning scores used for phenology records on walnut progenies at several fixed dates in both sites Ficulle (province of Terni, Umbria) and Ponteburiano (Province of Arezzo, Tuscany). Below is reported the ideographic scoring system established for walnut ( $P < 0.05$ ). *Test di Duncan applicato alle fasi fenologiche misurate sulle discendenze di noce a Ficulle e Ponteburiano ( $P < 0.05$ )*. In basso sono riportate le immagini e indicati i punteggi attribuiti alle diverse fasi su ciascuna pianta.

sidered traits. Where the selection index was greater than 10% then the average of the site was considered sufficient for selecting progenies. The same criterion was used for trees within progenies.

#### Testing models

Due to the geographical position, site fertility and flat topography, Ponteburiano was considered suitable to be transformed into a half-sib progeny seed orchard after selection of and within the best families. The genetic analysis was used in Ponteburiano to assess variation parameters within 5 possible seed orchard models:

- the total population in field, without considering *a priori* its structuration in progenies;
- the same population sub-divided into 12 half-sib progenies (including the ten common to both sites);
- the phenotypically best 6 families (50% of the total population);
- the best 4 families (30% of the total population);
- the best seedlings within the whole plantation (which allowed the selection of about 60 trees, about 25% of the total).

#### Genetic analysis with biochemical markers (isozymes)

Eight enzymes systems were analysed by horizontal starch-gel electrophoresis according to ARUSELKAR *et al.* (1985 and 1986), ALETÀ *et al.* (1993), MALVOLTI *et al.* (*op.cit.*) and RODRIGUEZ *et al.* (1989): IDH, PGI, GOT, MDH, DIA, PGD, SKDH and PGI were revealed for 19 *loci* and 40 alleles. Parameters estimated were: mean number of allele *per locus* (n), percentage of polymorphic *loci* ( $P_{5\%}$ ), average observed ( $H_o$ ) and expected ( $H_e$ ) heterozygosity, fixation indices (WRIGHT 1978) and genetic distances (NEI 1978). The *cluster analysis* (SNEATH and SOKAL 1973) was performed to explore the structure of genetic diversity and to permit the estimation of the inbreeding within progenies. Wright's indices and application of bootstrapping procedure (exact testing) for small samples (WEIR and COCKERHAM 1984; CAVALLI-SFORZA *et al.* 1994; RAYMOND and ROUSSET 1995) were carried out with F-STAT programme (GOUDET 2001). The Principal Component Analysis on the dispersion matrix, *via* the Correspondence Analysis (EL-KASSABY 1991), was carried out with NTSYS-pc (ROHLF 1994) software. The population tested in the field experiments was compared with an Italian refer-

ence population considered 'naturalised' since existing from long time.

## Results

#### Genetic analysis combined with phenotypic selection (Table 2)

##### a) The whole Ponteburiano 'population' and the Italian reference population

Among 19 *loci* examined, only 12 were polymorphic in both populations: IDH-2, PGI-2, GOT-2, GOT-3, SKDH-1, SKDH-2, DIA-1, DIA-3, 6PGD-2, MDH-2, MDH-3 and PGM-1. The allele distribution and the percentage of polymorphic *loci* ( $P_{5\%} = 70.6\%$ ) was similar in both populations.

In Ponteburiano the mean number of alleles per *locus* (n) was higher than in the reference population (2.2 vs. 2.1). The fixation index ( $F = 1 - H_o \cdot H_e^{-1}$ ) was positive both in Ponteburiano ( $F = .281$ ) and in the reference population ( $F = .335$ ) showing an excess of homozygotes.  $F_{ST} = .023$  showed that 97.5% of observed variability was common to the two populations and  $F_{IS} = .304$ , indicated that 70% of variability was among individuals. Some *loci* tended to allelic fixation (mean  $F_{IT} = .320$ ), in particular PGI-2 ( $F_{IT} = .553$ ), GOT-2 ( $F_{IT} = .544$ ), SKDH-2 ( $F_{IT} = .477$ ), GOT-3 ( $F_{IT} = .456$ ) and MDH-2 ( $F_{IT} = .411$ ). Only DIA-3 ( $F_{IT} = .071$ ) showed a lower value (nearer to equilibrium). Heterozygote deficiency is therefore common to all loci, except

**Tab. 2** - Genetic variation parameters within Ponteburiano plantation according to the different models of selection tested, compared with the pooled pseudo-natural local population near Ponteburiano.

*Principali parametri genetici stimati a Ponteburiano nei differenti livelli di selezione simulati. Sono comparati con la popolazione locale di noce.*

Model	N/l	n	$P_{5\%}$	$H_o$	$H_e$	F
1. Total plantation	263.6	2.2	70.6	0.235 0.045	0.327 0.057	0.281
2. Six best prog.	137.6	2.1	68.6	0.239 0.047	0.320 0.059	0.253
3. Four best prog.	89.3	2.1	69.1	0.241 0.048	0.323 0.060	0.254
4. Best trees within best progenies	32.5	2.1	70.6	0.231 0.047	0.322 0.059	0.283
5. Best trees within worst progenies	24.6	2.2	70.6	0.226 0.048	0.328 0.059	0.311
6. All trees with super.>10%	57.1	2.2	70.6	0.229 0.046	0.326 0.059	0.298
7. Reference pooled local population	69.9	2.1	70.6	0.204 0.042	0.307 0.053	0.335

N/l: Mean size of sample *per locus*.

$H_o$ : mean observed heterozygosity.

n: Mean number of alleles *per locus*.

$H_e$ : mean expected heterozygosity.

$P_{5\%}$ : % of polymorphic *loci* (*a locus* is considered polymorphic when frequency of the most common allele is < 95%).

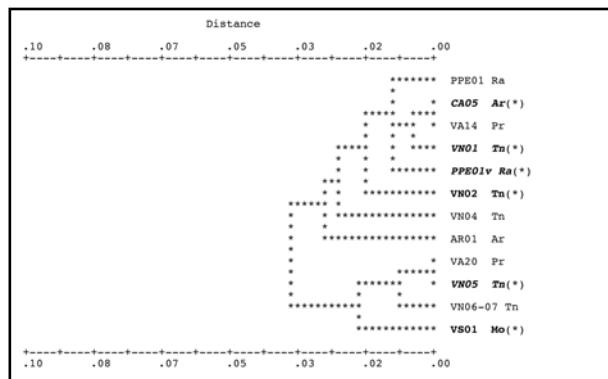
F:  $1 - (H_o / H_e)$

DIA-1 in Ponteburiano and DIA-3 and MDH-3 within the reference population. Deviation from the Hardy-Weinberg equilibrium was significant. The unbiased genetic distance (Nei *op. cit.*), was .019 and confirmed low differentiation between both populations.

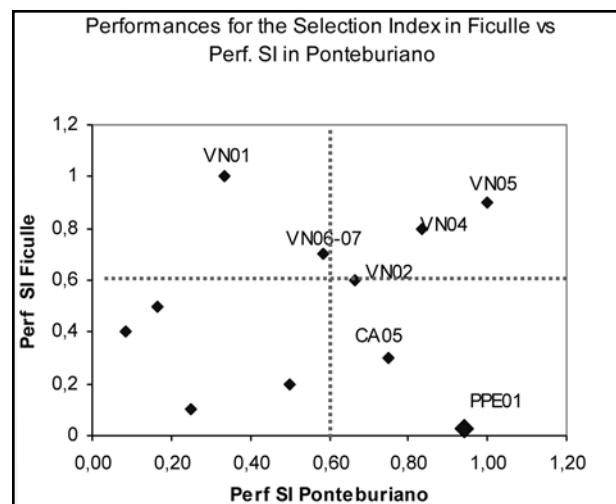
b) *The progenies*

The Ponteburiano trees were divided by family. Progenies CA05 and PPE01 had the highest mean number of alleles per *locus* ( $n = 2.2$ ), while PPE01, VN05, VA14 and VN02 showed the lower value  $n = 2.0$ . PPE01, VN05, VN02 with VA20 and VN06/07 showed lowest amount of polymorphic *loci* ( $P_{5\%} = 64.7\%$ ). For the remainder of trees,  $P_{5\%}$  was 70.6, as for Ponteburiano population. VA20, CA05 and PPE01 showed values close to Hardy-Weinberg equilibrium. All *loci* showed positive fixation index significantly higher than 0, except for diaphorase system. Most of the observed genetic variation was common to all progenies: indeed  $F_{ST}$  was 93%.  $F_{IS} = .226$ , confirmed that 77.4 % of variation is due to diversity among individuals within each progeny. Genetic distances among progenies were short, between .000 and .068 (Figure 3). A geographical gradient was not evident, and progenies were not clearly grouped by provenance.

It was possible to detect two clusters, one assembling materials from all the provenance areas, whilst the other grouped VN05 and VN06-07, VS01 and VA20 from Trentino and Emilia Romagna regions respectively. The principal component analysis indicated that the first 10 principal components explained only 53.6% of the total variance and confirmed the low degree of genetic variation in walnut. Alleles with higher dis-



**Fig. 3** - UPGMA clusters based on Nei (1978) unbiased genetic distances. All the 6 progenies with an (\*) were selected initially; progenies in Italics were used as the 4 final best ones  
*Dendrogramma UPGMA (Nei 1978) costruito sulle distanze genetiche. Le 6 discendenze contrassegnate con (\*) sono quelle selezionate nella prima fase, le 4 in corsivo sono le migliori in assoluto.*



**Fig. 4** - Best progenies selected on the base of their mean performance for multi-trait Selection Index (SI) in both the experimental sites.  
*Le migliori progenie selezionate sulla base del loro comportamento medio indicato dall'Indice di Selezione (IS) multi-carattere adottato (citato nel testo).*

minant meaning, in decreasing order, were: 6PGD-2a, DIA-3a, SKDH-2c, MDH-2a, DIA-3b, MDH-3c, PGM-1b, GOT-3b, GOT-3a e GOT-3b, with correlation between 0.64 and 0.49. The Correspondence Analysis did not indicate any significant homogeneous groups (*i.e.* by progenies). The genotype dispersion plot confirmed that the genetic variability depended from the variation of the 247 individuals .

c) *The six best progenies selected phenotypically, reduced the total population to about 50 %*

Six progenies from three regions were selected: VN01, VN02, VN05 from Trentino-Alto Adige, PPE01 and VS01 from Emilia-Romagna and CA05 from Tuscany (Figure 4).

The selected progenies demonstrated valuable traits in comparison with the remaining progenies and they showed lower *genotype x site* interaction as indicated by the Selection Index. Three progenies interacted most at Ponteburiano and Ficulle respectively. The progeny PPE01, planted only in Ponteburiano, was selected anyway because its good performance for growth, apical dominance and stem form. Considering that the *genotype x environment* interaction (Table 3) was similar for all progenies, it is hypothesised that PPE01 could perform similarly if planted at either of the sites.

In Ponteburiano, 8 years after planting, there were no significant differences between progenies for height growth. Differences among progenies were significant for stem diameter (P value 0.03) and very significant

**Tab. 3** - Performances indices for some of the main traits examined on progenies common to both the experimental sites. Numbers in bold characters evidence the best performances.  
*Indici di performance ricavati dalle graduatorie ottenute per alcuni caratteri. In grassetto le migliori performance in ciascun sito.*

Code	Flush. 19496 Pontebur.	Flush. 21497 Pontebur.	Flush 19496 Ficulle	Flush. 21497 Ficulle	H 92/93 Pontebur.	H92/93 Ficulle	H99 Pontebur.	H99 Ficulle	Dominance Pontebur.	Dominance Ficulle	Stem Form Pontebur.	Stem Form Ficulle	Frost Perf.PB
CA01 Ar													
CA02 Ar													
AR01 Ar	0.17	0.33	0.56	0.28	0.08	<b>0.61</b>	0.17	0.24	0.50	<b>0.65</b>	0.33	0.59	0.16
AR02 Ar													0.17
VTS01 Ar													
CA05 Ar	0.42	<b>0.67</b>	<b>0.67</b>	0.56	0.58	0.39	<b>0.92</b>	<b>0.71</b>	<b>0.75</b>	0.29	0.42	0.29	0.42
PPE02 Ra													
VS01 Mo	0.08	0.17	0.17	0.22	0.33	0.50	0.33	0.47	<b>0.67</b>	<b>0.94</b>	<b>0.75</b>	<b>0.76</b>	0.25
VN01 Tn	<b>0.67</b>	<b>1.00</b>	<b>0.72</b>	<b>0.78</b>	0.17	<b>1.00</b>	0.42	<b>0.82</b>	0.17	<b>0.82</b>	<b>0.67</b>	<b>1.00</b>	<b>0.83</b>
VN02 Tn	<b>0.92</b>	<b>0.83</b>	<b>0.61</b>		0.50	0.06	<b>0.83</b>	<b>0.94</b>	0.08	0.53	0.58	<b>0.82</b>	<b>0.67</b>
VN03 Tn													
VN04 Tn	<b>0.83</b>	0.25	<b>0.89</b>	<b>0.89</b>	<b>0.75</b>	0.56	0.08	<b>0.65</b>	<b>0.92</b>	0.35	<b>1.00</b>	<b>0.94</b>	<b>0.75</b>
VN05 Tn	<b>0.75</b>	<b>0.75</b>	<b>0.83</b>	<b>0.94</b>	<b>1.00</b>	<b>0.89</b>	<b>0.75</b>		0.25		<b>0.92</b>		<b>0.92</b>
VN06-07 Tn	<b>1.00</b>	<b>0.92</b>	<b>0.94</b>	<b>0.72</b>	<b>0.83</b>	<b>0.67</b>	0.25	0.53	0.58	0.12	0.17	<b>0.65</b>	<b>1.00</b>
VN08 Tn													
VA14 Pr	0.58	0.50	0.22	0.39	0.25	<b>0.94</b>	0.58	0.41	0.33	0.24	0.50	0.12	0.50
VA20 Pr	0.50	0.58	0.33	0.33	<b>0.92</b>	0.44	<b>0.67</b>	0.29	0.42	0.47	0.25	<b>0.71</b>	0.58
PPE01v Ra	0.25	0.08				0.42		<b>1.00</b>		<b>0.83</b>		<b>0.83</b>	0.08
PPE01 Ra	0.33	0.42				<b>0.67</b>		0.50		<b>1.00</b>		0.08	0.33

Flush.: late flushing/tardività dell'entrata in vegetazione.

H92/93: annual growth 1992-1993/incremento in altezza dopo la piantagione.

H99: height in 1999/altezza raggiunta nel 1999.

Frost perf.: late frost resistance/resistenza al gelo tardivo.

(P values between 0.05 and 0.01) for phenology, stem form and dominance. These characters are correlated (Table 4): indeed late bud breaking can allow to trees to avoid late frosts. Apical buds are not damaged by frost, therefore the likelihood of developing a well defined central axis and good dominance is higher. In progenies VN01, VN02 and VN05 the phenology trait had greatest influence, whilst for progenies PPE01v, CA05 and VS01 the shape indices were more important.

Phenology assessments based around April 20<sup>th</sup> appeared the best method in the discrimination of the progeny characteristics (Annex I and Figure 2). Indeed it was possible to distinguish a group of progenies from Bleggio (Alps region > 1000 m a.s.l, cold environment) from progenies sampled on the Apennine's (meanly 500 m a.s.l., relatively temperate with spring late frosts)<sup>2</sup>. Materials from Trentino were usually later to flush than other progenies. As an example of this criterion, these offsprings avoided frost damage between 17<sup>th</sup> – 19<sup>th</sup> April 1997, when temperatures of -7°C occurred for 14 hours. Correlation was negative and significant between frost damage and total height (H99) with flushing. This testify the advantage of late flushing material. Growth appears to be more

dependent on tree efficiency than length of the vegetation period. Families PPE01v and CA05 had the best growth index and, with VS01, had best dominance and shape indices.

It should be noted that CA05 and PPE01 are the progenies which showed the highest genetic variation levels within the experiment: 32 alleles were stained at 12 loci, the mean n. of alleles per locus was 2.2 and polymorphic loci varied respectively between 70.6% and 64.7%.

Among the 6 chosen progenies, genetic variability resulted low ( $F_{ST} = .056$ ). Poverty of heterozygotes is a common situation; all loci contributed to heterozygote deficiency, except DIA-1.

The first 10 components after the Principal Component Analysis explained only 58.9% of variance (Table 5).

Alleles showing higher discrimination power were: 6PGD-2a, IDH-1a, PGI-2a, PGI-2b, IDH-1c, MDH-2a, DIA-3a, MDH-3c. The Correspondence Analysis showed that the six progenies tended to be grouped in different quadrants. This is an important result, because it has been possible discriminate progenies by genetic markers and phenotypic traits.

<sup>2</sup> As for air temperature and growth period, one thousand meters of elevation in the Alpine region (Bleggio, Trentino) represents really harder conditions than in the Apennines at similar altitudes.

**Tab. 4** - Correlation matrix among the main traits recorded in Ponteburiano.

Correlazioni tra i principali caratteri rilevati a Ponteburiano, compreso fenologia e resistenza al gelo tardivo.

	Basal Diam.	Branch n.	Flushing 19 4 96	Flushing 9 5 96	Flushing 21 4 97	H99	DBH99	Branch Size	Stem Form	Domin.	Branch Angle	Frost Res. 17 4 97
<b>Basal Diam.</b>	1	.581(**)	0.057	0.102	0.059	.461(**)	.733(**)	-.250(**)	0.099	-0.091	.224(**)	0.051
n	287	285	287	287	275	284	273	273	273	273	262	287
<b>Branch n.</b>		1	-.216(**)	-0.061	-0.084	.248(**)	.435(**)	-.175(**)	.147(*)	-0.04	0.118	.197(**)
n		285	285	285	273	282	271	271	271	271	260	285
<b>Flushing 19 4 96</b>			1	.450(**)	.653(**)	.230(**)	0.101	.133(*)	-0.1	0.047	.184(**)	-.592(**)
n			287	287	275	284	273	273	273	273	262	287
<b>Flushing 9 5 96</b>				1	.492(**)	.529(**)	.170(**)	-0.025	0.044	-0.009	.190(**)	-.279(**)
n				285	275	284	273	273	273	273	262	287
<b>Flushing 21 4 97</b>					1	.181(**)	0.082	.192(**)	-0.028	0.095	.154(*)	-.465(**)
n						1	.844(**)	-.167(**)	0.08	-0.004	.154(*)	-.131(*)
<b>H99</b>							1	.285	.274	.274	.263	.285
n								1	.274	.274	.263	.285
<b>DBH99</b>								1	.274	.274	.263	.274
n									1	.274	.263	.274
<b>Branch Size</b>									1	-.142(*)	0.082	0.028
n										1	.274	.263
<b>Stem Form</b>											1	.150(*)
n												1
<b>Domin.</b>												1
n												
<b>Branch Angle</b>												1
n												
<b>Frost Resistance</b>												1
17 4 97												
n												288

\*\* correlation significant for  $P \leq 0.01$  - correlazione significativa per  $P \leq 0.01$ .

\* correlation significant for  $P \leq 0.05$  - correlazione significativa per  $P \leq 0.05$ .

n: number of examined trees - numero di alberi esaminati

d) A second selection reducing the selected population to 33%

Following a more strict criterion of selection, two other half-sib progenies were eliminated: VN02 and VS01. VN02 showed good growth and moderately good stem form indices but, especially in Ponteburiano, low dominance. Progeny VS01 was too early flushing and therefore more sensitive to late frost.

The decreasing from six to four progenies did not cause any loss of alleles, thanks to PPE01 and CA05 that had high number of alleles.

Within this four-progeny population the  $F_{ST} = 0.037$  indicated an increasing of genetic variation common to progenies.  $F_{IT}$  values were all positive, but heterozygote deficiency was lower compared to the initial population.

e) Final selection of superior phenotypes, reducing the selected population to 25%

Finally, and with the aim of sampling the maximum variation possible, single trees were selected within the Ponteburiano population. The requirement was a SI 10% higher than the average of the pooled plantation. H99, DBH99 and other performance traits were used as described above. Superior trees selected

would be used to establish a seed orchard model where progenies were in this case confounded. In total 58 trees were finally selected. No significant variations were observed for all the main genetic parameters compared to those of the Ponteburiano total population.

**Tab. 5** - Principal Components Analysis (absolute values  $> .70$  were considered). Main phenotypic and adaptive traits correlated to the first 4 components are reported.

Analisi delle Componenti Principali (soglia dei valori considerata  $> 0.70$ ). Sono riportati i principali caratteri fenotipici ed adattativi correlati alle prime 4 componenti.

	Principal Components			
	1	2	3	4
N. of Branches				
Branch Angle				
Strightness				
Phen. 19/4/96				.853
Phen. 9/5/96				
Phen. 21/4/97				.805
H99			.869	
DBH99			.879	
Dominance				
Mean angle				
Frost 17/4/97				-.735

## Discussion and conclusions

Most of the material selected in this experiment, with the aim to establish a possible seed orchard, was sourced from high altitude (about 1,000 m) in the region of Bleggio, near the Dolomite range. This result confirms the higher performance, already detected in other experimental fields, for this provenance. Indeed, in the framework of the European project W-Brains<sup>3</sup> this provenance performed relatively well in comparison with a wide range of accessions from all western Europe (FADY *et al. op. cit.*). Bleggio is also known as an Alpine area for walnut nut cultivation. A similar performance, with low interaction *genotype x environment*, was only present among the Apennine materials by the Tuscan progeny CA05 (named also Fabbri 3) from the Casentino valley near Arezzo.

Walnuts sourced from Western Europe, compared to Caucasian and Chinese populations, have been shown to have relatively good allele richness but low levels of heterozygosity (FORNARI *et al. op. cit.*). Centuries of selection for fruit production and for adaptability to different climatic conditions have led to a significant reduction of inter-population variation whilst the individual component has become more significant (DUCCI *et al. op. cit.*).

The very low intra-specific variation found in walnut populations in this study was similar to results of studies by MALVOLTI *et al. (op. cit.)* for Italian populations. Selection over time would have particularly favoured the best trees for fruit production. Compared to the probably wider initial gene pool, only few genotypes were used to create nut orchards; this genetic erosion favoured the fixation of some alleles. The reduction of genetic variation in walnut is evident in general, either using neutral markers, either morphological (architecture plant) or adaptive (phenology) traits. HANSCHE *et al. (op. cit.)* reported very high heritability values for this last trait. *Late flushing* has been considered an important character by breeders aiming to avoid damage by frosts. Nevertheless, in spite of the severe genetic erosion, a small amount of variation still exists within the remaining populations, but at single tree level only. It determines the variance component explained by individuals within populations. Some characters, and particularly adaptive traits (FADY *et al. op. cit.*), showed good variability

and a negative correlation among phenology, growth and frost resistance confirmed their importance for selection and improvement.

For this reason it will be possible to select late-flushing trees, and/or with low environment interaction, and/or with good stem form and vigour. The use of a selection multi-trait indices, even if simplified, was helpful in selecting material and the process confirmed its utility when the individual component of variance is high, and it allows the family selection.

Within the different models tested, the progressive reduction of progenies did not significantly modify the variation levels compared to the whole Ponteburiano and reference Italian population. Homozygosity levels are in general higher in walnut than in other species, at least using neutral markers as isozymes. Wright's F values decrease across different selection intensities when selection is carried out by progenies and increase when the individual selection is performed.

Homozygosity was lower within the selected progenies models than in the others. In this study four selected progenies showed greater allelic richness. A hypothetical seed orchard made with those progenies, at the same time, could provide material with good performance and supply variability similar to larger populations as the total plantation or the pseudonatural system chosen for comparison (Figure 5).

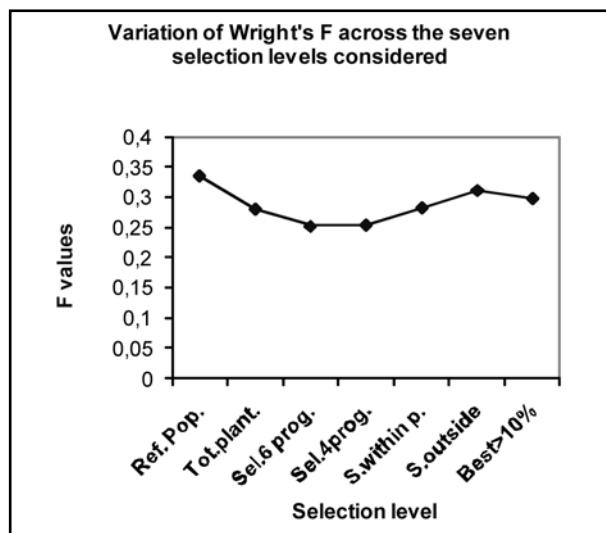


Fig. 5 - Variation of Wright's F across intensity levels of selection within Ponteburiano.

Variazione dell'indice F di Wright stimato per i differenti livelli di intensità di selezione in Ponteburiano.

<sup>3</sup> FAIR-CT96-1887 Network and Standards. Walnut - Basic Research for Agro-forestry and Industry.

**Annex 1.** Analysis of Variance for phenological phases at fixed dates in Ficulle and Ponteburiano. No significant differences were detected previously between blocks in Ficulle. Due to the inter-block variance in Ponteburiano, because of the differences in fertility between block, only the materials in the most homogeneous blocks were considered for the multiple comparison among averages.

*Analisi della varianza per i punteggi medi accumulati dalle progenie nei due test sperimentali di Ficulle e Ponteburiano relativamente al periodo di entrata in vegetazione a date fisse. In analisi preliminari i materiali in Ficulle non hanno mostrato differenze significative tra blocchi, né interazioni tra questi e le progenie, per cui si è proceduto all'analisi tra progenie direttamente. In Ponteburiano, invece, è stato necessario procedere alla stima della significatività delle differenze tra blocchi e dell'interazione tra questi e le discendenze. Quindi, dove l'interazione si è rivelata significativa si è preferito impiegare per i confronti tra medie i dati di due soli blocchi su tre. Le differenze sono probabilmente dovute alla differenza di fertilità e tessitura nella parcella, posta sulle rive dell'Arno.*

<b>FICULLE</b>					
<b>Trait: <u>Phen 19 Apr 96</u></b>					
Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Progenies	17	116,5147	6,8538	7,1329	,0000
Within Progenies	194	186,4099	,9609		
Total	211	302,9245			
<b>Trait: <u>Phen 9 May 96</u></b>					
Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Progenies	17	134,7003	7,9235	5,7521	,0000
Within Progenies	194	267,2383	1,3775		
Total	211	401,9387			
<b>Trait: <u>Phen 21 Apr 97</u></b>					
Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Progenies	17	120,3082	7,0770	5,6319	,0000
Within Progenies	190	238,7495	1,2566		
Total	207	359,0577			
<b>PONTEBURIANO</b>					
<b>Trait: <u>Phen 15 Apr 96</u></b>					
Source		Sum of Squares	DF	Mean Square	Sig of F
Progenies		79,894	11	7,263	8,564 ,000
Blocks		4,132	2	2,066	2,436 ,090
Progenies x Block		74,847	22	3,402	4,011 ,000
Residual		209,482	247	,848	
Total		367,420	282	1,303	
<b>Trait: <u>Phen 15 May 96</u></b>					
Source		Sum of Squares	DF	Mean Square	Sig of F
Progenies		3,448	11	,313	,379 ,964
Blocks		,654	2	,327	,395 ,674
Progenies x Block		21,974	22	,999	1,207 ,242
Residual		204,472	247	,828	
Total		230,502	282	,817	
<b>Trait: <u>Phen 15 April 97</u></b>					
Source		Sum of Squares	DF	Mean Square	Sig of F
Progenies		14,539	11	1,322	5,264 ,000
Blocks		,116	2	,058	,231 ,794
Progenies x Block		9,031	22	,410	1,635 ,040
Residual		59,260	236	,251	
Total		82,908	271	,306	

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