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Characterization of autochthonous Walnut germplasm in Friuli Venezia Giulia

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Abstract

Juglans regia is mostly distributed in the temperate and subtropical regions of the Northern Hemisphere. During the last ice age, the species survived only in refugial areas. In Europe it survived in the Italian and Balkan peninsulas. Friuli Venezia Giulia is the natural corridor where the two populations met during the re-colonization after the glaciation. In the present study 220 autochtonous accessions were sampled and georeferenced and a genetic analysis with 20 microsatellite loci taken from the literature was carried out. The local population shows a moderate genetic diversity and can be classified in two clusters. The first one shows a great mixture between genotypes coming from different areas, even particularly far away from each other. To this first cluster belongs the vast majority of the samples. The second cluster consist of three genotypes originated from an import of Walnuts from Russia at the beginning of the XX century. On the genotypes that during the investigation produced enough fruits carpometric analysis were performed. The best accessions were included in a ranking list compiled using a multivariate analysis considering all the traits valuable for breeding and selection, such as nut weight, kernel weight, shell thickness, kernel skin colour, ease of kernel extraction and fruit appearance. These best accessions will either be selected as they are for vegetative propagation or employed in breeding programs. For every genotype that produced fruits the oil content and its acidic spectrum were determined. Both turned out not to be influenced by the year of sampling. The studied wild walnut trees shown a significant linear relationship and a positive relationship between the minimum daily temperature and the oleic acid content. Many of the accessions could be used in breeding programs for the selections of cultivars with high oil contents and with differentiated acidic spectra, depending on the purpose of the oil. The last part of the investigation was about the diffusion in Friuli Venezia Giulia of two non-local walnut pests come from America, a leafminer belonging to the genus *Coptodisca* and the carpophagous *Rhagoletis completa*. *Coptodisca lucifluella* has been found in 55 out of the 219 considered sites. This is the first report of the species for the region. The species has been identified not only by typical leaf symptoms but also by means of mitochondrial DNA barcode. The DNA sequences were submitted to Genebank. The level of pest infestation in Friuli Venezia Giulia resulted lower than in other Italian areas and its diffusion was negatively correlated with the altitude. The species was observed in sites above 600 m a.s.l. The low infestation observed could be associated with the activity of native natural enemies, as parasitoids of the genus *Chrysocharis* (Hymenoptera, Encyrtidae) that were observed into the mines. *Rhagoletis completa* was found in 89 out of the 185 walnut trees with fruits. In many

sites, especially in lowland localities, the infestation level was very high, as to suggest treatments with insecticides. The level of infestation decreased with altitude: at sites above 700 m a.s.l. either there was no infestation or the infestation was very low. No correlation has been found between the average weight of the fruits and the level of infestation.

Sommario

La specie *Juglans regia* L. è distribuita principalmente nelle zone temperate e subtropicali dell'emisfero settentrionale. Durante l'ultimo fenomeno glaciale la specie è sopravvissuta in zone rifugio, identificabili in Europa nella penisola italiana e in quella balcanica. Il Friuli Venezia Giulia rappresenta il corridoio naturale dove le due popolazioni si sono sovrapposte durante la ricolonizzazione del periodo post-glaciale. Nel presente studio sono state campionate 220 accessioni autoctone di noce, gli individui sono stati georeferenziati e sottoposti ad indagine genetica con l'utilizzo di 20 microsatelliti individuati in letteratura. La popolazione mostra una moderata diversità genetica e risulta divisa in due cluster. Il primo comprende la maggioranza delle piante ed è caratterizzato da una forte mescolanza genetica tra individui provenienti da aree geografiche diverse e anche molto distanti. Il secondo è costituito da tre individui derivati da una importazione di noci dalla Russia all'inizio del XX secolo. Le accessioni che nel periodo di studio hanno fornito un numero sufficiente di frutti sono state sottoposte ad analisi carpometriche. Tramite un'analisi multivariata comprendente i caratteri valutabili per la selezione e il breeding (peso della noce, peso del gheriglio, resa allo sguscio, spessore del guscio, colore dell'episperma, facilità di estrazione del gheriglio, aspetto del frutto) è stata prodotta una ranking list che include le accessioni migliori. Queste saranno moltiplicate per via vegetativa e distribuite ai coltivatori o inserite in programmi di breeding. Di tutte le accessioni che hanno prodotto frutti sono stati determinati il contenuto in olio e il suo spettro acidico. Entrambi non sono risultati essere influenzati dall'annata. Negli individui considerati nello studio sono state osservate una relazione lineare significativa e una correlazione positiva tra la temperatura minima giornaliera e il contenuto in acido oleico. Molte delle accessioni interessate dallo studio potrebbero essere impiegate in programmi di breeding per selezionare varietà con alto contenuto in olio e con spettri acidici differenziati in base all'utilizzo finale dello stesso. L'ultima parte del lavoro ha riguardato la diffusione in Friuli Venezia Giulia di un fillominatore appartenente al genere *Coptodisca* e del carpofago *Rhagoletis completa*, due parassiti di *Juglans regia* introdotti dall'America. La specie di fillominatore, identificata come *Coptodisca lucifluella*, è stata rinvenuta in 55 dei 219 siti considerati dallo studio, primo caso di presenza della specie in regione. La specie è stata identificata non solo grazie ai tipici sintomi fogliari ma anche per mezzo del sequenziamento del DNA mitocondriale. Sono stati identificati due diversi aplotipi in accordo con quanto già merseo dalla letteratura. Le sequenze di DNA sono state depositate in Genebank. Il livello di infestazione in Friuli Venezia Giulia è risultato inferiore rispetto ad altre regioni italiane e la diffusione della specie è

risultata negativamente correlata con l'altitudine. Nelle zone più alte di 600 m s.l.m non è stato rinvenuto nessun esemplare. Il basso livello di infestazione può essere associato all'attività di antagonisti naturali, come parassitoidi del genere *Crhysoharis* (Hymenopterae, Encyrtidae) che sono stati osservati su parte delle larve campionate. *Rhagoletis completa* è stata rinvenuta in 89 dei 185 alberi di noce con frutti. In molti siti, in particolare in località di pianura, il livello di infestazione era molto elevato. Il livello dell'infestazione è risultato diminuire all'aumentare dell'altitudine: nei siti al di sopra dei 700 m s.l.m. l'infestazione era assente o molto contenuta. Non è stata trovata alcuna correlazione tra il peso medio dei frutti e il livello dell'infestazione.

Preface

This thesis is structured as a so-called cumulative Ph.D. thesis and as such includes the scientific papers to which I contributed during my Ph.D. The thesis is organized as follows. First of all, an introductory section provides some background information about the systematics of the walnut tree and the relevance of its cultivation in the world and in particular in Friuli Venezia Giulia, to give a context for the main topic of this work, the characterization of autochtonous walnut germplasm. The goals of the thesis are explained in more detail in the second section, where a general description of how the work was organized is provided, too.

The main part of the tesis consist of the four scientific papers realized with the results obtained from the studies carried out during the three years of the Ph.D. The first paper, already published in the journal *Forests*, presents the results of the activities concerning the genetic characterization of the collected Walnut population. The paper is included in its original and published form. In the second paper the results of the investigations of the morphological and carpological variability in the collected walnut population are presented. The paper has been submitted to the journal *Scientia Horticulturae* and is included in the form of the submitted manuscript. The third paper shows the results of the complete investigation of the walnut oil and the elements influencing its quality. This study was finalized very recently and is included as the current version of a manuscript that we plan to submit to the *Journal of the Science of Food and Agriculture* (Wiley). The fourth paper presents the activities concerning an entomological investigation that involves two walnut non-native phytofagi introduced from America. It is included in the form of a final manuscript that we are submitting to the journal *Bulletin of Insectology*.

The last section briefly summarizes all the results presented in the main part and gives an outlook in the light of the main findings of the thesis.

Contents

Abstract i

Sommario iii

Preface v

Table of contents vii

1. Introduction 1

1.1 Systematics of Juglandaceae 1

1.2 Relevance of the crop 2

1.3 Historical informations about Walnut presence and cultivation in Friuli Venezia Giulia 3

1.4 Why characterising the autochthonous walnut germoplasm in Friuli Venezia Giulia? 5

2. Aims of the work 9

3. Genetic diversity of Walnut (*Juglans regia* L.) in the Eastern Italian Alps 11

4. Morphological and carpological variability of Walnut germplasm (*Juglans regia* L.) collected in North-Eastern Italy and selection of superior genotypes 25

5. Determination of kernel oil content, oil composition and its modification by temperature in Walnut (*Juglans regia*) L. accessions from North-Eastern Italy 55

6. Distribution and damage of two non-native insect species (*Coptodisca lucifluella* and *Rhagoletis completa*) associated to common Walnut (*Juglans regia* L.) in Friuli Venezia Giulia (North-Eastern Italy) 83

7. Conclusion and outlook 103

1. Introduction

1.1 Systematics of Juglandaceae

The family of Juglandaceae is part of the Fagales, that belong to the dicotyledonous and therefore to the Angiospermae. It is a small family that consists of 70-80 arboreal and shrub-like species organized in eight genera (Manos *et al.* 2001). In Eurasia, the diffusion area spreads from the South-East of Europe and the central Asia to the South East of Asia. In America it goes from the South of Canada to the South America. The *Platycarya*, *Carya*, *Pterocarya*, *Cyclocarya* and *Juglans* genera are characteristic of the temperate zones. They are either forestry (*Carya cordiformis* Wan.) or decorative (*Pterocarya fraxinifolia* Lam.) plants (Schmidt 1995). With the exception of some belongings to the *Juglans* genus, an interesting fruit plant is the species *Carya Illinoiensis* Wan., also known as Pecan. Since it resists to low temperatures, it is possible to grow it even in the Mediterranean Europe. The Asian Juglandaceae that belong to the genera *Cyclocarya* and *Platycarya*, like *Cyclocarya paliurus* Bat. and *Platycarya strobilacea* Sie., are instead botanical rarities. The species that belong to the other three genera, *Alfaroa*, *Oreomunnea* (nine and two evergreen arboreal species hailing from the central America rainforest, respectively) and *Engelhardia* (about ten species hailing from China), are strictly tropical (VV. AA. 2008).

The genus *Juglans* is both the richest in species and the most known, due to the several uses of the fruits and of the wood of the species that belong to it. The name Juglans comes from latin Jovis glandis, that means Jupiter's acorn. The plants belonging to this genus are deciduous, monoecious and hail from temperate-cold zones. The genus is organized in four sections.

- Section Cardiocaryon: hailing from the North-East of Asia, very large leaves, soft wood, racemes with many fruits and thick walnut shell. To this section belong *J. ailantifolia* Car. and *J. mandshurica* Max.

- Section Rhysocaryon: hailing from America, known as "black walnuts". Some species have particularly hard wood. To this section belong *J. australis* Gri., *J. boliviana* C. DC. (regarding this latter, there are still some contestations about the systematic classification, since some authors, e.g. Manning, think that the differences between *J. boliviana* and *J. peruviana* are not so pronounced to justify the classification of the former as a proper species), *J. californica*, S.Wats. *J. Hindsii* Jep., *J. hirsuta* Man., *J. jamaicensis* G. DC., *J. major* Tor., *J. microcarpa* Ber., *J. mollis* Eng., *J. neotropica* Die., *J. olanchana* Sta., *J. peruviana*, *J. soratensis* Man., *J. steyermarkii* Man., *J. venezuelensis* Man., *J. nigra* L.

- Section Trachycaryon: hailing from North America, very large leaves (40-90 cm), soft wood, edible fruits that come in small clusters consisting of two or three of them and are characterized by very hard nutshell and winged crests. To this genus belongs *J. cinerea* L.

- Section Juglans: hailing from central Asia and from the South-East of Europe, large leaves (20-45 cm), particularly hard wood. To this section belong *J. sigillata* Dod. and *J. regia* L. (Manning 1978). The latter fruits are subglobose shaped with green and tannin rich husk. At ripening the husk gets longitudinally splitted and releases the woody endocarp. The kernel is big, palely coloured and can be extracted more easily than the ones of the others species belongig to this genus. The compound leaves are pinnate, consisting of 5-9 elliptically shaped leaflets, alternated, light green, up to 35 cm long, aromatic and smoothtly edged. The wood is solid, homogeneous and easily malleable and is the finest among the species of the same genus, with a colour varying from brown to light blond (VV. AA. 2008).

In the walnut case it is not easy to identify the borders of its last natural distribution (Beer *et al.* 2008). It seems likely that the species hails from Cina (Xinjiang, Tien Shan) and Himalaya (Huntley B. e Birks H.J.B. 1983). Subsequently, it spread from the refuge areas (Europe, China, Syria, Himalaya and Kyrgyzstan) after the last ice age (Beer et al. 2008). As proved by wrecks of fossil pollen that can be ascribed to Greek and Roman settlments, the origins of walnut plantation in Italy are really ancient (Being 1975).

1.2 Relevance of the crop

According to Food and Drug Administration (FAOSTAT) data, in 2010 the world walnut production was about 2.5 million tons, from about 846000 hectares of cultivated area. In the same year, with more than 1 million tons of production, China confirmed its positions as first producing country, followed by USA (458000 tons), Iran (270300 tons), Turkey (178142 tons) and Ukraine (87400 tons). This five countries contribute for the 80% to the world production and for the 66% to the world cultivated area. Since 2004 this product market has noticeably grown, both considering the cultivated area and regarding the production: comparing data from 2004 and from 2010 for the aforementioned five countries, the production has been increased by 89% and the cultivated area has been extended by 24%, according to the Food and Agriculture Organization Corporate Statistical Database (FAOSTAT).

In Europe there are 89256 cultivated hectares, from which about 170000 tons of nuts are produced. The greatest producer is Romania, with the 20.27% of the whole production, followed by France, with the 17.96%, Greece, with the 13.09%, Spain, with the 8.02%, and Italy (7.78%). The cultivated area in these countries is the 47% of

the whole european one. Between 2004 and 2010 the nut production in the European countries has been increased by the 28%, not regularly though. Among the five aforementioned countries, in France and Italy the production has instead registered a decrease in the period 2008-2010. Regarding Italy, the reduction has been lasting since 1990 and affects the 47% of the production (FAOSTAT).

According to 2007 data from the italian "Istituto di Servizi per il Mercato Agricolo Alimentare (ISMEA)", Italy imports a large amount of nuts, particularly of walnuts. In that year, Italy imported nuts for 482 million euro, the 8% of which was due to 19000 tons of walnuts, imported mostly from USA, that corresponded to the 14% of the imported nuts (FAOSTAT).

According to the 2007 data from the italian Istituto Nazionale di Statistica (ISTAT), in Italy walnut is cultivated on an agricultural land of about 8600 hectares, that is the 5% of the whole land allotted to the nuts cultivation. In 2012 more then 18540 tons of nuts were produced for 24 million euro (Piccirillo *et al.* 2013). The Italian regions leader for the production are Campania, with more than 11800 tons, and Veneto (where are located the only Farmers' organizations), with 2900 tons. In the South of Italy there is no particular specialization in terms of cultivars (the most cultivated ones are the "Sorrento" one, an open-pollinated seedling cultivar, and the "Malizia" one, obtained from a massal selection of the former) and the walnut cultivation does not play a main role, since it still mantains the old double purpose of bearing fruits and supplying wood at the end of the productive life of orchards. In the North of Italy, instead, the walnut cultivation has the main purpose of producing fruits and is notably highly specialized (density of plants in the order of 400/hectare, against the 90-100/hectare of the traditional ones), making use of plants coming from grafted Californian cultivars, like Hartley and Chander, and French ones, like Lara. The latter are preferred since they flower laterally, and are therefore more productive, and have been genetically improved, becoming particularly sought because of their commercial traits.

1.3 Historical informations about Walnut presence and cultivation in Friuli Venezia Giulia

Friuli Venezia Giulia region is located in the North-East part of Italy, on the border with Austria and Slovenia. It is notably characterized for being a transition zone between different kinds of environments: from the North-West to the South-East of the region, spanning 120 km, one can find an alpin environment, a transition one, a continental one and eventually a mediterranean one. In the region the average temperatures range between 7.4 and 15.5° Celsius and the rainfall between 993 and 3094 mm (OSMER data). The common walnut has been present in Friuli Venezia Giulia since last ice age, when the species hailing from south refuge zones reached the areas that had been freed by the ice age. In the eastern Alps and Pre-Alps zone the populations coming from the Italian peninsula overlapped the ones hailing from the Balkan area. This produces a very high genetic variability, still nowadays appreciable. Although the ideal growing

area is characterized by yearly mean temperatures between 10 and 17° C and rainfall levels higher than 700 mm (Forte 1993) and the species is more sensitive to late frosts and to minimum winter temperature than other fruit crops like apple, anyway the walnut found a wide variety of favourable environments. Whitin the region the species is indeed omnipresent: it can be found from the Carso region to the high mountains, sometimes even beyond 1000 above the sea level (e.g. Ravascletto – Carnic Alps, 1050 m above the sea level), with the exception of those areas where it is not present any more due to human causes. Suprisingly, it can survive to rather extreme climate conditions, like the minimum winter temperatures of Fusine (Julian Alps, -28° C in 1985, OSMER data) or the rainfalls level of Musi (Julian Prealpis, 6100 mm in 1960 registered by a local meteorological station, OSMER data). In Friuli Venezia Giulia there are no orchards expressely aimed to the walnut production and regarding the wood production, the dedicated area is negligible, in contrast to what happens in the close Slovenia, where there are some plants in which improved varieties are used (Solar 2002). Even if the walnut has always been considered a "secondary" fruit species, its cultivation has always been rather common in the region. Testimony of that is given by the many place names that recall the walnut cultivation. Particularly, this is the case for example of San Giorgio di Nogaro (South of Friuli), close to the sea, Nogaredo al Torre (in Comune di San Vito al Torre), Nogaredo di Prato (in Comune di Martignacco) and Nogaredo di Corno (in Comune di Coseano) in the plain, and Nojariis (in Comune di Sutrio), Nojarê (in Comune di Paularo) and Nojareit (in Comune di Comeglians) in the Carnic Alps (Nojâr and Nogaro in the local languages mean Walnut). Moreover, several local traditions are related to walnut, like the one of planting one walnut tree when a child was born. For people living in the alpine and pre-alpine regions (Alpi e Prealpi Carniche e Giulie) (Zandigiacomo 1998) the walnut used to have a fundamental role due to the nutrititive value and to the easy fruits storage and transport. In the pre-alpine zone it was common to offer walnuts to guests as a cordial welcome. In the past, the walnut cultivation was mostly developed in the mountain area, where the walnut was more present. In the 1885 literary work "Il comune rustico" by Giosuè Carducci, one of the verses is dedicated to the walnuts of the Carnia, that he was visiting in that period, that demonstrates how common the cultivation was (O noci della Carnia, addio! Erra tra i vostri rami il pensier mio sognando l'ombre d'un tempo che fu).

The 1929 agrarian real estate registry, that reported the mean production for the period 1923-1928, allows to have the first reliable data about the walnut cultivation. The data confirm that the cultivation was mostly diffused in the mountain region, with 1459 q. produced in Carnia, 338 in the Val Canale-Canal del Ferro, 236 in the carnic Prealps and 159 in the eastern montain region of Slavic Valleys. For the western, central and eastern hill area, 146, 94 and 176 q. were reported, respectively. The produced amount was less important in the plains area, with 72 q. in the low plain and in the San Vito al Tagliamento area, 139 in the west Fiuli plains, 110 in the middle area of Friuli and 16 in the area between Tagliamento and Cellina rivers, where actually also

the cultivation of other fruits crops was not very common (Zandigiacomo 1998).

In the same year, in the booklet "Lezioni di frutticoltura per contadini tenute dall'esperto per. Agr. Mario de Bortoli" (De Bortoli 1929) a section appears that is dedicated to the walnut cultivation, in which the author complains about the walnut reproduction being not very well managed and good only for plants obtained from seed and not from grafting. A possibly local variety, known as "di San Giovanni" is described, that had the attractive trait of flowering late in the season, therefore avoiding late frosts. In the highest production areas (Carnia and Pre-Alps), only the apple cultivation was more important than the walnut one. The walnut consumption was very common, since the fruit is particularly energetic and has high nutritional values. Moreover, once dried, it can be very easily stored without needing specific cares. The walnut cultivation for producing fruit lost importance only after the second world war, when the improvement of the life conditions caused the beginning of the depopulation of the mountain regions and the demolition of the highest trees for getting the wood, leading to an important loss of biodiversity.

1.4 Why characterising the autochthonous walnut germoplasm in Friuli Venezia Giulia?

One of the very fundamental resources for breeding programs of crops is genetic diversity. It comes from wild populations but also from cultivated ones, when not strongly selected by farmers. Selecting walnut is difficult mainly for two reasons, namely its allogamy and the long juvenile phase (because of that a long time is needed to evaluate the genotype, on contrary to what happens for other fruit crops). Moreover, the agamic propagation by grafting is difficult, too. To increase the chances of success and produce large amounts of grafted plants for cultivation, it is necessary to adopt the Hot callusing method (artificial heating of the grafting point), that needs specific technical knowledge (Achim 2001). These aforementioned difficulties in maintaining and diffusing the most promising genotypes caused the genetic diversity to be preserved in the walnut orchards up to nowadays. In particular, in Turkey and Iran it is possible to see a notable variability and to find many interesting genotypes (Aslantaş 2006 and Arzani 2008). The most appealing traits are the bearing habit (lateral or intermediate bearing), some nuts traits (nut weight, kernel percentage, kernel colour and shell thickness) and the late flowering. Several studies for characterizing the autochthonous germoplasm of walnut have been carried out also in India (Sharma and Sharma 1998), China (Gunn 2010) and Europe, in particular in Spain (Aleta and Ninot 1997), France (Germain 1997), Romania (S. Cosmulescu and Botu 2012), Albania (Zeneli 2005), Serbia (Cerovič 2010), Austria (Asadian and Pieber 2005) and Slovenia (Solar 1990). In some cases the collected material was not of particular interest (Akca and Sen 2001) but very often among the wild populations or the ones extensively cultivated it is possible to find many genotypes that are promising for cultivation or breeding. Some of these genotypes are superior than the improved cultivars (Mahmoodi 2016, Rouskas

2001). A large number of wild walnut trees and a wide variability regarding many traits (both nut traits and physiological ones) can be observed in Friuli Venezia Giulia, too. As in the other aforementioned locations, only the seedlings propagation has been adopted. In this region there are no intensive cultivations with improved cultivars yet and historical data regarding the commercialization of walnut trees are not found (this was on the contrary the case of other fruit crops, like for example apple, pear, cherry or chestnut). Only data about nuts trade are found (Zandigiacomo 1998). The resistance to diseases and pests is important, too. For breeding programs the antracnose resistance is of particular interest. The climate conditions and notably the high rainfall determine the development of the disease. In Friuli Venezia Giulia this situation occurs more than in other areas. A strong selective pressure from the antracnose promoted the diffusion of resistant or very tolerant genotypes.

In Friuli Venezia Giulia were still missing both a local walnut germoplasm characterization and investigations regarding introduced pests, like the husk fly (*Rhagoletis completa* Cre.), become common in many places (Aluja 2011). Of notable interest is the possible presence of leafminer belonging to the genus *Coptodisca*, recently introduced in Italy (Bernardo 2012). In the South of Italy a population has been found that has been identified as *C. lucifluella* Cle. The species is present also in the Veneto region (Bernardo 2015), though, and this leads to think it could have reached the Friuli Venezia Giulia, too.

References

Achim G. H. and Botu I. 2001. Results in Walnut propagation by using different methods. *Acta Hortic.* 544, 503-509.

Aleta N. and Ninot A. 1997. Field evaluation of Juglans regia selected clones from seedling populations of Mediterranean and Atlantic Spanish coast. *Acta Hort.* (ISHS), 442: 63-67.

Aluja M., Guillén L., Rull J., Höhn H., Frey J., Graf B., Samietz J. 2011. Is the alpine divide becoming more permeable to biological invasions? - Insights on the invasion and establishment of the Walnut Husk Fly, *Rhagoletis completa* (Diptera: Tephritidae) in Switzerland. *Bulletin of Entomological Research*, 101 (4): 451-465.

Akca Y. and S. M. Sen. 2001. Study on the selection of superior walnut trees in Hizan (Bitlis) populations. *Acta Hort.* (ISHS), 544: 115-118.

Arzani K, Ardakan H., Vezvaei A. and Roozban M. R. 2008. Morphological variation among Persian walnut (*Juglans regia*) genotypes from central Iran, *New Zealand Journal of Crop and Horticultural Science*, 36:3, 159-168.

Asadian G., Pieber K. 2005. Morphological variations in walnut varieties of the Mediterranean regions. *International Journal of Agriculture and Biology*, 7: 71-73.

Aslantaş R. 2006. Identification of superior walnut (*Juglans regia*) genotypes in northeastern Anatolia, Turkey, *New Zealand Journal of Crop and Horticultural Science*, 34:3, 231-237.

Beer R., Kaiser F., Schmidt K., Ammann B., Carraro G., Grisa E., Tinner W. 2008. Vegetation history of the walnut forests in Kyrgyzstan (Central Asia): natural or anthropogenic origin?. *Quaternary Science Reviews*, 27: 621–632.

Being H. J. 1975. Man as factor in the Vegetation History of the Balkan Peninsula. Proceedings of the 1st International Symposium on Balkan Flora and Vegetation. House of the Bulgarian Academy of Sciences, Varna (Bulgaria), 72/78.

Bernardo U., Sasso R., Gebiola M., Viggiani G. 2012. First record of a walnut shield bearer *Coptodisca* (Lepidoptera: Heliozelidae) in Europe. *Journal of Applied Entomology*, 136 (8): 638-640.

Bernardo U., van Nieukerken E. J., Sasso R., Gebiola M., Gualtieri L., Viggiani G. 2015. Characterization, distribution, biology and impact on Italian walnut orchards of the invasive North-American leafminer *Coptodisca lucifluella* (Lepidoptera: Heliozelidae). *Bulletin of Entomological Research*, 105 (2): 210-224.

Cerović S., Gološin B., Ninić Todorović J., Bijelić S., Ognjanov V. 2010. Walnut (*Juglans regia* L.) selection in Serbia. *Horticultural Science*, 37: (1) 1–5

Cosmulescu S., Botu M. 2012. Walnut biodiversity in south-western Romania - resource for perspective cultivars. *Pakistan Journal of Botany*, 44(1): 307-311.

De Bortoli M. 1929. Lezioni di frutticoltura per contadini tenute dall'esperto per. Agr. Mario de Bortoli. Consorzio provinciale di frutticoltura del Friuli - Udine. Sede presso la cattedra ambulante di agricoltura per la provincia di Udine.

Forte V. 1993. *Il noce*, Edagricole-Edizioni Agricole della Calderini s.r.l., Bologna.

Germain E. 1997. Genetic improvement of the Persian walnut (*Juglans regia* L). *Acta Horticulturae*, 442: 21-31

Gunn B. F., Aradhya M., Salick J. M., Miller A. J., Yongping Y., Lin L., Xian H. 2010. Genetic variation in walnuts (*Juglans regia* and *Juglans sigillata*; Juglandaceae): species distinctions, human impacts, and the conservation of agrobiodiversity in Yunnan, China. *Am. J. Bot.*

Huntley B. and Birks H. J. B. 1983. *An atlas of past and present pollen maps for Europe: 0-13000 years ago.* Cambridge University Press. N.Y.: p. 238-242.

Mahmoodi R., Hassani D., Amiri M. E., Jaffaraghaei M. 2016. Phenological and Pomological Characteristics of Five Promised Walnut Genotypes in Karaj, Iran. *Journal of Nuts*, 7(1):1-8.

Manning W. E. 1978. The Classification Within the Juglandaceae. *Annals of the Missouri Botanical Garden*, 65 (4), 1058-1087

Manos P. S., Stone D. E. 2001. Evolution, phylogeny and Systematics of the Juglandaceae. *Annals of the Missouri Botanical Garden*, 88, 231-269.

Piccirillo P, Deluca A. and Ciarmiello L. 2013. Consiglio per la Ricerca e Sperimentazione in Agricoltura - Unità di Ricerca per la Frutticoltura, Caserta. *FRUTTICOLTURA*, (6) 49.

Rouskas, D. and Zakynthinos G. 2001. Preliminary evaluation of seventy walnut (*Juglans regia* L.) seedlings selections in Greece. *Acta Hort.* (ISHS), 544: 61-72.

Schmidt O. 1995. Die Japanische Flugelnuss *Pterocarya rhoifolia*. *Forst und Holz*, 50, 677-678.

Sharma S. D., and Sharma O. C. 1998. Studies on the variability in nuts of seedling walnut (*Juglans regia* L.) in relation to the tree age. *Fruit Variety Journal*, 52: 20-23.

Solar A. 1990. Phenological and pomological characteristics of walnut cultivars in northeastern Slovenia. *Acta Hort.* (ISHS), 284, 167-174.

Solar A., Ivancic A., Stampar F. and Hudina M. 2002. Genetic resources for walnut (*Juglans regia* L.) improvement in Slovenia. Evaluation of the largest collection of local genotypes. *Genetic Resources and Crop Evolution*, 49(5): 491-501.

VV. AA. 2008. *Beitrage zur Walnuss*, LWF Wissen, 61.

Zandigiacomo P. 1998. Peraries, melaries, brombaries e nujârs. Evoluzione della frutticoltura in Carnia negli ultimi due secoli. In Adduca G., Molfetta P., Pellegrini P., Sulli F., Violino C, and Zandigiacomo P. *Perarias melaries... Frutticoltura in Carnia*, Lithostampa-Pasian di Prato (UD), 13/39.

Zeneli G., Kola H., Dida M. 2005. Phenotypic variation in native walnut populations of Northern Albania. *Scientia Horticulturae*, 105: 91–100.

http://www.fao.org/faostat/en/#data/QC (accessed 12/15/16).

http://www.ismea.it/istituto-di-servizi-per-il-mercato-agricolo-alimentare (accessed 12/15/16).

http://www.istat.it/en/(accessed 12/15/16).

http://www.osmer.fvg.it/clima/clima_fvg/02_documenti_descrittivi_e_approfondimenti/04_PIOGGE (accessed 12/12/16).

2. Aims of the work

To achieve the goals of this work (see below) sampling sites spread all over the regional area where the species is present were considered. 220 genotypes were sampled, that became the target of the main lines of the present investigation. Genotypes of possibly non local origin (like for instance commercial varieties) were not considered. In every site the oldest available plants were sampled. When possible, the farmers or the owners of the sampled plants were interviewed, to confirm the local origin of the trees.

The first study carried out in the present work is a molecular analysis of all the genotypes. The aim is to characterize the genetic diversity, to understand the structure of the local walnut population and to identify non autocthonus genotypes.

The second study is a carpological and physiological characterization, to identify the most interesting genotypes. Nut traits, bearing habit and resistance to antracnose are analyzed for a possible use in cultivation or breeding programs. The high mean rainfall of the region allows to easily and certainly detect genotypes that show resistance or low susceptibility to the Antracnose disease.

The third study is the characterization of the oil yield and the acidic profile of the sampled genotypes. The interesting aspect is to employ the intrinsic population variability to get cultivars with differentiated oil content and acidic profile depending on the different uses. Influence by environmental factors is investigated, too.

The topic of the fourth study is an entomological investigation that involves two walnut phytofagirecently introduced from America. Regarding the first species, *Rhagoletis completa* Cresson or Walnut husk fly, the purpose is to explore the regional distribution and factors limiting its diffusion. Concerning the second pest, belonging to the genus *Coptodisca*, recently become common in Italy, the goal is to identify the exact species of the found individuals. Additionally, the study is aimed to the examination of the distribution of the species, also in this case detecting potential limiting factors that could help in its containment.

forests

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Article

Genetic Diversity of Walnut (*Juglans Regia* L.) in the Eastern Italian Alps

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Abstract: *Juglans regia* L. is distributed primarily across temperate and subtropical regions of the Northern Hemisphere. During the last glaciation, the species survived in refugial areas that in Europe included the Balkans and the Italian peninsula, two areas joined by a corridor represented by the Friuli Venezia Giulia region, where two germplasm reservoirs met and likely intercrossed during re-colonization after the last glaciation. In this work, two hundred and fifteen wild accessions native to the area were sampled, georeferenced, and genotyped with 20 microsatellite loci selected from the literature. The local accessions of this study displayed moderate genetic diversity with 80 alleles identified. The number of alleles/loci was 4.0 (4.7 alleles for the genomic SSRs (Simple Sequence Repeats) and 2.7 alleles per EST (Expressed Sequence Tag)-derived SSR, on average). An analysis of molecular variance (AMOVA) revealed that most of the molecular diversity was between individuals (nearly 98% of variation explained). The model-based clustering algorithms implemented either in STRUCTURE and GENELAND software revealed two clusters: The first one encompassed most of the samples and showed a great genetic admixture throughout the five sampling areas defined on the base of orographic characteristics of the region. The second cluster represented a small island with three samples traced back to an introduction from Russia at the beginning of the 20th century.

Keywords: walnut germplasm; *Juglans* spp.; germplasm; genetic diversity; SSR genotyping; spatial structure genetics

1. Introduction

Persian walnut (*Juglans regia* L.) is one of approximately 21 species in the genus *Juglans* L. [1]. The genus originated in the late Paleocene, diversified during the Eocene [2], and maintained its presence in the area between the 45° and 65° paleolatitudes during evolution of the climate to cooler conditions [3]. *Juglans regia* L. is nowadays distributed primarily across the temperate and subtropical regions of the Northern Hemisphere from Central Asia to the Mediterranean basin [4]. Because of the presence of large walnut (*J. regia* L.) forests in countries of Central Asia such as Kyrgyzstan, Uzbekistan, and Tajikistan, and the large phenotypic diversity recorded in those countries, Vavilov considered

Central Asia to be the primary center of diversity of the species [5], but the subject is still debated [6]. It is likely that prior to the Pleistocene glaciations the species had a wide distribution in Eurasia, but during the glacial periods the distribution was contracted to refugial areas in China, the Himalayan slopes, Southern-Central Asia, the Balkans, and the Iberian and Italian peninsulas [7–10]. Following the last glaciation, recolonization of the species' original areas of diffusion and other neighboring areas with compatible climates has likely masked the preglacial centers of origin of this species. Yet, this spontaneous spread was accompanied 2000 years ago by artificial diffusion through human silvo-pastoral practices, as has been demonstrated in the Fergana Valley where walnut forest stands are a mosaic of natural and planted trees [9,11–13]. Nevertheless, disregarding the problem of the original center of diffusion of the species, and considering only the recent spread after the last glacial maximum (LGM), the Southern Balkan and Italian peninsulas were two likely reservoirs of germplasm from which the species recolonized northern latitudes [7]. However, the severe reduction in the walnut populations in these reservoirs that has been claimed by some authors would have favored the re-introduction of the species from Western Asia [14]. The above-mentioned peninsulas, which are separated from each other by the Adriatic Sea, are joined to the north by a corridor represented by the Friuli Venezia Giulia Region and neighboring areas such as Istria. These are known to be areas where different germplasm populations, which had retreated during the last glaciation, came into contact again, thus creating new genetic diversity. The botanical richness of these regions evidences the intermingling of the flora of the two glacial refuges [15]. Walnut fossils are commonly found in the Neolithic sites of northeastern Italy [16]. Therefore, a wide genetic diversity is expected in the corridor represented by the Friuli Venezia Giulia region, so a wide exploration of wild walnut was undertaken in that area by sampling 215 old trees propagated by seed alone according to information collected from local people. The genetic diversity was investigated with 20 Simple Sequence Repeat (SSR) markers selected from the literature.

2. Materials and Methods

2.1. Plant Materials

The work plan aimed to sample walnut trees (*Juglans regia* L.) originating from seedlings in the Friuli Venezia Giulia region. To achieve a good representation of the native walnut population, the region was initially split into geographically homogeneous areas, represented by the eastern Alpine highlands ('Alpi Giulie', pop 1), the western Alpine highlands ('Alpi Carniche', pop 2), the Friuli plains (pop 3), the valleys of the Torre and Natisone rivers (pop 4), and the Trieste karst (pop 5) (Figure 1). At each location, the trees sampled were among the oldest individuals in the area. In some areas that were poorly populated, additional younger trees were sampled when their seed origins were known with confidence. A total of 215 accessions were collected. The list of the accessions analyzed is reported in the Supplementary Material (Table S1).

2.2. DNA Extraction

Leaves were collected from young shoots at the beginning of the season or mature leaves later in the season. DNA was extracted using the DNeasy 96 Plant Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol.

2.3. SSR Genotyping

A review of the SSRs present in the literature [10,12,17–26] suggested the selection of twenty-two SSR markers, which were tested initially in a small panel of accessions. The control of amplification, the goodness of signal recorded by the automatic sequencer, and the number and quality of the true peaks suggested that only 20 of those SSR markers be retained for the analysis of the whole set of accessions. Furthermore, the neutrality of selected loci was checked with the Ewens-Watterson Test (1000 permutations) [27] using PopGene software [28]. Markers from different literature sources

are collectively reported in Table 1 with primers and annealing temperatures for the convenience of the reader.

(**A**)

(**B**)

Color	Geographic Area
	Western Alpine Highlands
	Eastern Alpine Highlands
	Torre Natisone Valleys
	Friuli Plains
	Trieste Karst

Figure 1. (**A**) General map with a red circle representing the region of study; (**B**) Explosion of the sampling areas, for the color code see the legend below.

Table 1. List of the 20 Simple Sequence Repeat (SSR) markers selected from the literature for this study.

SSR Code	Repeat Array	Forward Primer	Reverse Primer	Tanneal (°C) [a]	Reference
WGA001	(GA)5GCA(GA)3GCA(GA)3	attggaagggaagggaaatg	cgcgcacatacgtaaatcac	48–58	Dangl et al., 2005 [18]
WGA004	(GT)5,(GA)15,(GA)11	tgttgcattgacccacttgt	taagccaacatggtatgcca	50–58	Woeste et al., 2002 [17]
WGA009	(GA)16	catcaaagcaagcaatggg	ccattgctctgtgattggg	48–58	Dangl et al., 2005 [18]
WGA027	(GA)30	aaccctacaacgccttgatg	tgctcaggctccacttcc	51–55	Woeste et al., 2002 [17]
WGA069	(GA)4ATATAA(GA)16	ttagttagcaaacccacccg	agatgcacagaccaaccctc	45–58	Woeste et al., 2002 [17]
WGA071	(GA)6,(G)12	acccgagagatttctgggat	ggacccagctcctcttctct	45	Woeste et al., 2002 [17]
WGA072	(CT)14	aaaccacctaaaaccctgca	acccatccatgatcttccaa	55–58	Woeste et al., 2002 [17]
WGA089	(GT)13(GA)21	acccatctttcacgtgtgtg	tgcctaattagcaatttcca	53–58	Dangl et al., 2005 [18]
WGA118	(GA)18(GT)11	tgtgctctgatctgcctcc	gggtgggtgaaaagtagcaa	55–62	Dangl et al., 2005 [18]
WGA225	(AG)14	caatccctctcctgggcag	tgttccactgaccacttcca	n.r.	Dangl et al., 2005 [18] [b]
WGA321	(GA)14	tccaatcgaaactccaaagg	tgtccaaagacgatgatgga	50–58	Dangl et al., 2005 [18]
WGA331	(GA)13	tcccctgaaatcttctcct	cggtggtgtaaggcaaatg	53–58	Dangl et al., 2005 [18]
WGA349	(CT)14	gtggcgaaagtttatttttttgc	acaaatgcacagcagcaaac	n.r.	Dangl et al., 2005 [18] [b]
WGA376	(AG)2AA(AG)6	gccctcaaagtgatgaacgt	tcatccatatttacccctttcg	n.r.	Dangl et al., 2005 [18] [b]
Contig_40	(CTGT)5	tgggctgagctggattgccgt	tccaccgtcatggtttccacg	59	Zhang et al., 2010 [22]
Contig_156	(TTTG)6	tgcaagagtggcgcaggcactg	tggtagcctaatctcatggctcg	60	Zhang et al., 2010 [22]
Contig_642	(CAG)7	tgaaaggttttggcctccaatgg	tgagatcatgggctgcctgtagg	59	Zhang et al., 2010 [22]
Contig_721	(CTT)8	acccctggtttgaactgcgac	agatccaactttcgcgtggaac	57	Zhang et al., 2010 [22]
Contig_1528	(CCT)7	ccgaagagatcctaagctcaacc	gaggtggaaatgatggtggggtg	59	Zhang et al., 2010 [22]
Contig_1681	(TTC)8	agagatttctccaggaaggctcc	tctggtggccaacgatagccga	62	Zhang et al., 2010 [22]
Contig_1692	(CCA)6	caatggtcagtttccgtccgatc	cgagctcgaatacttctcgtcg	58	Zhang et al., 2010 [22]

[a] The annealing temperature from the literature, n.r. = not reported; [b] The core repeat for these markers was recovered from the NCBI (National Center of Biotechnology Information) database of walnut sequences.

The forward primers were tailed by adding a 19-base M13 oligo sequence (M13 tail) labeled with FAM or HEX dye to the 5′ end [29,30]. The PCR reaction was carried out in a 10 µL solution containing 10 ng genomic DNA, 1 × Mg-free PCR buffer solution, 0.20 mM·dNTPs (Deoxynucleotide Triphosphates), 3.0 mM·MgCl2, 0.10 pmol forward primer, 0.30 pmol reverse primer, 0.30 pmol M13-labeled primer, 1.0 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), and dH_2O. Amplification was performed in a 9700 Thermal Cycler (Applied Biosystems) with the following cycles: 10 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 45 s at 57 °C, 45 s at 72 °C; eight cycles of 30 s at 94 °C, 45 s at 53 °C, 45 s at 72 °C; and a final extension cycle of 30 min at 72 °C. The PCR products were separated in an ABI 3730 DNA automatic sequencer (Applied Biosystems) and fragments sized by means of the GeneScan™ 500 LIZ® Size Standard (Applied Biosystems). Data were analyzed and alleles called with GeneMarker software version 2.2.0 (SoftGenetics, College Station, TX, USA).

2.4. Data Analysis

2.4.1. Genetic Variation and Related Metrics

Diversity parameters were estimated for each SSR locus, including the number of alleles, the number of unique genotypes, observed heterozygosity (Ho), expected heterozygosity or gene diversity (GD), polymorphism information content (PIC), and the major allele frequency (MAF). The parameters were calculated with PowerMarker v3.25 software [31]. The Hardy-Weinberg equilibrium (HW) and the probability of identity (PID) for both unrelated genotypes and full sibs [32] were calculated using Cervus (http://www.fieldgenetics.com) [33,34]. The estimation of the expected frequency of null alleles (Fnull) was calculated in Cervus using an iterative algorithm (10 iterations) based on the observed and expected frequencies of the various genotypes [35]. The kinship coefficient (Fij) was estimated according to J. Nason as described in [36] using SPAGeDi 1.5 [37], and statistical significance was determined by Jackknifed after 20,000 permutation estimators [38].

2.4.2. The Comparison among Populations

Genetic diversity was measured for each population across all loci by calculating the actual (A) and the effective (Ne) number of alleles/loci, observed (Ho) and expected heterozygosity (He), using GenAlEx 6.3 software [39]. Allelic richness (Rs) was calculated by using the rarefaction method with HP-Rare software [40,41]. The estimates of Rs were standardized on a minimum sample size of six individuals.

2.4.3. Distance-Based Clustering

Distance matrixes of pairwise combinations of populations and individual accessions were calculated for co-dominant data by the Codom-Genotypic distance option in GenAlEx 6.1 software [39,42]. The matrixes generated were used for subsequent PCA and analysis of molecular variance (AMOVA) analyses with 999 permutations. A Mantel test, as implemented in GenAlEx, was performed using genetic and geographical distances to test for isolation by distance [43]. Cluster analysis was carried out on genetic distances by the neighbor joining method [44] using MEGA 6 software [45]. The results were visualized in the form of a circular tree using TreeView v1.6.6 [46].

2.4.4. Model-Based Clustering

We applied the model-based clustering algorithm implemented in the software, STRUCTURE [47]. STRUCTURE was run independently 20 times for each K value (range 1–20) using 250,000 iterations for burn-in and 1,000,000 iterations for MCMC (Markov chain Monte Carlo). We used the admixture model option with the correlated allele frequencies [48]. Parameters were set to their default values, as advised in the package documentation [49]. All accessions were treated as having unknown origin (USEPOPINFO = 0). The inference of true K (number of populations) was calculated based

on the second order rate of change of the likelihood (ΔK) [50]. The STRUCTURE results were processed using STRUCTURE HARVESTER web version v0.6.94 (http://taylor0.biology.ucla.edu/structureHarvester/) [51].

2.4.5. Landscape Genetics

We also inferred population structure using a Bayesian Monte Carlo Markov Chains method implemented in the Geneland package, version 4.0.5 [52], under the R Language and Environment for Statistical Computing software, as described by Guillot et al. [53–55] and Guillot [56]. Twenty independent Markov Chain Monte Carlo runs were performed by Geneland with the following settings: 1,000,000 iterations with 100 thinning intervals and a burn-in period of 250,000, using the correlated allele frequencies model. The maximum number of populations was unknown and hence treated as simulated variable along the MCMC simulations allowed to vary between 1 and 10. The run displayed a clear mode at K = 2 which was thus the maximum a posteriori estimate of K, confirming the number of sub-populations (K) estimated by the empirical statistic K [50]. Therefore, we did a second run with the maximum number of populations set to 2. A map of posterior probabilities (membership) was obtained by inputting PostProcessChain and PostTessellation functions into Geneland by tessellating the landscape at a resolution of 1 m. The posterior probabilities of population memberships were plotted on Google maps as reported in the Geneland package version 4.0.5. Null alleles were also calculated with Geneland 4.0.5 [55], but are not reported here.

3. Results and Discussion

3.1. SSR Marker Polymorphism and Genetic Metrics

Several SSR markers of walnut reported in the literature were unable to provide reliable outputs, despite their repeated citation. Lack of polymorphism, multilocus amplification, a large frequency of null alleles, strong peak stuttering, and inconstant amplifications were the main drawbacks. After a preliminary amplification test, twenty markers that provided good amplifications and clear fragment separation were selected and used to genotype the whole set of samples. The allelic profiles of the collected accessions are reported in the Supplementary Material (Table S1). The number of alleles per locus ranged from two to nine (mean 4.0). The observed heterozygosity (Ho) ranged from 0.051 to 0.712 (mean 0.462), and the genetic diversity ranged from 0.059 to 0.745 (mean 0.516), which in most cases closely followed the observed heterozygosity. The polymorphism information content (PIC) ranged from 0.058 to 0.698 (mean 0.438), which also followed the observed and expected heterozygosity (Table 2). Contig_721 and Contig_1692 were the two least informative markers due to the very high frequency of the major allele (0.97 and 0.96, respectively). Generally speaking, SSR markers derived from genomic libraries were more informative and discriminated better among accessions compared to those derived from EST (Expressed Sequence Tag) libraries, with the mean number of alleles being 4.7 vs. 2.7, the observed heterozygosity 0.558 vs. 0.367, the gene diversity 0.589 vs. 0.381, and the PIC 0.505 vs. 0.313, respectively (Table 2). In spite of this low polymorphism of EST-derived markers, the cumulative probability of identity (PID), which is the probability that two individuals would share by chance the same profile over all loci, was appreciably low, ranging from 2.93×10^{-11} for unrelated individuals to 1.075×10^{-5} for full sibs (Table 2). The estimated frequency of null alleles was below 12%, except in the case of WGA349 where the frequency was rather higher (a. 23%). The estimates provided by Cervus and Geneland were similar in magnitude but different in values due to the different algorithms adopted by the two packages. It is noteworthy that the null allele frequency is estimated indirectly because the value simply indicates an excess of homozygotes, which are not necessarily linked to the presence of null alleles. For this we took the estimate of null alleles as a warning about the use of a given marker in genotyping individuals. None of the 215 accessions showed identical profiles to any other accession of the dataset (data not shown).

Table 2. Genetic metrics of the 20 SSR markers tested on the whole set of walnut accessions: number of alleles, allele size ranges, number of genotypes, observed heterozygosity (Ho), gene diversity (GD), polymorphism information content (PIC), major allele frequency without null alleles (MAF), Hardy-Weinberg equilibrium (HW), estimated frequency of null alleles (Fnull), and probability of identity (PID) for unrelated individuals and full-sibs.

SSR Marker	Samples n	Alleles n	Size Range bp [(b)]	Genotypes n	Ho	GD	PIC	MAF	HW	F_{null}	P_{ID} Unrelated	P_{ID} Full-Sibs
Genomic SSR												
WGA004	215	2	228–238	3	0.619	0.482	0.365	0.60	**	−0.125	0.385	0.606
WGA009	215	3	229–245	6	0.619	0.639	0.561	0.40	NS	+0.016	0.208	0.483
WGA027	212	2	206–210	3	0.533	0.488	0.368	*0.58*	NS	−0.046	0.382	0.602
WGA069	209	9	160–182	18	0.656	0.745	0.698	*0.32*	NS	+0.063	0.111	0.406
WGA071	209	3	205–209	4	0.498	0.503	0.381	*0.54*	NS	+0.002	0.369	0.591
WGA072	214	5	128–142	6	0.411	0.467	0.368	*0.64*	NS	+0.058	0.383	0.613
WGA089	215	4	212–220	7	0.712	0.673	0.599	0.33	NS	−0.029	0.180	0.459
WGA118	215	7	185–207	11	0.581	0.581	0.490	0.48	NS	−0.004	0.267	0.527
WGA225	194	3	190–202	6	0.567	0.644	0.566	*0.40*	NS	+0.063	0.204	0.480
WGA321	215	5	223–245	12	0.642	0.674	0.608	0.41	NS	+0.025	0.172	0.457
WGA331	214	4	273–279	6	0.481	0.484	0.373	0.60	NS	+0.003	0.377	0.603
WGA349	205	6	262–276	11	0.405	0.646	0.591	*0.51*	***	+0.226	0.180	0.473
WGA376	204	8	231–256	17	0.525	0.634	0.594	*0.56*	*	+0.094	0.174	0.477
Mean		*4.7*		*8.5*	*0.558*	*0.589*	*0.505*	*0.49*				
EST-derived SSR												
Contig_40	215	3	210–223	4	0.349	0.340	0.286	0.79	NS	−0.005	0.490	0.703
Contig_156	215	2	300–313	3	0.433	0.493	0.371	0.56	NS	+0.064	0.379	0.599
Contig_642	215	2	262–281	3	0.488	0.494	0.371	0.56	NS	+0.004	0.379	0.598
Contig_721	215	3	369–390	4	0.051	0.059	0.058	0.97	ND	+0.068	0.887	0.942
Contig_1528	215	3	154–163	6	0.553	0.562	0.463	0.50	NS	+0.008	0.290	0.542
Contig_1681	215	4	197–210	9	0.619	0.647	0.576	0.46	NS	+0.023	0.196	0.476
Contig_1692	215	2	208–211	2	0.074	0.072	0.069	0.96	ND	−0.010	0.864	0.930
Mean		*2.7*		*4.4*	*0.367*	*0.381*	*0.313*	*0.69*				
General Mean [(a)]		*4.0*		*6.4*	*0.462*	*0.516*	*0.438*	*0.59*			*2.93E-11*	*1.07E-5*

[(a)] In the case of probability of Identity (PID) the reported value is the combined non-exclusion probability; [(b)] The fragment sizes do not include the 19 pigtail bases.

3.2. Hierarchical and Model-Based Clustering of Walnut Accessions

The clusters that could be identified in the tree topology, according to the level of similarity selected (Figure 2), did not group individuals according to their geographical origin. Frequently, accessions sampled from adjacent locations were clustered far away from each other. Although such evidence has already been reported for Italian walnut germplasm [24], it is not easy to explain such an unexpected outcome. From the evidence of the current study it would seem likely that the accession clustering is the result of the traditional ways that walnuts have been distributed across the region. In previous centuries, and especially in the 19th and 20th centuries, fruits collected in the alpine valleys were traditionally sold in markets across the entire region during local festivals. We have found traces of this traditional nut market in local books and leaflets (cited in [57]). The analysis of molecular variance (AMOVA) confirmed that the differences between the populations accounted for only 2.12% of the entire variance, while the variability within populations accounted for 97.87% (Table 3). The Mantel test did not reveal any significant correlation between geographic and genetic distances among accessions (data not shown). The pairwise comparisons of populations for FST did not show any apparent subdivision of the whole population in the study (Table 4), thus confirming the large genetic admixture observed with other analyses. A further analysis carried out by splitting the whole population into the five geographic sub-populations confirmed that the whole population should be correctly treated as a non-subdivided population. The only new information obtained from such an analysis was the existence of private alleles, that is alleles carried by a single individual accession (Table 5). The existence of accessions with private alleles could have the significance of an incipient genetic isolation of those accessions from the original population or sub-population, but when private alleles of a single accessions are one or very few, as this is the case, no prediction can be made on the evolution of population fragmentation. The situation could not be permanent and gene flow could change by the modification of the genetic and geographical barriers. The genetic structure was investigated by two further approaches: the first one based on the Bayesian cluster analysis implemented in STRUCTURE [47] and a second one represented by the spatial clustering model as implemented in Geneland. The advantage of using spatial vs. non-spatial clustering models lies in the ability to obtain more accurate results when the number of loci is relatively small and data are characterized by low levels of genetic differentiation [52,53]. In the first case, the analysis strongly supported two clusters with K = 2 corresponding to the maximum ΔK (delta K) (Figure 3A,B). We did not find any apparent common feature, such as the geographic origin, among the samples belonging to the same cluster (Figure 4). The results of Geneland analyses were consistent with STRUCTURE by clearly showing that the same two distinct clusters can be identified in the study area (Figures 4 and 5A). In addition, Geneland indicated that the genetic differentiation between the two subpopulations (clusters 1 and 2) is small (FST = 0.033), thereby showing a similar genetic structure. In particular, cluster 1 showed a higher FIS value (0.21) with respect to cluster 2 (0.05), indicating a higher deficit of heterozygotes in the cluster 1. It is noteworthy that cluster 1 is composed of the individuals W156, W157, and W216 (Figure 5B,C). The kinship coefficient (Fij) between W156 and W157 was 0.25, indicating a full sib origin. The W216 individual has a Fij equal to 0.02 with individual W157, and a negative value with all the other individuals analyzed. Therefore, even if the relationship between the W216 and W157 individuals is very low, this is sufficient to explain the presence of W216 in cluster 1.

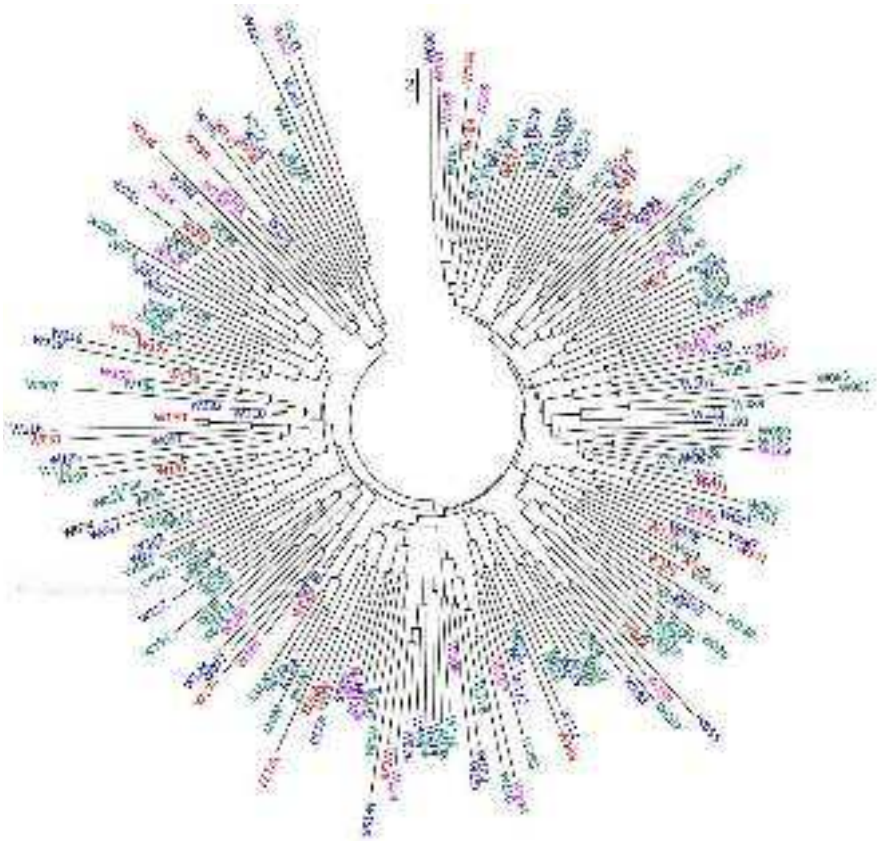

Figure 2. UPGMA (Unweighted Pair Group Method with Arithmetic Mean)-based circular dendrogram showing genetic similarities between the 215 walnut (*Juglans regia* L.) accessions analyzed as listed in the Supplementary Material, Table S1. Accessions from the same geographic area have the same color (see the legend in Figure 1B for association of the colors with the geographical areas).

Table 3. Analysis of Molecular Variance (AMOVA) of five walnut populations analyzed with 20 SSRs.

Source of Variation	d.f.	Variance Components	% Variation
Among populations	4	28.18	2.12
Among individuals	210	1296.91	97.87
Total	214	1325.09	

Table 4. Pairwise Genetic distances (FST) between sampling locations (the number of samples is provided in brackets), d.f. = degrees of freedom

Population	WAH	EAH	TNV	FP	TK
Western Alpine Highlands (WAH) (95)					
Eastern Alpine Highlands (EAH) (32)	0.005				
Torre-Natisone Valley (TNV)(25)	0.008	0.012			
Friuli Plains (FP) (59)	0.005	0.005	0.010		
Trieste Karst (TK) (4)	0.046	0.042	0.052	0.048	

Table 5. Genetic diversity within the five populations of Persian walnut (between brackets the no. of samples) collected in the Friuli Venezia Giulia Region assessed with 20 SSR loci. A = no. of alleles, Ne = effective no. of alleles, Rs = allelic richness, Ho = observed heterozygosity, GD = gene diversity, Pa = private alleles (the private allele length is listed in brackets).

Population	A	Ne	Rs	Ho	GD	Pa
Western Alpine Highlands (95)	3.050	2.257	2.25	0.483	0.506	WGA118 (220), WGA331 (298)
Eastern Alpine Highlands (32)	3.200	2.279	2.29	0.489	0.511	WGA069 (178, 198), WGA118 (212), WGA331 (294), WGA376 (268)
Torre-Natisone Valley (25)	2.950	2.301	2.29	0.519	0.514	WGA072 (156), WGA118 (222)
Friuli Plains (59)	3.400	2.256	2.27	0.488	0.515	WGA069 (186, 188), WGA071 (224), WGA072 (148), WGA349 (284, 291)
Trieste Karst (4)	2.350	1.978	2.23	0.554	0.431	WGA376 (257)

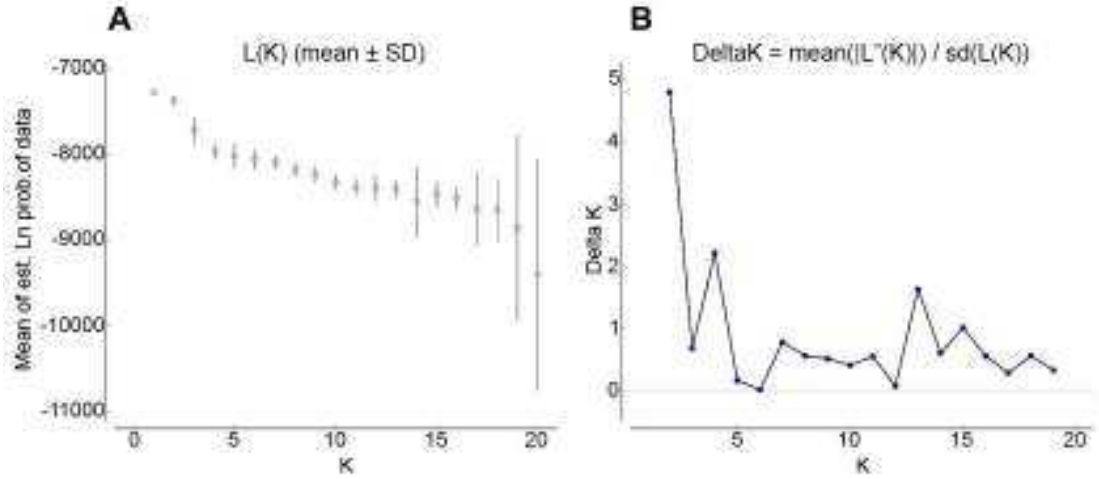

Figure 3. Inference of K, the most probable number of clusters, using STRUCTURE software, based on microsatellite analysis of 215 walnut samples. (**A**) Plot of mean likelihood L(K) and variance per K value from STRUCTURE on a dataset containing 215 individuals genotyped for 20 polymorphic microsatellite loci over 20 runs for each K value; (**B**) Plot of the ad hoc statistic ΔK, which is based on the rate of change of the log-likelihood as K is increased) [50] ΔK tends to peak at the value of K that corresponds to the highest level of hierarchical substructure. The modal value of this distribution is the true K, here two clusters.

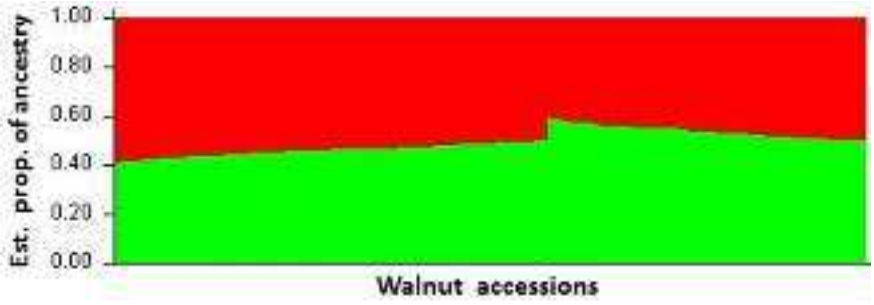

Figure 4. Population structure inference for 215 walnut (*Juglans regia* L.) accessions as assigned using STRUCTURE [47] and the admixture option for K = 2 (see text for details). Vertical bars represent individual accessions. The length of segment color in each vertical bar represents the proportion contributed by each of the two populations in the model (represented by different colors) to that individual.

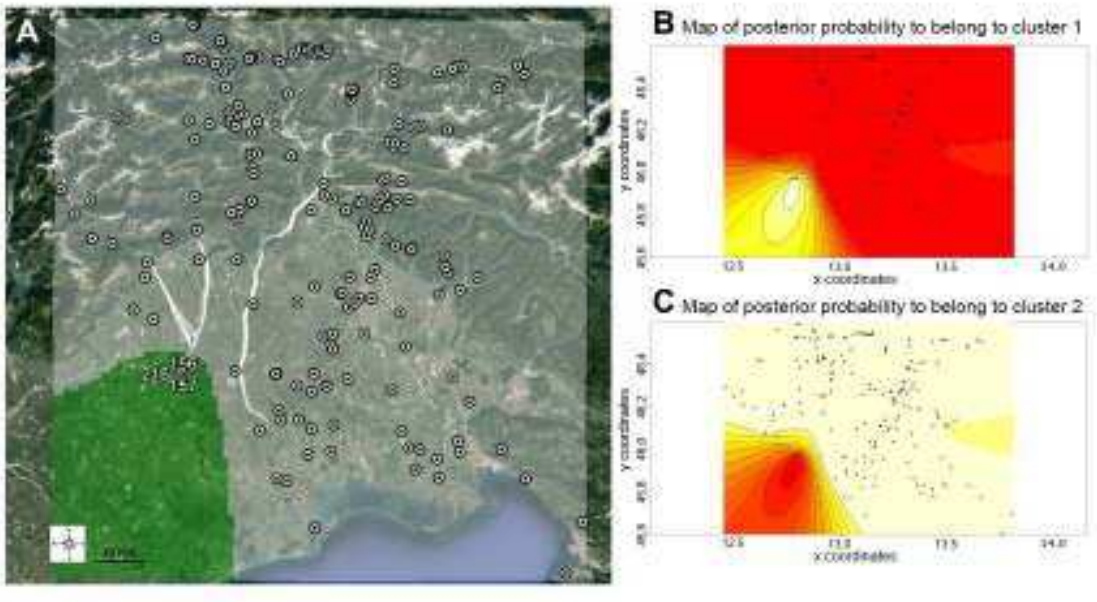

Figure 5. Results of Geneland analysis of 215 walnut (*Juglans regia* L.) accessions for K = 2. (**A**) Map of estimated population membership plotted on Google maps, with cluster 1 in green and cluster 2 in white. The accession IDs of the three individuals in cluster 1 are recorded (see text for details); (**B**) and (**C**) are maps of posterior probabilities of cluster 1 and cluster 2, respectively. Contour lines represent posterior probabilities of belonging to cluster 1 (**B**) and 2 (**C**).

4. Conclusions

Considering the large number of accessions collected in the sampling area, we are confident that we have sampled a good representation of the genetic diversity of walnuts in the Friuli Venezia Giulia region. If we exclude the EST-derived markers, which showed very low polymorphism, the genetic metrics observed in the 13 genomic markers of the study were similar to those reported by Pollegioni et al. [24]. These authors reported that the common markers analyzed in 456 walnut trees sampled in Italy were substantially comparable with earlier reports that considered large regional or national sampling [18,22]. As stated by other authors, the genetic diversity of *J. regia* investigated primarily on a national or regional scale across Eurasia has revealed low levels of genetic differentiation among populations and no robust geographic patterns of genetic diversity [10,12,24], and this has been reinforced by the current work, even though it was carried out on a limited geographical scale. The reasons for such moderate genetic diversity are not easily determined. One hypothesis is that the reservoirs of walnut genetic diversity in Europe were largely eroded during the last glacial maximum [14]; the second hypothesis, which is not in contrast with the first one, rules out that the re-introduction of walnut from the Western Asia by humans suffered a bottleneck [12], and has not been mitigated by the few generations that have passed in the intervening 2000 years. Historians report a well-developed walnut industry in the sub-alpine arch surrounding the Friuli plain. The people of Carnia and the Torre and Natisone valleys attended local festivals bringing and selling their nuts collected from the wild or grown in their courtyards from heirloom seed [57]. Walnut seeds were for human consumption, but they were occasionally planted at the birth of children in looking ahead to their eventual marriages, at which time the trees were cut to craft furniture for the new family. This dispersal of seeds could explain the inability of the dendrogram of genetic distances among accessions to group samples according to neighborhood, but instead it distributed the local samples into numerous clusters that did not represent any of the geographical areas identified at the time of planning the study. The walnut admixture we report as a result of human traditions and heirloom

management has a strong homology with the kin groups or 'households' reported for the walnut population structure of germplasm studied in Tibetan villages by Gunn et al. [10].

Supplementary Materials: The following are available online at www.mdpi.com/1999-4907/8/1/17/s1, Table S1: Database of the accessions and their molecular profiles at 20 SSR markers analyzed in this study. The 19 bp of the primer pigtail have been removed to make the SSR fragment sizes comparable with those in the literature. Missing data are reported as 0 (zero).

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Author Contributions: M.V., L.P., and R.T. conceived and designed the experiments; M.V., C.C., S.R., L.P., and R.M. performed the DNA genotyping and the first control on data; M.V., L.P., P.E., G.C., D.P., C.V., and R.T. analyzed the data; M.V., L.P., G.C., C.V., and R.T. wrote the paper. All authors contributed to the revision of the manuscript and approved the version submitted.

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References

1. Manning, W.E. The classification within the Juglandaceae. *Ann. MO. Bot. Gard.* **1978**, *65*, 1058–1087. [CrossRef]
2. Manchester, S.R. The fossil history of the Juglandaceae. In *Monographs in Systematic Botany*; Missouri Bot Garden: St. Louis, MO, USA, 1987; Volume 21, pp. 1–137.
3. Willis, K.J.; McElwain, J.C. *The Evolution of Plants*; Oxford University Press: New York, NY, USA, 2002; p. 378.
4. McGranahan, G.H.; Leslie, C. Walnuts (*Juglans*). In *Genetic Resources of Temperate Fruit and Nut Crops*; Moore, J.N., Ballington, J.R., Eds.; International Society for Horticultural Science: Wageningen, The Netherlands, 1991; pp. 907–951.
5. Vavilov, N.I. The wild relatives of fruit trees of the Asian part of the USSR and Caucasus and problems of origin of fruit trees. *Tr. Po Prikl. Bot. Genet. Sel.* **1931**, *23*, 343–360. (In Russian)
6. Molnar, T.J.; Zaurov, D.E.; Capik, J.M.; Eisenman, S.W.; Ford, T.; Nikolyi, L.V.; Funk, C.R. Persian walnuts (*Juglans regia* L.) in Central Asia. *Annu. Rep. North Nut. Grow Assoc.* **2011**, *101*, 56–69.
7. Carrión, J.S.; Sanchez-Gomez, P. Palynological data in support of the survival of walnut (*Juglans regia* L.) in the western Mediterranean area during last glacial times. *J. Biogeogr.* **1992**, *19*, 623–630. [CrossRef]
8. Figueiral, I.; Terral, J. Late Quaternary refugia of Mediterranean taxa in the Portuguese Estremadura: Charcoal based palaeovegetation and climatic reconstruction. *Quat. Sci. Rev.* **2002**, *21*, 549–558. [CrossRef]
9. Beer, R.; Kaiser, F.; Schmidt, K.; Ammann, B.; Carraro, G.; Grisa, E.; Tinner, W. Vegetation history of the walnut forests in Kyrgyzstan (Central Asia): Natural or anthropogenic origin? *Quat. Sci. Rev.* **2008**, *27*, 621–632. [CrossRef]
10. Gunn, B.F.; Aradhya, M.; Salick, J.M.; Miller, A.J.; Yongping, Y.; Lin, L.; Xian, H. Genetic variation in walnuts (Juglans regia and J sigillata; Juglandaceae): Species distinctions, human impacts, and the conservation of agrobiodiversity in Yunnan, China. *Am. J. Bot.* **2010**. [CrossRef] [PubMed]
11. Epple, C. A vegetation study in the walnut and fruit-tree forests of Southern Kyrgyzstan. *Phytocoenologia* **2001**, *31*, 571–604. [CrossRef]
12. Pollegioni, P.; Woeste, K.E.; Chiocchini, F.; Olimpieri, I.; Tortolano, V.; Clark, J.; Hemery, G.E.; Mapelli, S.; Maria, E.M. Landscape genetics of Persian walnut (*Juglans regia* L.) across its Asian range. *Tree Genet. Genomes* **2014**. [CrossRef]
13. Pollegioni, P.; Woeste, K.E.; Chiocchini, F.; Del Lungo, S.; Olimpieri, I.; Tortolano, V.; Clark, J.; Hemery, G.E.; Mapelli, S.; Malvolti, M.E. Ancient Humans Influenced the Current Spatial Genetic Structure of Common Walnut Populations in Asia. *PLoS ONE* **2015**. [CrossRef] [PubMed]
14. Bottema, S. The Holocene history of walnut, sweet-chestnut, manna-ash and plane tree in the Eastern Mediterranean. *Pallas* **2000**, *52*, 35–59.
15. Poldini, L. *Atlante Corologico Delle Piante Vascolari Nel Friuli-Venezia Giulia: Inventario Floristico Regionale*; Arti Grafiche Friulane: Udine, Italy, 1991; p. 899.

16. Rottoli, M.; Castiglioni, E. Prehistory of plant growing and collecting in northern Italy, based on seed remains from the early Neolithic to the Chalcolithic (c. 5600–2100 cal b.c.). *Veg. Hist. Archaeol.* **2009**, *18*, 91–103. [CrossRef]
17. Woeste, K.; Burns, R.; Rhodes, O.; Michler, C. Thirty polymorphic nuclear microsatellite loci from black walnut. *J. Hered.* **2002**, *93*, 58–60. [CrossRef] [PubMed]
18. Dangl, G.S.; Woeste, K.; Ardhya, M.K.; Koehmstedt, A.; Simon, C. Characterization of 14 microsatellite markers for genetic analysis and cultivar identification of walnut. *J. Am. Soc. Hortic. Sci.* **2005**, *130*, 348–354.
19. Foroni, I.; Rao, R.; Woeste, K.; Gallitelli, M. Characterization of Juglans regia L. through SSR markers and evaluation of genetic relationships among cultivars and the 'Sorrento' landrace. *J. Hortic. Sci. Biotechnol.* **2005**, *80*, 49–53. [CrossRef]
20. Wang, H.; Pei, D. Genetic diversity and structure of walnut populations in Central and Southwestern China revealed by microsatellite markers. *J. Am. Soc. Hortic. Sci.* **2008**, *133*, 197–203.
21. Pollegioni, P.; Woeste, K.; Mugnozza, G.S.; Malvolti, M.E. Retrospective identification of hybridogenic walnut plants by SSR fingerprinting and parentage analysis. *Mol. Breed.* **2009**, *24*, 321–335. [CrossRef]
22. Zhang, R.; Zhu, A.D.; Wang, X.L.; Yu, J.; Zhang, H.R.; Gao, J.S.; Cheng, Y.J.; Deng, X.X. Development of *Juglans regia* SSR Markers by Data Mining of the EST Database. *Plant Mol. Biol. Rep.* **2010**, *28*, 646–653. [CrossRef]
23. Ebrahimi, A.; Fatahi, R.; Zamani, Z. Analysis of genetic diversity among some Persian walnut genotypes (*Juglans regia* L.) using morphological traits and SSRs markers. *Sci. Hortic.* **2011**, *130*, 146–151. [CrossRef]
24. Pollegioni, P.; Woeste, K.; Olimpieri, I.; Marandola, D.; Cannata, F.; Malvolti, M.E. Long-term human impacts on genetic structure of Italian walnut inferred by SSR markers. *Tree Genet. Genomes* **2011**, *7*, 707–723. [CrossRef]
25. Ruiz-Garcia, L.; Lopez-Ortega, G.; Fuentes Denia, A.; Frutos Tomas, D. Identification of a walnut (*Juglans regia* L.) germplasm collection and evaluation of their genetic variability by microsatellite markers. *Span. J. Agric. Res.* **2011**, *9*, 179–192. [CrossRef]
26. Zhang, Z.Y.; Han, J.W.; Jin, Q.; Wang, Y.; Pang, X.M.; Li, Y.Y. Development and characterization of new microsatellites for walnut (*Juglans regia*). *Genet. Mol. Res.* **2013**, *12*, 4723–4734. [CrossRef] [PubMed]
27. Manly, B.F.J. *The Statistics of Natural Selection on Animal Populations*; Chapman and Hall: London, UK, 1985; pp. 272–282.
28. Yeh, F.C.; Boyle, T.E. Population genetic analysis of co-dominant and dominant markers and quantitative traits. *Belg. J. Bot.* **1997**, *129*, 129–157.
29. Schuelke, M. An economic method for the fluorescent labeling of PCR fragments. *Nat. Biotechnol.* **2000**, *18*, 233–234. [CrossRef] [PubMed]
30. Boutin-Ganache, I.; Raposo, M.; Raymond, M.; Deschepper, C.F. M13-tailed primers improve the readability and usability of microsatellite analyses performed with two different allele-sizing methods. *Biotechniques* **2001**, *31*, 24–28. [PubMed]
31. Liu, K.; Muse, S.V. PowerMarker: An integrated analysis environment for genetic marker analysis. *Bioinformatics* **2005**, *21*, 2128–2129. [CrossRef] [PubMed]
32. Waits, L.P.; Luikart, G.; Taberlet, P. Estimating the probability of identity among genotypes in natural populations: Cautions and guidelines. *Mol. Ecol.* **2001**, *10*, 249–256. [CrossRef] [PubMed]
33. Marshall, T.C.; Slate, J.; Kruuk, L.E.B.; Pemberton, J.M. Statistical confidence for likelihood-based paternity inference in natural populations. *Mol. Ecol.* **1998**, *7*, 639–655. [CrossRef] [PubMed]
34. Kalinowski, S.T.; Taper, M.L.; Marshall, T.C. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Mol. Ecol.* **2007**, *16*, 1099–1106. [CrossRef] [PubMed]
35. Kalinowski, S.T.; Taper, M.L. Maximum likelihood estimation of the frequency of null alleles at microsatellite loci. *Conserv. Genet.* **2006**. [CrossRef]
36. Loiselle, B.A.; Sork, V.L.; Nason, J.; Graham, C. Spatial genetic structure of a tropical understory shrub, *Psychotria officinalis* (Rubiaceae). *Am. J. Bot.* **1995**, *82*, 1420–1425. [CrossRef]
37. Hardy, O.J.; Vekemans, X. SPAGeDi: A versatile computer program to analyse spatial genetic structure at the individual or population levels. *Mol. Ecol. Notes* **2002**, *2*, 618–620. [CrossRef]
38. Sokal, R.R.; Rohlf, F.J. *Biometry: The Principles and Practice of Statistics in Biological Research*, 3rd ed.; W.H. Freeman, and Company: New York, NY, USA, 1995; 887p.

39. Peakall, R.; Smouse, P.E. GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Mol. Ecol. Notes* **2006**, *6*, 288–295. [CrossRef]
40. Kalinowski, S.T. Counting alleles with rarefaction: Private alleles and hierarchical sampling designs. *Conserv. Genet.* **2004**, *5*, 539–543. [CrossRef]
41. Kalinowski, S.T. HP-Rare: A computer program for performing rarefaction on measures of allelic diversity. *Mol. Ecol. Notes* **2005**, *5*, 187–189. [CrossRef]
42. Peakall, R.; Smouse, P.E. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research—An update. *Bioinformatics* **2012**, *28*, 2537–2539. [CrossRef] [PubMed]
43. Mantel, N.A. The detection of disease clustering and a generalized regression approach. *Cancer Res.* **1967**, *27*, 209–220. [PubMed]
44. Saitou, N.; Nei, M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **1987**, *4*, 406–425. [PubMed]
45. Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Mol. Biol. Evol.* **2013**, *30*, 2725–2729. [CrossRef] [PubMed]
46. Page, R.D. TreeView: An application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* **1996**, *12*, 357–358. [PubMed]
47. Pritchard, J.K.; Stephens, M.; Donnelly, P. Inference of population structure from multi-locus genotype data. *Genetics* **2000**, *155*, 945–959. [PubMed]
48. Falush, D.; Stephens, M.; Pritchard, J.K. Inference of population structure using multilocus genotype data: Linked loci and correlated allele frequencies. *Genetics* **2003**, *164*, 1567–1587. [PubMed]
49. Pritchard, J.K.; Wen, W.; Falush, D. *Documentation for Structure Software: Version 2.3*; Department of Human Genetics, University of Chicago: Chicago, IL, USA, 2010; p. 38.
50. Evanno, G.; Regnaut, S.; Goudet, J. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Mol. Ecol.* **2005**, *14*, 2611–2620. [CrossRef] [PubMed]
51. Earl, D.A.; vonHoldt, B.M. STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv. Genet. Res.* **2012**, *4*, 359–361. [CrossRef]
52. Guillot, G.; Leblois, R.; Coulon, A.; Frantz, A.C. Statistical methods in spatial genetics. *Mol. Ecol.* **2009**, *18*, 4734–4756. [CrossRef] [PubMed]
53. Guillot, G.; Estoup, A.; Mortier, F.; Cosson, J.F. A spatial statistical model for landscape genetics. *Genetics* **2005**, *170*, 1261–1280. [CrossRef] [PubMed]
54. Guillot, G.; Mortier, F.; Estoup, A. Geneland: A computer package for landscape genetics. *Mol. Ecol. Notes* **2005**, *5*, 712–715. [CrossRef]
55. Guillot, G.; Santos, F.; Estoup, A. Analysing georeferenced population genetics data with Geneland: A new algorithm to deal with null alleles and a friendly graphical user interface. *Bioinformatics* **2008**, *24*, 1406–1407. [CrossRef] [PubMed]
56. Guillot, G. Inference of structure in subdivided populations at low levels of genetic differentiation. The correlated allele frequencies model revisited. *Bioinformatics* **2008**, *24*, 2222–2228. [CrossRef] [PubMed]
57. Youssef, J.; Nassimbeni, P.L. La frutticoltura nel Friuli-Venezia Giulia. Origine, situazione e prospettive (Fruit-growing in Friuli-Venezia Giulia. Origin, situation and prospects) (IN ITALIAN). *Frutticoltura* **1977**, *39*, 19–45.

MORPHOLOGICAL AND CARPOLOGICAL VARIABILITY OF WALNUT GERMPLASM (*JUGLANS REGIA* L.) COLLECTED IN NORTH-EASTERN ITALY AND SELECTION OF SUPERIOR GENOTYPES

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Abstract

Nuts collected from wild accessions of Persian walnut (*Juglans regia* L.) in the Friuli Venezia Giulia region (North Eastern Italian Alps) were evaluated during 2013 to 2015. The analyses carried out were mainly on fruit traits, the only morphological traits that could be observed and statistically analysed, being less dependent on the area of sampling, considering geographic features such as altitude, slope, soil, and climate. Such fruit traits proved to be very variable. Nut weight ranged from 2.2 to 17.3 g, shell thickness from 0.35 to 2.30 mm, the color of kernel skin varied from light to amber. The fruit nut appearance, evaluated by a panel of consumers, varied from 2.50 to 6.83 in a scale from 0 (very bad) to 10 (very attractive). A multivariate analysis carried out considering traits

valuable for breeding and selection, such as nut weight, kernel weight : nut weight ratio, shell thickness, kernel color, and fruit appearance, produced a ranking list, that included, at best, accessions that could be either selected as such for vegetative propagation and distribution to growers or used in breeding programs. A disadvantage of these selections is their terminal bearing habit which is not appreciated by breeders, who prefer the most productive genotypes with lateral bearing habit.

Keywords Persian walnut, genetic resources, genetic diversity, pomological description, nut descriptors

1. Introduction

The Persian walnut (*Juglans regia* L.) is monoecious, bearing staminate and pistillate flowers separately on the same tree (McGranahan and Leslie 1990). It is self-compatible, but with pronounced protandry and in some cases protogyny (Mert 2010) and because the period of pollen shedding does not completely overlap female receptivity, the rate of cross pollination could be high in certain circumstances (Luza and Polito 1988).

Genetic heterozygosity is therefore maintained and considerable variability in phenological and carpological traits, such as fruit size and shape, shell and kernel color and thickness, oil content and kernel taste is often observed in natural walnut populations (McGranahan and Leslie 1990; Korac et al. 1997; Solar et al. 2002, Zeneli et al. 2004).

This variability has led to extensive investigation of germplasm native to the main centers of origin, such as Iran (Atefi 1997), Central Asia (Molnar et al. 2011), India and the Himalayan foothills (Sharma and Sharma 2001), China (Gunn et al. 2010) and the refugial areas into which the species retreated during the last glaciation, such as the Iberian peninsula (Carrión and Sanchez-Gomez 1992), Italy (Pollegioni et al. 2011), the Balkans

(Solar and Štampar 2006), Greece and the Aegean islands (Rouskas and Zakynthinos 2001), and the Anatolian plateau (Sen and Tekintas 1992; Yarilgac et al. 2001). In several countries such investigations have resulted in the selection of superior genotypes (Akça and Sen 2001; Aletà and Ninot 1997; Balci et al. 2001; Simsek et al. 2010), occasionally used in breeding programs (Germain 1997).

In the Friuli Venezia Giulia region (North-Eastern Alps), walnuts are reasonably common and can be readily found in scrub and forest edges, standing isolated in grassland, and along roadsides in the vicinity of villages. Walnut trees are also common in farm courtyards as they used to be planted at the birth of a child to provide wood for furniture at the eventual time of marriage (Zandigiacomo 1998). Moreover, nuts collected from the wild were sold during local festivals and in local markets thus supplementing family incomes and favoring genetic dispersal.

A survey carried out in Friuli Venezia Giulia during 2013-2015, when more than 200 walnut trees were sampled and georeferenced, revealed great molecular diversity among individuals with no genetic subpopulations as evidence of a large admixture of genotypes due to human activity (Vischi et al. 2017). In the present paper we report the plant and fruit characteristics of those walnut accessions and a preliminary tentative ranking of superior genotypes on the basis of a multivariate index taking into account the main characteristics valued by the market.

2. Materials and methods

2.1 Walnut tree sampling

The purpose of the investigation over three years (2013-2015) was to explore the fruit diversity of the common walnut in the Friuli Venezia Giulia region (North-Eastern

Italian Alps) and to identify genotypes with superior fruit characteristics. The sampling area is shown in Fig. 1.

Seedling trees, possibly over 30 years old, were sampled. In certain areas, like in the Friuli plains, old walnut trees were found in low number. In such areas younger trees were sampled to get adequate numbers of samples.

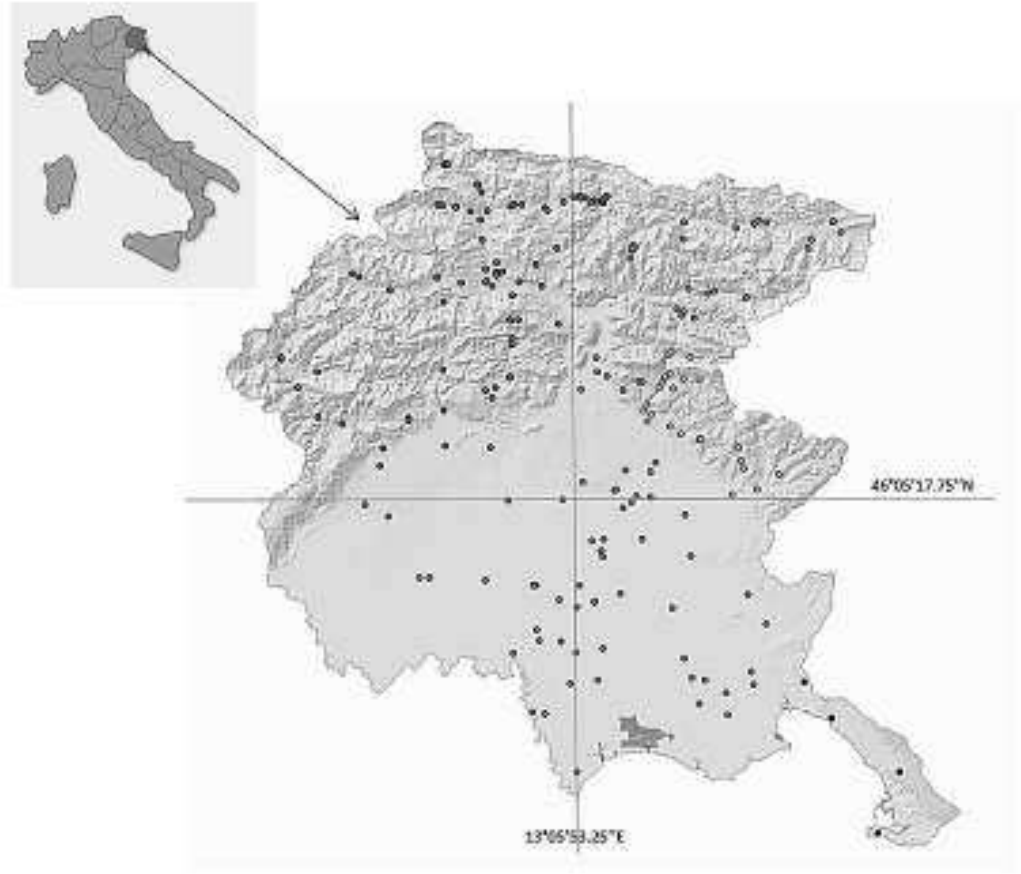

Fig. 1. Sample collection sites.

Individual trees were georeferenced with GPS coordinates recorded together with altitude and slope (estimated). A code was assigned starting with W (standing for walnut) followed by a progressive number of three digits. Further data were collected such as the age of the tree (if known by the owner, otherwise estimated), height, trunk circumference at 1.0 m above ground, bearing habit (lateral/apical bearing), vigor, productivity and health

(visual inspection). Twenty to forty nuts were randomly collected at maturity before nut fall, and brought to the laboratory for analysis.

2.2 Fruit measurements and other traits

Samples stored in a dry room were inspected regularly and when the husk was open, fruit were dehusked, the nut was washed in 1% sodium hypochlorite, dried in a heater at 30°C for 3 days and stored in a cold room.

The following measurements were taken from 20-nut samples: nut weight (g) and kernel weight (g) measured with a 0.01 g precision balance and shell thickness (mm) measured with a caliper in the middle of the shell as recommended in the UPOV Walnut Descriptor Bulletin (available at www.upov.int). The ratio kernel weight : nut weight was then calculated. The ease of kernel removal and anthracnose susceptibility were determined according to the UPOV walnut descriptors. The color of the kernel skin was evaluated following the color chart of the Dried Fruit Association of California (https://www.walnuts.org/).

The whole nut and the halved nut and kernel of each accession were photographed, separately and together. Data were analysed using GraphPad InStat 3 for MacIntosh (version 3.1.a).

3. Results and discussion

3.1 Geographical survey

Altogether, 220 walnut individual plants were sampled during the survey. The trees had not undergone pruning or other horticultural treatments (fertilization, irrigation) and

had not been previously inspected or sprayed for any pest or disease. Sampling was carried out in all five geographic areas into which the region was split, although several areas in the plains had few trees because of extensive felling by local wood factories after World War 2. In such areas, younger seedling trees were sampled to get more samples.

3.2 Characteristics of sampled trees

Most of the 220 trees studied were ≥ 30 years old. Only 26 were younger and at least 20 were 100 years or older. They grew between 1 and 1,073 meters above sea level, demonstrating the adaptability of walnuts to altitude (see McGranahan and Leslie 1991 for review).

Trunk circumference ranged from 735 to 3,990 mm. Tree height varied between 6 and 25 m. Trunk circumference and height are influenced not only by age but also by elevation, slope and soil characteristics so that the correlation between the tree age and trunk circumference, although clear, showed considerable scatter (Fig. 2).

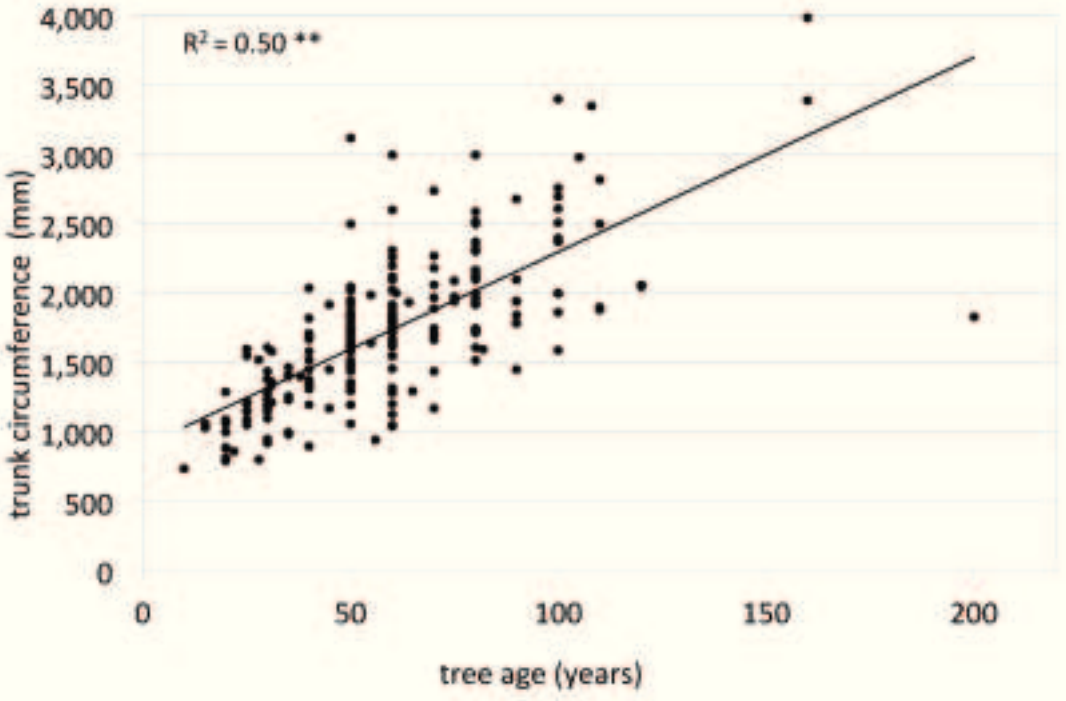

Fig. 2. Relationship between tree age and trunk circumference at 1.0 m above ground of walnut accessions collected in the Friuli Venezia Giulia region (North-Eastern Italian Alps).

Fruiting in lateral branches – popularly known as lateral bearing – is a trait sought by breeders because it results in higher productivity in comparison with only terminal fruit bearing (Solar and Štampar 2003). In our survey, all plants turned out to be terminal bearing. A small proportion (< c. 5 %) of lateral-bearing genotytpes has been observed in several countries, such as Iran (Atefi 1997), southern Romania (Botu et al. 2010) and the former Jugoslavia (Korac et al. 1997; Solar et al. 2002). Only in Greece have higher proportions of lateral-bearing genotypes been reported, but in that case plant material was a panel of selected genotypes (Rouskas and Zakynthinos 2001).

Tolerance to anthracnose was very variable among accessions spanning the complete range of 0 to 9 according to the UPOV descriptors (www.upov.int). Three quarters of all genotypes collected showed only very low anthracnose symptoms and one showed no symptoms at all, despite growing in a location with heavy rainfall (about 2,300 mm annually). Great variability in anthracnose susceptibility has also been reported in other countries (Atefi 1997; Balci et al. 2001; Aslantaş 2006; Arzani et al. 2008). There is no evidence that accessions do differ in anthracnose susceptibility but the very different environments in which accessions were growing (e.g., temperature, air humidity, rainfall) indicate the need for caution in drawing conclusions.

Phenological data, such as time of bud burst, flowering time, the overlap female and male flowering, and fruit maturity were not recorded because they would almost certainly be affected by the very different climatic conditions under which the trees sampled grew.

3.3 Nut and kernel properties

3.3.1 Nut weight, kernel weight and the ratio kernel weight : nut weight

Nut weight ranged widely from 2.2 (W024) to 17.3 g (W156) (Table 1). If a minimum weight of 12 g is accepted, as recommended for marketing by McGranahan and Leslie (1990) and Sharma and Sharma (1998), only 9 (4.9%) of the 184 trees that bore fruit exceeded this threshold.

Table 1 – Characteristics of nuts collected from walnut wild accessions of the Friuli Venezia Giulia region, in the Italian North-Eastern Alps.

Variable	Unit	Mean	Std Dev	CV %	Range
Nut weight	g	7.6	2.4	32.0	2.2 ÷ 17.3
Kernel weight	g	3.0	0.9	28.7	1.0 ÷ 6.2
Kernel : nut ratio	-	0.40	0.06	15.8	0.25 ÷ 0.58
Shell thickness	mm	1.3	0.3	25.0	0.4 ÷ 2.3
Easy of kernel removal	(a)	2.0	2.0	101.0	1.0 ÷ 9.0
Kernel skin color	(b)	1.9	0.9	48.0	1.0 ÷ 4.0
Anthracnose susceptibility	(c)	1.4	1.2	84.0	0.0 ÷ 9.0

(a) 1 = very easy, 3 = easy, 5 = intermediate, 7 = difficult, 9 = very difficult

(b) 1 = extra light, 2 = light, 3 = light amber, 4 = amber

(c) 0 = no sign of disease, 1 = very low, 3 = low, 5 = intermediate, 7 = high, 9 = very high

Thus, the wild native accessions sampled in the region of study had a mean weight and range similar to those found in germplasm from the former Yugoslavia (Korac et al. 1997) but a mean weight lower than observed, for instance, in Greece (Rouskas and

Zakynthinos 2001), Iran (Atefí 1997; Arzani et al. 2008; Ghasemi et al. 2012; Khadivi-Khub et al. 2015), the Himalayan foothills (Sharma and Sharma 1998), Romania (Cosmulescu and Botu 2012), Slovenia (Solar et al. 2002; Solar and Štampar 2006), and Turkey (Sen and Tekintas 1992; Akça and Sen 2001; Yarilgac et al. 2001; Balci et al. 2001; Simsek et al. 2010). The most likely explanation is that the surveys in Italy and the former Yugoslavia were initial surveys of genetic diversity, whereas in all other cases the plant material consisted of advanced selections.

Kernel weight is in some way related to the whole nut weight, but the relationship was not so strict and depended on the extent to which the kernel occupied the interior of the nut. We observed cases of large nuts with only small kernel inside giving a low kernel weight : nut weight ratio. The kernel weight : nut weight ratio in our samples varied between 0.25 and 0.58. If it is accepted that for high breeding value, the kernel to nut ratios should be greater than 0.48 - 0.50 (Germain 1997; Korac et al. 1997; Sharma and Sharma 1998; Arzani et al. 2008) only 21 of our samples (11.4%) reached that threshold.

Plotting together the nut weight and the kernel weight : nut weight ratio resulted in a graph where samples with large nut weight and high kernel weight : nut weight ratio, which would represent the best accessions from a commercial point of view and the candidate parents for breeding at least for these combined traits, are missing (Fig. 3).

3.3.2 Shell thickness

Shell thickness ranged between 0.4 mm (W137) and 2.3 mm (W213). Shell thickness affects ease of shell removal and from a commercial point of view, the easier the removal the better the quality of the nut (Sharma and Sharma 2001). A shell thickness of between 0.7 and 1.5 mm is considered desirable (Zhadan and Strukov 1977), although some selections are reported to have shells close to 0.7 mm or even less (Arzani et al.

2008; Aslantaş 2006; Yarilgac et al. 2001; Kazankaya et al. 2001). Among the samples considered in our study, 6 had a shell thickness less than 0.7 mm and 142 had shells within the recommended range. Considering ease of shell removal, 136 sampled fell in the class of "very easy removal", 22 in the class "easy", 12 "intermediate", 8 "difficult" and only 6 "very difficult".

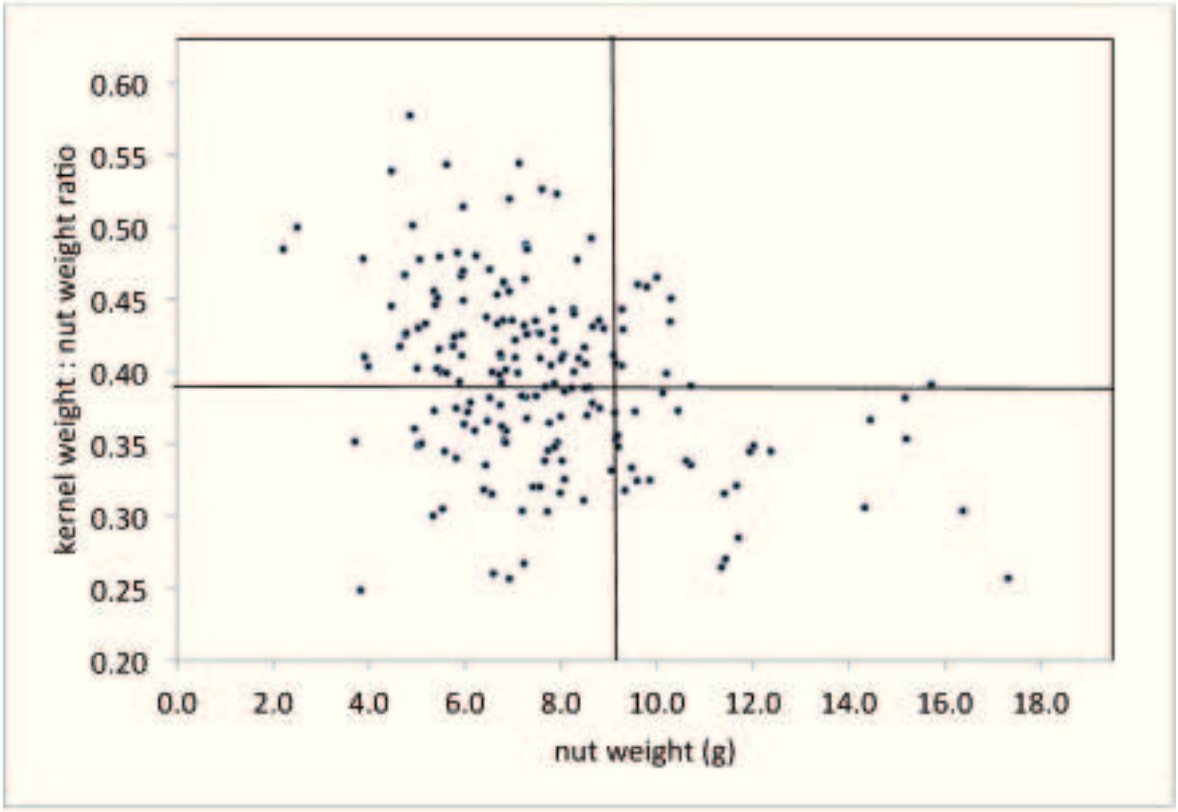

Fig. 3. Relationship between nut weight (g) and shell : nut ratio) in the walnut accessions collected in the Friuli Venezia Giulia region (North-Eastern Italian Alps)

3.3.3 Kernel skin color

Commercially acceptable walnuts have kernels which are 'extra light' or 'light' as defined by the color chart of the Dried Fruit Association of California (https://www.walnuts.org/). Seventy-three of our samples were classified as 'extra light', 66 as 'light', 27

as ‘light amber’ and only 18 as ‘amber’. Thus 139 of our samples (74%) would be considered commercially acceptable in terms of kernel skin color.

3.4 Correlations between variables

Correlations between variables are shown in Table 2. As expected, we found two strong correlations. The first, positive, was between nut weight and kernel weight (r = 0.85 **) and has also been observed by other authors (Arzani et al. 2008; Bayazit 2012; Gahsemi et al. 2012; Cosmulescu and Botu 2012; Mahmoodi et al. 2016). The correlation clearly indicates that a selection based on the external aspect of the nut is a rough approximation of kernel weight, although variation in the kernel weight : nut weight ratio could make selection less efficient.

The second strong correlation is negative: between shell thickness and kernel weight : nut weight ratio (r = - 0.62 **). A similar correlation has been previously reported (Sharma and Sharma 2001; Aslantaş 2006; Arzani et al. 2008; Bayazit 2012; Cosmulescu and Botu 2012; Khadivi-Khub et al. 2015). This means that shell thickness can explain part of the variance in nut weight and this must be taken into account if selection is on nut weight alone.

Table 2 – Correlation matrix among variables observed in walnut wild accessions of the Friuli Venezia Giulia region, in the Italian North-Eastern Alps.

Variable	1	2	3	4	5
1. Nut weight	-				
2. Kernel weight	0.85 **	-			
3. Kernel:nut ratio	- 0.36 **	0.15 *	-		
4. Shell thickness	0.44 **	0.16 *	- 0.62 **	-	
5. Kernel skin color	0.01 ns	0.10 ns	0.22*	0.17 *	-

* P ≤ 0.05, ** P ≤ 0.01, ns = not significant

Other correlations were in some way linked to those described above. Among the noteworthy are the negative correlation between kernel weight : nut weight ratio and nut weight (- 0.36**) and the positive correlation between shell thickness and nut weight (0.44**).

3.5 Selection indexes

The walnut ideotype, the ideal walnut to which a breeder should aim during selection should weigh between 12 and 18 g, have a clean, strong and thin shell with tight seal and an easily removed light kernel, clean and plump weighing at least 50% of the intact nut (Sharma and Sharma 1998; McGranahan and Leslie 1990). Other authors have concentrated their attention on fewer traits, namely shell thickness and ease of kernel removal since these are considered to be the key traits for preliminary selection of superior genotypes (Aslantaş 2006; Arzani et al. 2008; Cosmulescu and Botu 2012; Khadivi-Khub et al. 2015).

We applied a multivariate selection index for scoring and ranking our accessions. The procedure consisted of two steps: (a) accessions not meeting the threshold for any given parameter (Table 3) were excluded; (b) a selection index using the variables of Table 3 was applied. Each variable was standardized to the unit, so that each variable had the same relative weight, and multiplied by the weighting coefficient (Kx). The formula of the selection index (SI) was:

SI = K1*((WEIGHT-min1)/R1) + K2*((YIELD-min2)/R2) + K3*((SHELL-min3)/R3) + K4*(1-((COLOR-min4)/R4)) + K5*((VIEW-min5)/R5)

where

- K1, K2, K3, K4, K5 are the weighting coefficient assigned to the variables of Table 3
- min1, min2, min3, min4, min5, are the minimum values assumed by the variables of Table 3
- R1, R2, R3, R4, R5 are the ranges of variation of the variables of Table 3.

A similar two-step selection is reported in Simsek et al. (2010). We applied different K coefficients during a set of simulations by giving more weight to given variables at the expense of others. In Table 4 we report the results obtained using the coefficient K = 20 for all five variables of the equation.

Table 3 – Variables adopted for the multivariate selection index, minimum and maximum value in the population, range of variation, threshold for exclusion from the selection, and relative weighting coefficient in the selection index

Variabile	min	max	range (R)	threshold for exclusion	Weighting coefficient
K1 Nut weight (WEIGHT)	2.20	17.33	15.13	< 6.00	20
K2 Kernel : nut ratio (YIELD)	0.25	0.58	0.33	none	20
K3 Shell thickness (SHELL)	0.35	2.30	1.95	> 1.80	20
K4 Kernel skin color (COLOR)	1.00	4.00	3.00	> 3.50	20
K5 Fruit appearance (VIEW)	2.50	6.83	4.33	< 3.50	20

4. Conclusions

A survey carried out on wild walnut accessions of the Friuli Venezia Giulia region (North-Eastern Italian Alps) revealed large variability in fruit traits, the only morphological traits that could be reasonably observed, being less dependent on the area of sampling, considering geographic features such as altitude, slope, soil, and climate.

A multivariate analysis carried out considering traits valuable for breeding and selection, such as nut weight, kernel weight : nut weight ratio, shell thickness, kernel skin color, and nut appearance, produced a ranking list, that included, at best, accessions that could be either selected as such for vegetative propagation and distribution to growers or used in future breeding programs. A disadvantage of these selections is their terminal bearing habit as breeders prefer the more productive genotypes with lateral bearing habit.

Table 4 – Ranking list of the best 20 walnut selections following the procedure of multivariate selection described in the text. The origin of the accession can be checked with the code in the data base reported in the supplementary file S1.

Code	Nut weight g	Kernel weight : nut weight ratio	Shell thickness mm	Kernel skin color	Appearance (a)	Selection Index
W110	6.93	0.52	0.57	1.00	6.17	77.26
W048	7.13	0.54	0.70	1.00	5.67	75.38
W142	7.87	0.43	1.13	1.00	6.83	70.41
W223	10.71	0.39	1.27	1.00	6.67	69.56
W047	7.27	0.49	0.98	1.00	5.67	69.29
W181	7.30	0.48	0.78	1.10	5.17	68.22
W193	8.51	0.41	0.99	1.00	6.17	68.13
W041	8.05	0.41	1.06	1.00	6.33	67.95
W144	10.00	0.47	1.08	1.80	6.00	66.72
W184	9.29	0.43	1.17	1.00	5.67	66.50
W197	15.72	0.39	1.54	1.00	5.00	65.77
W145	8.63	0.49	1.08	1.60	5.50	65.56
W222	9.14	0.41	1.15	1.10	5.83	65.15
W096	7.86	0.42	1.34	1.10	6.33	64.74
W165	7.29	0.49	0.88	1.70	5.50	64.73
W216	9.79	0.46	1.11	2.00	6.00	64.38
W177	6.84	0.35	1.01	1.00	6.50	63.95
W152	8.66	0.43	1.57	1.00	6.17	63.94

W006	6.81	0.46	1.21	1.00	5.50	63.87
W186	8.01	0.41	1.06	1.00	5.50	63.85

(a) Evaluation of the appearance of the nut: range 1-10 with 1 = not acceptable, 10 = largely acceptable

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References

Aletà, N., Ninot, A., 1997. Field evaluation of *Juglans regia* selected clones from seedling populations of Mediterranean and Atlantic Spanish coast. Acta Hort. 442, 63-67.

Akça, Y., Sen, S.M., 2001. Study on the selection of superior walnut trees in Hizan (Bitlis) populations. Acta Hort. 544, 115-118.

Arzani, K., Mansouri-Ardakan, H., Vezvaei, A., Reza Roozban, M., 2008. Morphological variation among Persian walnut (*Juglans regia*) genotypes from central Iran. New Zealand J. Crop & Hort. Sci., 36 (3), 159-168. DOI: 10.1080/01140670809510232

Aslantaş R., 2006. Identification of superior walnut (*Juglans regia*) genotypes in north-eastern Anatolia, Turkey. New Zealand J. Crop & Hort. Sci., 34 (3), 231-237.

Atefi, J., 1997. Study on phenological and pomological characters on walnut promising clones in Iran. Acta Hort. 442, 101-108.

Balci, I., Balta, A., Kazankaya, A., Sen, S.M., 2001. Promising native walnut genotypes (*Juglans regia* L.) of the east Black Sea region of Turkey. J. Am. Pomol. Soc. 55(4), 204-208.

Bayazit, S., 2012. Determination of relationships among kernel percentage and yield characteristics in some Turkish walnut genotypes by correlation and path analysis. The Journal of Animal & Plant Sciences 22 (2), 513-517.

Botu, M., Tudor, M., Botu, I., Cosmulescu, S., Papachatzis, A., 2010. Evaluation of walnut cultivars in the conditions of the Oltenia's hill area regarding functioning potential. Analele Universitatii din Craiova, Biol Hort Tehnolo prelucrarii produselor agricole Ingineria mediului 15, 94-103.

Carrión, J.S., Sanchez-Gomez, P., 1992. Palynological data in support of the survival of walnut (*Juglans regia* L.) in the western Mediterranean area during last glacial times. J. Biogeography 19, 623-630.

Cosmulescu, S., Botu, M., 2012. Walnut biodiversity in south-western Romania. Resource for perspective cultivars. Pakistan J. Bot. 44(1), 307-311.

Ghasemi, M., Arzani, K., Hassani, D., 2012. Evaluation and identification of walnut (*Juglans regia* L.) genotypes in Markazi province of Iran. Crop Breeding J. 2(2), 119-124.

Germain, E., 1997. Genetic improvement of the Persian walnut (*Juglans regia* L). Acta Hort. 442, 21-31

Gunn, B.F., Aradhya, M., Salick, J.M., Miller, A.J., Yongping, Y., Lin, L., Xian, H., 2010. Genetic variation in walnuts (*Juglans regia* and *J. sigillata*; Juglandaceae): species distinctions, human impacts, and the conservation of agrobiodiversity in Yunnan, China. Am. J. Bot., doi: 10.3732/ ajb.0900114.

Kazankaya, A., Koyuncu, M.A., Koyuncu, F., Yarilgac, T., Sen, S.M., 2001. Some nut properties of walnuts (*Juglans regia* L.) of Edremit country. Acta Hort. 544, 97-100.

Khadivi-Khub, A., Ebrahimi, A., Sheibani, F., Esmaeili, A., 2015. Phenological and pomological characterization of Persian walnut to select promising trees. Euphytica 205, 557–567.

Korac, M., Cerovic, S., Golosin, B., Miletic, R., 1997. Collecting, evaluation and utilization of walnut (*Juglans regia* L.) in Yugoslavia. Plant Gen. Res. Newsletter 111, 72-74.

Luza, J.G., Polito, V.S., 1988. Microsporogenesis and anther differentiation in *Juglans regia* L.: a developmental basis for heterodichogamy in walnut. Bot. Gaz. 149 (1), 30-36.

Mahmoodi, R., Hassani, D., Amiri, M.E., Jaffaraghaei, M., 2016. Phenological and pomological characteristics of five promised walnut genotypes in Karaj, Iran. J. of Nuts 7(1), 1-8.

McGranahan, G.H., Leslie, C., 1990. Walnuts (*Juglans*). In: Moore, J.N., Ballington, J.R. (Eds.) Genetic Resources of Temperate Fruit and Nut Crops. (Acta Hort. 290) International Society for Horticultural Science. Wageningen, Netherlands, pp. 907-951.

Mert, C., 2010. Anther and pollen morphology and anatomy in walnut (*Juglans regia* L.). HortScience 45 (5), 757-760.

Molnar, T.J., Zaurov, D.E., Capik, J.M., Eisenman, S.W., Ford, T., Nikolyi, L.V., Funk, C.R., 2011. Persian Walnuts (*Juglans regia* L.) in Central Asia. NNGA 101st Ann. Rep. Sept, 56-69.

Pollegioni, P., Woeste, K., Olimpieri, I., Marandola, D., Cannata, F., Malvolti, M.E., 2011. Long-term human impacts on genetic structure of Italian walnut inferred by SSR markers. Tree Genet Genomes 7, 707-723.

Rouskas, D., Zakynthinos, G., 2001. Preliminary evaluation of seventy walnut (*Juglans regia* L.) seedlings selections in Greece. Acta Hort. 544, 61-72.

Sen, S.M., Tekintas, F.E., 1992. A study on the selection of Adilcevaz walnuts. Acta Hort. 317, 171-174.

Sharma, S.D., Sharma, O.C., 1998. Studies on the variability in nuts of seedling walnut (Juglans regia L.) in relation to the tree age. Fruit Var. J. 52, 20-23.

Sharma O.C., Sharma, S.D., 2001. Genetic divergence in seedling trees of Persian walnut (*Juglans regia* L.) for various metric nut and kernel characters in Himachal Pradesh. Sci. Hortic. 88, 163-171.

Simsek, M., Yilmaz, K.U., Demirkiran, A.R., 2010. Selection and determination of some significant properties of superior walnut genotypes. Scientific Research and Essays, 5(19), 2987-2996.

Solar, A., Ivancic, A., Stampar, F., Hudina, M., 2002. Genetic resources for walnut (*Juglans regia* L.) improvement in Slovenia. Evaluation of the largest collection of local genotypes. Genet. Res. Crop Evol. 49 (5), 491-501.

Solar, A., Štampar, F., 2003. Genotypic differences in branching pattern and fruiting habit in common walnut (*Juglans regia* L.). Ann. Bot. 92, 317-325.

Solar, A., Štampar, F., 2006. Evaluation of some perspective walnut genotypes in Slovenia. Acta Hort. 705, 131-136.

Vischi, M., Chiabà, C., Raranciuc, S., Poggetti, L., Messina, R., Ermacora, P., Cipriani, G., Paffetti, D., Vettori, C., Testolin, R., 2017. Genetic diversity of walnut (*Juglans regia* L.) in Italian Eastern Alpes. Forests 8, 81; doi:10.3390/f8030081

Yarilgac, T., Koyuncu, F., Koyuncu, M.A., Kazankaya, A., Sen, S.M., 2001. Some promising walnut selections (*Juglans regia* L.). Acta Hort. 544, 93-96.

Zhadan, V.M., Strukov, M.V., 1977. Breeding walnut for fruit size. Plant Breed. Abstracts 47(11), 10805.

Zandigiacomo, P., 1998. Melaries, peraries, brombaries e nujârs: pp 13-39. In: Adduca, G., Molfetta, P., Pellegrini, P., Sulli, F., Violino, C., Zandigiacomo, P. (Eds) Perarias

melarias ... Futticoltura in Carnia. Associazione culturale Cjarnie Culture, Cercivento, Udine: 164 pp

Zeneli, G., Kola, H., Dida, M., 2004. Phenotypic variation in native walnut populations of Northern Albania. Sci. Hortic. 105, 91-100. [doi:10.1016/ j.scienta.2004. 11.003]

Supplemental file

S1.xls: Database of the 220 accessions of walnut collected in the Friuli Venezia Giulia region (North-Eastern Italian Alps). The data base includes the accession code (W followed by a progressive number), the location, the google coordinates and information on morphological and carpological characteristics of sampled individuals. The first sheet includes a heading with definition of the traits observed and measurement units/scale.

	unit	description
code		W followed by a progressive number (e.g. W004)
province		UD Udine, GO Gorizia, PN Pordenone, TS Trieste
municipality		
GOOGLE latitude		
GOOGLE longitude		
altitude	m	altitude above sea level
ground slope	1-4	1 = flat to 4 = steep slope
age	years	
hight	m	
trunk circumference	mm	trunk circumference measured at 1.0 m height from the ground level
vigour	1-5	1 = weak , 5 = very vigorous
tolerance to antracnose (Marssonina juglandis)		0 = no sign, 1 = very low, 3 = low, 5 = intermediate, 7 = high, 9 = very high
nut weight	g	
kernel weight	g	
kernel/nut ratio		ratio kernel weight : nut weight
shell thickness	mm	
kernel skin colour		1 = extra light, 2 = light, 3 = light amber, 4 = amber. See color chart of the Dried Fruit Association of California

code	prov	municipality	GOOGLE latitude	GOOGLE longitude	ground slope	altitude m asl	age years	trunk circ mm	vigour	height m	tolerance to antracnose	nut weight g	kernel weight g	kernel weight : nut weight ratio	shell thickness mm	kernel skin colour
W001	UD	Paularo	46.53667355	13.10653562	1	845	60	1,280	2	12	1	14.3	4.4	0.31	1.3	1
W002	UD	Paularo	46.53517938	13.11305600	3	714	110	1,900	4	18	1	8.0	3.0	0.37	1.1	3
W003	UD	Paularo	46.53250967	13.12528923	1	682	50	1,620	3	16	1	7.9	2.8	0.35	1.1	4
W004	UD	Paularo	46.53413151	13.12485522	1	726	56	945	2	8	1	5.6	3.1	0.54	0.5	1
W005	UD	Paularo	46.53852471	13.12603422	1	830	60	2,025	4	15	1	7.5	3.3	0.44	1.0	1
W006	UD	Paularo	46.53876135	13.12619562	2	842	60	1,620	3	12	1	6.8	3.1	0.46	1.2	1
W007	UD	Paularo	46.53862900	13.12635668	3	842	75	1,975	3	12	1	6.8	2.4	0.35	1.6	1
W008	UD	Paularo	46.53818746	13.12467643	1	813	60	1,550	4	12	1	5.0	1.8	0.35	1.2	1
W009	UD	Paularo	46.53200556	13.09857373	1	836	50	1,340	4	12	1					
W010	UD	Ligosullo	46.54146063	13.07522321	2	1020	30	1,095	4	13	1					
W011	UD	Treppo Carnico	46.53204206	13.0394471	1	659	30	1,380	4	13	1					
W012	UD	Cercivento	46.52311998	12.99873660	1	556	60	1,835	3	15	1	6.2	3.0	0.48	1.2	1
W013	UD	Ravascletto	46.52703339	12.94837856	2	804	50	1,440	4	16	1					
W014	UD	Ravascletto	46.52490772	12.92583632	3	982	110	2,820	3	20	1	4.6	1.9	0.42	1.1	1
W015	UD	Ravascletto	46.52495177	12.92429607	3	953	60	1,040	2	12	1	5.4	2.0	0.37	1.3	2
W016	UD	Ravascletto	46.52674735	12.93150354	3	1044	80	1,930	3	15	1	6.9	2.5	0.36	1.7	1
W017	UD	Ravascletto	46.52651789	12.92976500	2	1040	60	1,670	3	16	1	7.4	2.4	0.32	1.3	1
W018	UD	Ravascletto	46.52637803	12.92949649	2	1035	100	2,760	3	20	1	6.7	2.7	0.40	1.2	1
W019	UD	Ravascletto	46.52738567	12.93146626	1	1052	90	1,940	4	18	1					
W020	UD	Cercivento	46.52714381	12.97883423	1	607	60	1,920	3	22	1					
W021	UD	Moggio Udinese	46.45141595	13.18746393	1	463	45	1,450	2	12	1	5.4	2.4	0.45	1.1	2
W022	UD	Moggio Udinese	46.46379355	13.19105144	2	524	50	1,300	3	14	1	9.1	3.4	0.37	1.3	1
W023	UD	Moggio Udinese	46.46456150	13.19180745	2	565	60	1,205	2	9	1	9.2	3.2	0.35	1.5	1
W024	UD	Moggio Udinese	46.46846318	13.19210804	2	628	50	1,300	3	18	1	2.2	1.1	0.48	0.4	2

W025	UD	Moggio Udinese	46.46928241	13.1926931	2	615	50	1,200	2	10	1				
W026	UD	Moggio Udinese	46.46895622	13.19597325	2	713	100	2,700	3	18	1	8.6	3.3	0.39	1.8	1
W027	UD	Moggio Udinese	46.46654213	13.19241913	2	597	60	2,310	2	15	1	6.8	3.1	0.46	1.0	2
W028	UD	Pontebba	46.48168556	13.30078220	3	523	100	3,400	3	18	1	6.2	2.2	0.36	1.5	2
W029	UD	Pontebba	46.50625507	13.30261037	1	568	105	2,980	4	12	1	5.6	1.9	0.35	1.2	2
W030	UD	Malborghetto	46.49976973	13.41510121	2	702	70	1,170	2	8	1	6.4	2.0	0.32	1.8	2
W031	UD	Valbruna	46.50620750	13.45410769	1	776	80	2,500	4	13	1	7.3	3.1	0.43	1.3	2
W032	UD	Valbruna	46.50960136	13.47969025	2	775	80	2,370	3	12	1	3.8	1.0	0.25	1.4	3
W033	UD	Camporosso	46.50993871	13.52748017	3	792	80	3,000	3	14	1					
W034	UD	Tarvisio	46.49568541	13.64182604	2	738	50	2,050	2	10	1	8.1	2.6	0.33	1.5	3
W035	UD	Ugovizza	46.51115797	13.46417394	2	776	40	1,310	3	10	1	6.1	2.3	0.37	0.9	2
W036	UD	Forni Avoltri	46.58500000	12.77916667	3	875	50	1,060	3	12	1	6.8	2.5	0.36	1.2	1
W037	UD	Forni Avoltri	46.58500000	12.77888889	1	865	40	900	2	9	1	8.0	2.5	0.32	1.6	1
W038	UD	Forni Avoltri	46.58583333	12.78444444	2	929	50	1,300	3	10	1					
W039	UD	Forni Avoltri	46.58500000	12.78500000	2	916	100	1,590	3	13	1	5.3	1.6	0.30	1.4	2
W040	UD	Rigolato	46.55611111	12.85055556	1	749	120	2,050	4	18	1	7.3	3.1	0.43	1.3	2
W041	UD	Rigolato	46.55472222	12.85194444	1	762	120	2,060	4	18	1	8.1	3.3	0.41	1.1	1
W042	UD	Rigolato	46.55555556	12.85166667	2	766	55	1,990	4	25	1	10.4	3.9	0.37	1.3	2
W043	UD	Rigolato	46.54472222	12.86083333	1	777	70	1,440	2	12	1	6.5	3.1	0.47	1.1	2
W044	UD	Comeglians	46.51638889	12.87416667	1	580	80	1,750	3	18	1	12.0	4.2	0.35	1.9	1
W045	UD	Comeglians	46.51666667	12.87416667	1	586	100	2,400	4	18	1	6.1	2.3	0.38	1.0	2
W046	UD	Prato Carnico	46.52333333	12.76694444	1	788	100	1,865	4	18	1	7.7	2.7	0.35	1.6	1
W047	UD	Prato Carnico	46.52305556	12.76805556	1	767	70	1,665	4	15	1	7.3	3.5	0.49	1.0	1
W048	UD	Prato Carnico	46.52138889	12.80583333	2	670	50	1,580	3	14	1	7.1	3.9	0.54	0.7	1
W049	UD	Prato Carnico	46.51472222	12.83722222	1	602	90	1,785	3	18	1	5.9	2.4	0.41	1.3	1
W050	UD	Ovaro	46.50305556	12.85944444	2	526	80	1,715	3	17	1	6.8	2.8	0.41	1.2	2

W051	UD	Ovaro	46.50305556	12.85972222	2	524	80	1,735	3	18	1	8.5	3.2	0.37	1.5	1
W052	UD	Ovaro	46.47350000	12.86444444	1	536	100	2,000	4	20	1	7.9	4.1	0.52	1.0	2
W053	UD	Villa Santina	46.41208333	12.94650000	1	341	60	1,635	3	15	1	7.3	2.8	0.38	1.4	2
W054	UD	Villa Santina	46.41219444	12.94650000	1	340	50	1,515	2	13	1	5.9	2.8	0.47	1.1	2
W055	UD	Reana	46.14700049	13.25236245	0	160	28	1,520	4	10	1	6.7	2.8	0.41	1.2	4
W056	UD	Udine	46.09508405	13.24280029	0	115	200	1,830	3.5	8	1	4.9	1.8	0.36	1.1	2
W057	UD	Basiliano	46.02751066	13.11854879	0	82	50	1,750	5	12	1	7.3	2.7	0.37	1.5	2
W058	UD	Tolmezzo	46.40573848	12.99668554	1	325	70	2,180	3	13	1	6.5	2.5	0.38	1.1	3
W059	UD	Enemonzo	46.40382056	12.89025682	1	377	70	2,740	1	14	1					
W060	UD	Enemonzo	46.41149599	12.87610324	2	402	50	1,950	3	13	1	3.9	1.6	0.41	1.5	4
W061	UD	Enemonzo	46.41030388	12.87443202	1	405	80	2,590	3	14	1	5.9	2.5	0.43	1.4	1
W062	UD	Medìis	46.40763605	12.82173771	1	528	50	1,840	2	12	3	6.7	3.0	0.45	1.4	1
W063	UD	Socchieve	46.42555556	12.89694444	1	390	90	2,680	2	18	1	5.5	2.2	0.40	1.1	4
W064	UD	Socchieve	46.42555556	12.90972222	1	381	75	2,090	2	14	3	3.7	1.3	0.35	1.3	4
W065	UD	Forni di Sotto	46.55916667	12.68361111	3	774	90	2,100	3	16	1					
W066	UD	Forni di Sopra	46.41678595	12.58639589	2	877	75	1,940	2	10	3	5.4	2.5	0.45	0.9	2
W067	UD	Forni di Sopra	46.41245000	12.60000000	2	900	108	3,350	3	25	1	11.4	3.6	0.32	1.3	1
W068	UD	Ampezzo	46.41415000	12.76850000	2	713	80	2,300	2	18	1	7.8	3.5	0.44	1.2	1
W069	UD	Ampezzo	46.41296667	12.80321667	1	616	50	3,120	4	20	1					
W070	UD	Socchieve	46.43861111	12.98305556	1	405	50	2,020	3	15	1	8.3	3.7	0.44	1.1	2
W071	UD	S. Maria la longa	45.92986316	13.29501526	0	38	35	1,256	4	9	1	14.5	5.3	0.37	1.6	3
W072	UD	Cervignano	45.82289120	13.36833006	0	5	50	1,620	4	11	1	9.3	4.1	0.44	1.2	2
W073	TS	Villa Opicina	45.69116735	13.78757047	0	312	60	1,750	4	9	1	6.8	2.7	0.39	1.2	1
W074	TS	Muggia	45.59889601	13.74322004	0	168	110	1,880	4	8	1	7.7	2.3	0.30	1.6	2
W075	TS	Sistiana	45.76984597	13.63999304	0	81	90	1,940	4	8	1	9.5	3.2	0.33	1.6	1
W076	GO	Doberdò del lago	45.82314246	13.57892328	0	43	50	1,450	4	10	1	7.2	3.4	0.46	1.2	1
W077	GO	Gradisca d'Isonzo	45.90863293	13.49689380	0	44	20	790	4	8	1	9.1	3.2	0.35	1.4	2
W078	GO	Cormons	45.95303704	13.45542099	0	41	30	1,230	5	7	1	7.7	2.6	0.34	1.4	3

W079	UD	Buttrio	46.00822564	13.33182340	0	77	60	2,120	4	9	1	8.9	3.8	0.43	1.0	2
W080	UD	Udine	46.03152660	13.22610477	0	93	30	1,152	4	8	1	8.0	2.7	0.34	1.5	2
W081	UD	Udine	46.09628859	13.21137610	0	127	80	2,000	5	16	1	11.7	3.3	0.28	2.0	2
W082	UD	Fagagna	46.11568665	13.09528196	1	212	30	1,440	3	14	1	7.0	3.0	0.42	1.2	3
W083	UD	Paluzza	46.52012263	13.00516943	0	560	60	2,098	4	18	1	7.8	2.8	0.36	2.2	2
W084	UD	Zuglio	46.46275876	13.02646370	0	410	60	2,260	4	18	1					
W085	UD	Trava	46.44001667	12.89761667	0	679	60	3,000	5	20	3	6.0	2.8	0.47	1.1	1
W086	UD	Passons	46.07851667	13.18368333	0	117	25	1,150	4	11	1	9.5	3.6	0.37	1.3	2
W087	UD	Silvella	46.08830000	13.05236667	0	128	20	1,087	5	9	1	8.4	3.4	0.41	1.6	3
W088	PN	Arba	46.16375000	12.79803333	0	238	30	950	3	10	1	6.5	2.4	0.37	1.4	3
W089	PN	Cimolais	46.28625000	12.43800000	1	652	90	1,844	4	12	1	8.3	3.6	0.44	1.2	2
W090	PN	Cimolais	46.28816667	12.43725000	0	649	100	2,370	3	14	3	8.5	3.3	0.39	1.0	1
W091	PN	Claut	46.26836667	12.51660000	0	619	60	1,830	5	18	1	3.9	1.9	0.48	0.8	1
W092	PN	Cellino di Sotto	46.24411667	12.47601667	0	506	55	1,643	4	13	1	6.0	2.2	0.36	1.3	4
W093	PN	Arcola	46.20068333	12.52120000	0	433	70	1,708	3	13	1	5.0	2.2	0.43	1.1	3
W094	PN	Barcis	46.19161667	12.57300000	3	406	50	1,200	5	15	1					
W095	PN	Montereale	46.15810000	12.66401667	0	307	65	1,295	4	10	1	6.8	3.0	0.44	1.5	3
W096	PN	Meduno	46.21658333	12.79108333	0	295	80	1,518	5	14	3	7.9	3.3	0.42	1.3	1
W097	PN	Grizzo	46.13155000	12.65816667	0	273	20	890	3	8	1	8.5	2.6	0.31	1.5	3
W098	PN	S. Martino di Campagna	46.07301667	12.62795000	0	170	60	1,750	3	10	3	6.0	2.7	0.45	1.1	4
W099	PN	Marsure	46.08370000	12.59620000	0	189	82	1,595	4	13	1					
W100	PN	S. Quirino di Pordenone	46.05653333	12.67900000	0	152	25	1,050	4	8	1	7.8	3.2	0.40	1.2	4
W101	PN	Valvasone	45.96435000	12.89146667	0	57	50	1,360	4	12	1	7.2	2.8	0.38	1.6	4
W102	UD	Bressa	46.03101667	13.14320000	0	87	50	1,640	2	10	1	5.1	1.8	0.35	1.3	2
W103	UD	Remanzacco	46.07010000	13.31625000	0	104	60	1,810	2	15	1	5.5	2.3	0.42	1.1	1
W104	UD	Ponte S. Quirino	46.11003333	13.47208333	0	155	40	1,450	4	12	1	8.1	3.1	0.39	1.5	2
W105	UD	Tarpezzo-Clastra	46.13246667	13.51733333	0	344	20	1,001	4	11	3	9.3	3.7	0.40	1.4	2

W106	UD	Torreano di Cividale	46.14075000	13.44196667	0	230	100	1,995	3	15	1	5.7	2.4	0.42	1.3	1
W107	UD	Forgaria nel Friuli	46.35111111	13.03555556	2	296	40	1,700	2	10	3	7.9	3.1	0.39	1.9	3
W108	PN	Clauzetto	46.23725000	12.89625000	1	564	60	1,800	3	14	3	7.2	3.1	0.43	1.2	2
W109	PN	Clauzetto	46.24806667	12.88128333	2	568	80	2,370	4	11	0	6.6	1.7	0.26	1.5	1
W110	PN	Frisanco	46.20453333	12.71613333	1	424	50	1,600	3	12	1	6.9	3.6	0.52	0.6	1
W111	PN	Frisanco	46.19998333	12.71613333	1	435	50	1,630	2	12	1	11.4	3.1	0.27	1.0	4
W112	PN	Lestans	46.16381667	12.89563333	1	175	50	1,700	2	14	3	6.4	2.8	0.44	1.6	1
W113	UD	Dignano	46.08451667	12.93645000	1	111	30	1,270	2	10	1	8.7	3.3	0.38	1.6	4
W114	UD	Campoformido	46.01255000	13.13838333	0	64	25	1,205	4	10	1	8.8	3.8	0.44	1.3	2
W115	UD	Orgnano	46.00399888	13.14323990	0	76	45	1,920	4	12	1	5.5	1.7	0.31	1.3	3
W116	UD	Pozzecco	45.96032556	13.09395525	0	43	35	1,410	4	11	1	7.9	3.1	0.39	1.5	2
W117	UD	Bertiolo	45.93842660	13.05130889	0	29	20	885	5	9	1	10.1	3.9	0.39	1.2	3
W118	UD	Roveredo	45.89326972	13.00442309	0	20	35	1,000	5	12	5	7.2	1.9	0.27	1.2	4
W119	UD	Varmo	45.87662352	13.01037990	0	14	60	1,715	5	14	9	10.7	3.6	0.34	1.4	2
W120	PN	Morsano al Tagliamento	45.85720874	12.95490869	1	11	40	1,340	4	10	1	4.9	2.8	0.58	1.0	4
W121	UD	Latisana	45.76987958	12.99884926	1	9	60	1,785	5	14	5	6.0	3.1	0.51	0.5	3
W122	UD	Lignano	45.68165075	13.09814072	0	0	20	800	5	8	5	10.2	4.1	0.40	1.7	2
W123	UD	Lignano	45.76708000	13.02620157	0	0	30	1,170	3	8	5	6.9	2.8	0.40	1.5	4
W124	UD	Palazzolo dello Stella	45.81331894	13.07901460	0	1	80	2,165	5	16	1	7.9	2.7	0.35	1.5	2
W125	UD	Pozzuolo	45.93715901	13.12661278	0	33	20	815	4	8	1	7.7	3.0	0.39	1.6	2
W126	UD	Pozzuolo	45.93688343	13.12737027	0	34	50	1,485	5	13	1	10.3	4.6	0.45	1.3	2
W127	UD	Paradiso	45.86765496	13.14638192	0	13	22	860	4.5	8	1	5.0	2.0	0.40	1.0	2
W128	UD	Mortegliano	45.94979944	13.18134743	0	45	30	1,385	5	9	1	7.5	3.2	0.43	1.4	2
W129	UD	Codroipo	45.95973044	12.99928873	0	43	31	1,580	5	18	3					
W130	UD	Codroipo	45.95946258	12.99531062	0	43	31	1,210	5	18	3	7.6	2.4	0.32	1.8	4
W131	UD	Codroipo	45.95968412	12.99562526	0	42	31	1,360	4.5	18	9	11.4	3.0	0.26	1.7	3
W132	UD	Codroipo	45.95955464	12.99593966	0	42	31	1,335	5	20	1					
W133	UD	Codroipo	45.95924797	12.99641531	0	42	10	735	4.5	12	3					
W134	UD	Rivignano	45.87660168	13.05608778	0	22	40	1,335	3	12	1					

W135	UD	Cividale	46.10170557	13.41728447	0	144	60	1,905	4	12	1	9.1	3.7	0.41	1.2	2
W136	UD	Torreano di Cividale	46.17183770	13.42982175	4	655	60	1,455	4	14	3	8.3	4.0	0.48	1.2	1
W137	UD	Cividale	46.15156581	13.43547491	1	261	60	1,905	4	10	1	2.5	1.3	0.50	0.4	1
W138	UD	Resia	46.37678848	13.28956680	1	442	60	1,820	2	14	1					
W139	UD	Resia	46.37398463	13.30493249	3	479	70	1,970	3	16	3	4.8	2.0	0.43	1.2	2
W140	UD	Resia	46.36444544	13.32879735	1	435	20	1,290	3	10	1	5.1	2.4	0.48	0.9	1
W141	UD	Resia	46.36781506	13.30098902	1	499	50	1,830	2	15	1	6.9	3.2	0.46	1.4	3
W142	UD	Gemona	46.30107651	13.11881823	1	219	50	1,750	2	11	3	7.9	3.4	0.43	1.1	1
W143	UD	Gemona	46.28003389	13.12097961	1	198	15	1,060	3	9	3	10.3	4.5	0.43	1.3	3
W144	UD	Gemona	46.27485905	13.14188437	2	252	25	1,550	3	9	1	10.0	4.7	0.47	1.1	2
W145	UD	Montenars	46.25376519	13.17719945	1	453	60	2,200	2	15	1	8.6	4.3	0.49	1.1	2
W146	UD	Montenars	46.26786911	13.21138192	3	608	35	980	3	9	3	9.6	3.1	0.32	1.8	2
W147	UD	Montenars	46.26654372	13.21928925	3	595	40	1,520	3	11	1	7.2	2.2	0.30	1.6	3
W148	UD	Tarcento	46.22372779	13.22434421	1	238	30	1,300	2	12	1	9.2	3.3	0.36	1.9	1
W149	UD	Tarcento	46.20817866	13.23092534	2	221	40	1,380	3	9	1	8.8	3.3	0.37	1.4	2
W150	UD	Nimis	46.20112593	13.28122322	2	211	25	1,090	4	8	1	5.8	2.4	0.42	1.0	1
W151	UD	Attimis	46.18986149	13.30495276	1	200	50	1,920	3	10	1	7.0	2.9	0.41	1.6	1
W152	UD	Attimis	46.18313311	13.34508182	1	692	60	1,550	3	9	1	8.7	3.7	0.43	1.6	1
W153	UD	Attimis	46.18297346	13.34772995	1	694	60	1,320	3	8	5	16.4	5.0	0.30	1.5	1
W154	UD	Attimis	46.18377957	13.34808156	2	694	50	1,760	4	15	1	5.4	2.2	0.40	1.4	1
W155	UD	Torreano di Martignacco	46.10345331	13.16380093	0	137	64	1,935	5	16	1	9.3	3.0	0.32	1.7	4
W156	PN	Zoppola	45.96580000	12.77031000	1	36	25	1,220	3	12	1	17.3	4.5	0.26	1.8	3
W157	PN	Zoppola	45.96580000	12.77031000	1	36	20	1,080	3	10	1	15.2	5.4	0.35	1.8	3
W158	UD	Tarcento	46.23326667	13.22960000	2	273	40	1,200	3	12	1	7.6	3.2	0.43	1.5	2
W159	UD	Lusevera	46.25893333	13.25468333	2	314	60	1,130	1	13	1					
W160	UD	Lusevera	46.26636667	13.26026667	1	328	35	1,230	2	14	1	4.9	2.5	0.50	0.7	2
W161	UD	Lusevera	46.30795000	13.27315000	1	525	15	1,030	3	10	1	6.6	2.1	0.32	2.0	1
W162	UD	Lusevera	46.31168333	13.28065000	1	597	60	2,600	4	15	1	5.8	2.0	0.34	1.9	2
W163	UD	Lusevera	46.30471667	13.32166667	1	780	25	1,600	2	12	1	5.4	2.4	0.46	1.0	2
W164	UD	Lusevera	46.27453333	13.26753333	2	467	80	2,320	3	12	1	11.9	4.1	0.34	1.4	1
W165	UD	Lusevera	46.27976667	13.27790000	1	502	40	1,580	2	9	1	7.3	3.5	0.49	0.9	2

W166	UD	Lusevera	46.25775000	13.28666667	1	614	60	1,860	3	12	1	4.0	1.6	0.40	2.0	3
W167	UD	Taipana	46.27385000	13.30815000	1	627	35	1,400	2	9	1	7.0	3.0	0.44	1.6	2
W168	UD	Taipana	46.27175000	13.34230000	1	541	28	800	3	8	1	6.9	1.8	0.26	1.4	2
W169	UD	Tarcento	46.21933333	13.23965000	1	331	90	1,450	2	10	1	8.3	3.3	0.40	1.5	2
W170	UD	Chiusaforte	46.40735000	13.31445000	1	387	70	2,060	4	17	1	5.6	2.2	0.40	1.4	2
W171	UD	Chiusaforte	46.40093333	13.35560000	1	485	30	920	3	9	1	5.2	2.2	0.43	0.5	1
W172	UD	Chiusaforte	46.40456667	13.36998333	1	550	80	2,120	1	18	1					
W173	UD	Chiusaforte	46.39616667	13.43938333	2	741	45	1,170	1	10	1					
W174	UD	Tarvisio	46.47190000	13.57125000	2	797	25	1,150	1	8	1					
W175	UD	Tarvisio	46.48351667	13.57968333	2	773	60	1,050	0	10	1					
W176	UD	Verzegnis	46.35471667	12.94695000	1	955	80	2,520	2	18	1	5.8	2.2	0.37	1.2	1
W177	UD	Verzegnis	46.35443333	12.92968333	1	951	80	1,920	2	13	1	6.8	2.4	0.35	1.0	1
W178	UD	Verzegnis	46.35440000	12.92971667	1	947	80	2,120	2	18	1	4.5	2.0	0.45	0.9	1
W179	UD	Verzegnis	46.35321667	12.93066667	1	942	50	1,870	3	10	3	4.8	2.0	0.43	1.0	1
W180	PN	Vito d'Asio	46.31890000	12.93698333	1	490	80	2,170	3	15	1	4.7	2.2	0.47	1.1	1
W181	PN	Vito d'Asio	46.32765000	12.93603333	1	409	40	1,670	3	9	1	7.3	3.5	0.48	0.8	1
W182	PN	Vito d'Asio	46.26911667	12.93180000	1	597	40	1,710	3	12	1	7.1	2.8	0.40	1.2	1
W183	PN	Vito d'Asio	46.26878333	12.93211667	1	505	70	1,750	2	20	1					
W184	PN	Vito d'Asio	46.26921667	12.93240000	1	520	40	2,041	2	10	1	9.3	4.0	0.43	1.2	1
W185	PN	Vito d'Asio	46.26935000	12.93235000	2	514	100	2,512	2	15	1	5.8	2.8	0.48	1.0	1
W186	PN	Clauzetto	46.25310000	12.90275000	1	683	40	1,820	3	10	1	8.0	3.3	0.41	1.1	1
W187	PN	Tramonti di sotto	46.27681667	12.78735000	0	368	70	1,890	3	12						
W188	UD	Socchieve	46.37861667	12.78521667	1	507	70	2,270	2	15		11.7	3.7	0.32	1.4	1
W189	UD	Tarvisio	46.51204000	13.62452000	1	755	160	3,990	1	20						
W190	UD	Tarvisio	46.51242000	13.62492000	1	762	160	3,390	1	15						
W191	UD	ligosullo	46.53993000	13.08580000	2	1073	50	1,610	1	6						
W192	UD	ligosullo	46.54080000	13.07542000	2	969	50	1,800	1	8						
W193	UD	treppo carnico	46.54094000	13.06101000	2	920	100	2,610	1	6		8.5	3.5	0.41	1.0	1
W194	UD	raveo	46.42942000	12.87458000	1	467	80	2,100	1	10		6.6	2.6	0.40	0.9	2
W195	UD	raveo	46.42953000	12.87460000	1	466	80	1,610	1	10		6.7	2.5	0.38	1.1	2
W196	UD	raveo	46.42939000	12.87480000	1	459	80	1,960	1	10						
W197	UD	raveo	46.42112000	12.89804000	1	395	50	1,600	1	6		15.7	6.2	0.39	1.5	1

W198	UD	osoppo	46.25387000	13.08643000	1	186	50	1,720	1	10		7.6	3.1	0.41	1.2	3
W199	UD	Torreano di Martignacco	46.10519000	13.16777000	0	140	50	1,660	1	8						
W200	UD	paularo	46.54040000	13.12830000	4	916	110	2,500	1	8		4.5	2.4	0.54	0.8	1
W201	UD	Strassoldo	45.85560000	13.32111667	0	8	50	2,500	4.5	15	1	8.2	3.2	0.39	1.3	2
W202	UD	Aquileia	45.78745000	13.35611667	0	2	30	1,610	4	12	1	8.5	3.5	0.42	1.3	1
W203	UD	Fiumicello	45.77255000	13.41700000	0	0	61	2,004	5	12	1					
W204	UD	Fiumicello	45.80538333	13.41270000	0	5	50	1,680	4	9	1	9.6	4.4	0.46	1.3	3
W205	GO	Ronchi dei Legionari	45.81890000	13.47153333	2	9	30	1,200	3	9	1	5.9	2.3	0.39	1.2	2
W206	GO	Fogliano/Pieris	45.83743333	13.46658333	0	12	20	1,060	3	8	1	6.4	2.2	0.34	1.3	2
W207	UD	Cervignano	45.82603333	13.33973333	1	3	38	1,400	4	8	1	9.9	3.2	0.32	1.6	4
W208	UD	Muzzana	45.82028333	13.13846667	1	10	35	1,465	3	9	1	9.1	3.0	0.33	1.5	3
W209	UD	Pocenia	45.86041667	13.08911667	0	8	38	1,500	4	10	1	7.6	4.0	0.53	1.0	2
W210	UD	Flambro	45.92758333	13.08866667	0	28	45	1,740	4	10	1	10.6	3.6	0.34	1.9	1
W211	UD	Colugna	46.08760000	13.20061667	0	106	25	1,455	4	9		7.5	2.9	0.38	1.2	4
W212	UD	Prato Carnico	46.52264000	12.77763000	1	767	153	2,400	3	15		5.5	2.6	0.48	0.9	1
W213	UD	Prato Carnico	46.52056000	12.80549000	2	674	50	1,230	3	12		12.4	4.3	0.34	2.3	1
W214	UD	Ribis	46.13346000	13.24184000	0	143	93	1,755	5	14		6.7	2.9	0.43	1.1	1
W215	UD	Pagnacco	46.13393000	13.18768000	0	184	35	1,718	4	14		15.2	5.8	0.38	1.5	3
W216	PN	Zoppola	45.96578333	12.75013333	1	34	30	1,225	1	10		9.8	4.5	0.46	1.1	2
W222	UD	Paularo	46.54355200	13.13252800	2	987	25	1,778	3	8		9.1	3.7	0.41	1.2	1
W223	UD	Ligosullo	46.53927900	13.07691100	3	954	80	1,652	2	12		10.7	4.2	0.39	1.3	1
W224	UD	Verzegnis	46.39165800	12.93258200	1	566	50	1,620	2	8						
W225	UD	Verzegnis	46.39139200	12.93369200	1	571	50	1,220	1	9						

Determination of kernel oil content, oil composition and its modification by temperature in Walnut (*Juglans regia* L.) accessions from North Eastern of Italy

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ABSTRACT

Background: the use of walnut oil is currently limited by its own poor oxidative stability due to the high percentage of polyunsaturated fatty acids. Modifying the oil composition may be a goal in walnut breeding to increase the interest for this crop. Exploring the natural variability and identifying the main environmental factors affecting the oil quality are the first steps in crop selection. Thus, 190 wild accessions distributed throughout the Friuli Venezia Giulia Region (Eastern Italian Alps) were collected and evaluated during 2013 and 2014 for the variability in oil content and its fatty acids profile and compared with five commercial cultivars (Lara, Franquette, Hartley, Howard and Sorrento) taken as references.

Results: High variation in kernel oil content and in fatty acid composition was found in the native walnut accession. Kernel oil content ranged from 54.2 to 72.2%, with a negligible environmental effect. The major fatty acids were linoleic (range 46.9 – 68.6), oleic (10.0 – 25.1), linolenic (6.9 – 17.6), palmitic (3.9 – 11.4)

and stearic acid (1.1 – 5.2). Some accessions had an oil with a fatty acid ratio very different from the reference commercial cultivars, especially for oleic/polyunsatured fatty acids (PUFAs) ratio. Oil quality of the wild accessions with the highest oleic acid content in the oil (>20%) did not show any significant variation across years, unlike the accessions with a lower oleic acid content. A significant linear relationship and a positive correlation between the daily minimum temperature and the oleic acid content was observed in the wild walnuts studied. Every 1°C increase in minimum temperature (in a range between 9.5 and 13.0 °C) caused an increase in oleic acid content of around 1.5%. The oleic acid content was strongly and negatively related to PUFAs content and to a lesser extent to linoleic and linoleic acids, individually.

Conclusions: The large variation in fatty acid content and composition allows to select superior accessions for the diffusion among growers. Hence, several of these selections with high oil content in the kernel and a high relative content in oleic acid could be exploited as a source of genotypes for breeding purposes. A proper strategy to achieve this goal would be to make a selection against PUFAs content rather than just for high oleic. In addition, the selected high oleic accessions, before being utilized per se or as donor parents of high oleic, have to demonstrate not to be adversely affected by the environment.

Keywords: walnut germplasm, fatty acid composition, PUFAs, oleic acid content, walnut breeding

Introduction

Common or Persian walnut (*Juglans regia* L.) is an important fruit tree grown in Europe and Asia since ancient time. Nowadays, walnut is commercially cultivated throughout southern Europe, northern Africa, eastern Asia, USA and western South America, with a total surface of about 1 Mha in 2014 (FAOSTAT, 2017). Walnut represents a valuable lipid food source; in fact the oil content, ranging in commercial walnut varieties from 620 to 740 g kg^{-1} kernel (Martìnez et al., 2010), represents the predominant component of this nut crop (Amaral et al., 2003; Pereira et al., 2008; Martinez et al., 2010). Walnut oil is naturally rich in polyunsaturated fatty acids (PUFAs), mainly linoleic (55 – 70%) and linolenic (10 – 18%) acid, and consequently, poor in monounsaturated fatty acid (MUFA), mainly represented by oleic acid (10 – 20%) and in saturated fatty acids (Peirera et al., 2008; Martìnez et al., 2010; Hayes et al., 2016; Sena-Moreno et al., 2016). Walnuts are mainly consumed as fruits and their nutritional qualities and use depend mainly on oil fatty acid profile. Walnut oil is excellent if fresh used directly for edible purposes, because rich in the so-

called ω-3 and ω-6 fatty acids that are essential fatty acids for humans diet. On the contrary, given its high PUFAs content, the walnut oil is very susceptible to oxidation and rancidity and consequently unsuitable for long storage, refining, deep frying and any other cooking applications, because of its low smoking point. Its short shelf life limits many food and no food applications. Changes in oil composition is currently a goal in many oil seed crops breeding programs. Besides that, several novel products, mainly in the food industry, have been developed from several oil crops through conventional breeding approaches. Thus, several crops have been brought to similar fatty acid composition through breeding. An example is the high oleic acid content in sunflower, soybean and canola, as well as the high stearic acid content in sunflower and soybean and low saturated fatty acids in soybean and canola (Baldini et al., 2014). Breeding to modify the quality of walnut oil have received little attention until recently, but changing the oil composition on the basis of nut end-use would contribute to the expansion of walnut oil utilization. The development of alternative walnut vegetable oil feedstocks with improved functionality, maintaining at the same time the nutritional quality, has therefore become a priority for the food market. The specific goal in walnut breeding could be the improving of oxidative stability through the reduction of PUFAs and the relative increase of the MUFA (Martìnez et al., 2010; Hayes et al., 2016; Sena-Moreno et al., 2016). The same objective of an improved stability of the oil could be reached by rising the saturated fatty acids content, but that can have adverse effects on human health, due to the increase of low-density lipoprotein (LDL) in blood caused by high levels of palmitic acid (C16:0) consumption (Minihanea and Harlanda, 2007). Genotypes with high oleic acid content are naturally present in walnut. A content in oleic acid higher than 400 g kg^{-1} (Ozkan and Koyuncu, 2005) and up to 300 g kg^{-1} (Zwarts et al., 1999) have been reported for walnuts grown in Turkey and in New Zealand, respectively.

Genetic resources are the base of crop improvement, thus the characterization of wild accessions, with the identification of genetic variability for the target traits, is the first step in new cultivars development. However, no information is reported about environmental effects on these genotypes. Significant variation in linoleic, linolenic and oleic acid content naturally exists due to plant genotype and environmental factors occurred during fruit growth (Zwang, 1999). Some variations recorded in seed fatty acid profile within cultivars were associated to location (Zwang et al., 1999) and irrigation management. Environmental factors such as temperature, latitude, drought, radiation, and maturing stage/harvest date may also affect oil composition in most oil crops. The increase in the oleic/linoleic acid ratio with increasing temperature during

grain filling has been widely reported in several oil seed crops such as sunflower, maize (Canvin, 1965; Nagao and Yamazaki, 1984; Sobrino et al., 2003) and soybean (Byfield and Upchurch, 2007). Among environmental factors, temperature is known as the main factor affecting oil composition in oil crops genotypes (Baldini et al., 2014). Many authors have worked on oil quality as affected by temperature in sunflower (Izquierdo et al., 2006), where minimum temperature seems to vary oleic: linoleic acid ratio, but no studies on temperature effect on fatty acid composition in walnut can found in the literature. The aim of this work was: i) to characterize wild walnut accessions distributed throughout the Friuli Venezia Giulia region for oil content and fatty acid composition in comparison with popular commercial varieties, ii) to evaluate the effect of different daily temperature regimes during the nut development on oil fatty acid profile.

Materials and Methods

Walnut Samples Collection

166 nut samples were collected from wild walnut accessions in 2013 and 58 in 2014 throughout the Friuli Venezia Giulia region, in North-Eastern Italian Alps (Figure 1.a). 35 of these accessions were common to both years (Figure 1.b). Most trees were more than 30 years old. They grew between 1 and 1,073 meters above sea level and were genetically characterized in a previous paper (Vischi et al., 2017). The sampled trees never underwent either pruning or other horticultural practice (fertilization and irrigation) or pests inspect. Nuts of five commercial cultivars were included as reference. "Lara", "Franquette", "Hartley" and "Howard" were sampled from commercial orchards, while "Sorrento" was supplied by the National Council for Agricultural Research and Economics, CREA-FRUT of Rome. All genotypes were characterized for oil content and oil fatty acids composition.

Meteorological data

Meteorological data were provided by Osmer (Friuli Venezia Giulia Meteorological Service) and as suggested by the same service, Friuli Venezia Giulia region was divided into six climatic zones, namely Carnia Alps, Pre-Alpine belt, foothills, the Friuli plain, Tarvisio Alps and Canal del Ferro valley (ARPA FVG – OSMER, 2015; Figure 1b). Minimum, average and maximum daily temperature of both years of

experimentation, average mean temperature and rainfall of the last 25 years and altitude above sea level for each location were obtained from the regional meteorological service (Table 1).

Sample preparation

40 nuts were sampled before nut fall from each accession and brought to the laboratory for the analyses. Samples stored in a dry room were inspected regularly and when the husk was open, fruit were dehusked, the nut washed in water with 1% sodium hypochlorite , dried in a heater at 30 °C for 3 days and stored at 4 °C in a cold store at 60% of relative humidity. At the time of analysis, nuts were manually cracked, shelled, and kernel chopped in a MKM 6000 coffee mill (Bosch, Germany).

Moisture content

Analyses of moisture was carried out in duplicate. Moisture was determined on 5 g sample in a stove at 105 ± 2 °C until constant weight was reached.

Oil content determination

Oil content was determined by Nuclear Magnetic Resonance (NMR) on whole kernel. NMR is a non–destructive method mainly used in oil crops breeding (FGIS-USDA, 2009). A preliminary trial has been carried out to compare Soxhlet oil extraction method to NMR and the results are reported as supporting information.

Fatty acids determination

Lipids were extracted in 1 mL of n-hexane. Fatty acids were converted in fatty acid methyl esters (FAMEs) by transesterification with a methanolic potassium hydroxide solution (2N). Composition was determined by gas chromatography with flame ionization detection (GC–FID) and every fatty acid was expressed as a percentage of the total detected in the oil. The gas chromatograph was fitted with a 60 m HP-88 capillary column (Agilent Technologies, USA). Helium was used as carrier gas, and the injector, detector and oven temperatures were 230, 250 and 200 °C, respectively. 5 µL of sample were injected in split mode. A 5.0 mm ID precision inlet split liner was used. Different FAMEs were identified by comparison with known standards.

Statistical analysis

Descriptive metrics, such as mean, standard deviation and range of data distribution were calculated. Non-parametric bootstrap analysis was applied to select the best temperature predictor for fatty acid composition (Izquierdo et al., 2006). Statistical analysis was performed using R version 2.15.0 (R Development Core Team, 2012), utilizing Shapiro–Wilk normality test to analyze the normality condition. 10,000 random samplings with replacement were performed. The sample size of each bootstrap iteration was the same as the original one. Several temperature parameters were evaluated by comparing the distribution of the 10,000 R^2 values.

Results and discussion

Moisture content

Although the kernel moisture could vary as a function of season, harvest time and environmental condition, both wild accessions and commercial cultivars had the same moisture content of about 3.5% (data not shown) at harvest time and suitable for long storage (Kader and Thompson, 2002). These values were in agreement with those reported by Peirera et al. (2008) and Amaral et al. (2003) and lower than those reported in other study (Savage, 2001; Christopoulos and Tsantili, 2014).

Oil content

Oil content is reported in Table 2. Commercial varieties generally showed a mean oil content higher than wild genotypes, but the difference was pretty small (about 4%) (Table 2). The oil content of the commercial varieties was comparable with the data of the literature for the same cultivars (Peirera et al., 2008; Martínez et al., 2010; Christopoulos and Tsantili, 2014). However, there were some differences between commercial varieties. Howard was the cultivar with the highest oil content, whereas Hartley showed the lowest one. "Sorrento" recorded an oil content lower than that reported by Martinez et al. (2010) (72-74%) and higher than that reported by Malvolti et al. (2010) (61%). Kernel oil content of the wild accessions ranged from 56.2% to 71.6% in 2013 and from 54.2% to 72.2% in 2014. The lowest level in oil content was found in the genotype W160 (Pre-alpine belt) in both years, whereas the highest oil content was recorded for the

accessions W122 and W123 (Friuli plain, close to the sea coast) in 2013 and 2014, respectively; both the latter accession were located in the same environmental area. (Table 3).

Kernel oil concentration was slightly higher on average than those reported for other walnut collections in New Zealand (Savage et al., 1999; Zwarts et al., 1999) and Portugal (Amaral et al., 2003). Also Malvolti et al. (2010) obtained a kernel oil content, as average of 190 walnut genotypes grown throughout the Italian peninsula, lower than those obtained in the present study. The range obtained for the wild accessions in the present survey was similar to those reported by Caglarirmak (2003), Dogan and Akgul (2005), Ozkan and Koyuncu (2005), Yerlikaya et al. (2012), who analysed selections collected in Anatolia (57-71%).

The oil content in walnut kernel is determined by the genotype, but it may also be influenced by environmental conditions and irrigation management (Zeneli et al., 2005; Pereira et al., 2008; Martinez et al., 2010). However, no significant relation was found between altitude and oil content (Fig 4.).

Fatty acid composition

Data on oil fatty acid composition are reported in Table 2. Walnut kernel oil was composed mainly by five fatty acids, namely palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) acid. The most representative one was linoleic acid in both wild accessions and commercial varieties (Table 2), followed by oleic acid (10.0-25.1%) and linolenic (6.9-17.6%). The data obtained indicate that the accessions harvested in Friuli Venezia Giulia region shown a higher variability in oil composition compared to commercial cultivars. In particular, some accessions shown a significantly higher content in oleic acid (>20%) and lower content in linoleic (<56%) and linolenic acid (<11%) (Table 2).

PUFAs were the main group of fatty acids in walnut oil in both wild accessions (62.9 to 79.1%) and commercial varieties (70 to 76%). On the contrary, saturated fatty acids were the minor group, ranging from 5.8 to 13.8%, with palmitic and stearic acids the two main saturated fatty acids present in walnut oil, totaling on average 7.5 and 2.5% of total fatty acids respectively (Table 2). Oleic acid concentration in the seed oil ranged from 11.2 to 25.1% in 2013 and from 10.0 to 23.3% in 2014, respectively, with averages and range of variation very similar in both year (Table 2). 15 accessions of the 35 analysed in both years shown a

negligible variation in oleic acid content across years (approx. 2%); the remainders showed 5% of variation across the same years (Table 3).

Accessions were divided into three classes on the basis of their oleic acid content. Oleic classes were defined as follows: <12%, 12-20% and >20%. The class limits were drawn using the five commercial cultivars as reference. Most of the wild accessions had an oleic acid content falling into the intermediate class, with the exception of four accessions, namely W037, W046, W089 and W201, which shown an oleic acid content stably higher than 20% in both years with little influence of environmental conditions (Table 3). For instance, the accessions with high oleic acid content W037 and W046 were located in Alpine area, characterized by low mean temperature), whereas accessions W089 and W201 were located in the plain. These accessions may be used in breeding programs targeted to enhance oleic acid content in walnut kernel oil and to expand the utilization of walnut oil, by improving the stability during storage period (long-term storage), allowing the utilization as frying oil and enhancing the shelf life of walnut nuts consumed as fruit. For instance, in sunflower, breeders have incorporated the high-oleic trait into confectionary sunflower varieties to increase the self-life of sunflower kernels (Miller and Fick, 1997).

Significant variations in linoleic and linolenic acid across the years were observed just in few accessions (Tables 2 and 3).

Relationship between fatty acids

The coefficients of correlation between fatty acids of the wild walnuts oil are reported in Table 4. A significant and positive correlation was observed between the palmitic and stearic saturated fatty acids. The stronger relationship between fatty acid concentrations was the negative one between oleic acid and PUFAs content (Table 4). The strong negative relationship between PUFAs and oleic acid content can be seen in the linear regression analysis (p-value < 0.001) (Figure 2). Oleic acid showed also a negative relationship, although of lesser intensity, with linoleic and linolenic acid (table 4), as already observed in several herbaceous oil-crops, such as sunflower (Izquierdo et al., 2002, 2006; Izquierdo and Aguirrezábal, 2008) and soybean (Pham et al., 2011, 2012). Our results are comparable with those obtained by Malvolti et. al (2010), who reported a significant correlation between oleic and linoleic acid content (r = -0.88) and between oleic

and linolenic (r = -0.67) acid content. Despite that, in our experiments the magnitudes of the relationships were generally lower for both linoleic (R = -0.55) and linoleic fatty acid content (R = -0.49), which would claim interferences from environmental factors, such as temperatures, rainfall and light, as reported elsewhere (Zwang et al., 1999; Peirera et al, 2008).

Temperature and fatty acid composition

We analysed the effect of temperature (the daily average, maximum and minimum), during the whole fruit development period, starting from the end of flowering and ending to physiological maturity, on fatty acid composition. In Friuli-Venezia Giulia this period lasts about 2 months, beginning in June and ending in August, with about few days of difference according with the altitude. No relationship between any daily temperature and saturated fatty acids, linoleic acid and PUFAs content was found (data not shown). On the other side, R^2 values of the linear functions between oleic acid concentration and temperature ranged from 0.08 to 0.65, depending on the temperature used. The best positive linear function was obtained considering the daily minimum temperatures ($R^2 = 0.65$) evidencing that in the range 9.5 -13.0 °C, an increase of 1 °C of the minimum temperature determined an increase in the oleic acid content of 1.5% (Figure 3a). Average and maximum temperatures gave worse correlation coefficients (R^2=0.46 and 0.08 respectively). The latter, due to the very low correlation value, was discarded from further investigations. R^2 values of the linear functions between linolenic acid concentration and temperature ranged from 0.18 to 0.65, depending on the temperature used and again, daily minimum temperature gave the best linear fitting function ($R^2 = 0.65$), but in this case, the linolenic acid content decreases when temperature increases (Figure 3b). The relationship between oleic acid and maximum temperature was once again low ($R^2 = 0.18$) and therefore disregarded in further investigations. The biological and physiological mechanisms through which the daily minimum temperature influences oleic and linolenic acid concentrations are still unknown and it would deserve investigation, provided that the knowledge of environmental factors affecting fatty acids accumulation need to be considered during germplasm selection and new genotypes development (Izquierdo et al. 2006).

Bootstrap samples

Table 5 reports the results of bootstrapping on coefficient of determination (R^2) of linear functions between average and minimum temperatures and fatty acid content. The goal of bootstrapping is to determine how the

content of the fatty acid is influenced by different temperature regimes and to compare the different type of temperature (minimum *vs.* average). Regarding the temperature, the oleic and linolenic fatty acids contents were affected more by the daily minimum temperature than by the daily average one. Several authors have reported comparable results for herbaceous oil crops (Harris et al., 1978; Izquierdo et al. 2002). Izquierdo et al. (2002) reported for instance that in sunflower, oleic acid percentage was positively related to night minimum temperature.

Conclusions

A considerable phenotypic variability in kernel oil content and oil fatty acid composition was found within walnut accessions sampled throughout Friuli Venezia Giulia region in two years of observations. Kernel oil content recorded for some wild accessions was comparable to cultivars of the market. These and other accessions shown significant differences also in oil fatty acid composition compared to commercial cultivars.

It would be possible to develop cultivars with a higher oleic acid (>20%) and lower PUFAs content than commercial varieties, since the two characters are negatively and strongly related. In this case, the first step in a breeding program would be to select against PUFAs rather than linoleic or linolenic acid individually or against saturated fatty acids. Secondarily, the selected new genotypes, before being recommended to growers or selected as donor parents, have to show a high and stable level of oleic acid across years and environments. These genotypes could be of great interest for the market since they would combine the unchanged nutritional characteristics with an increased oxidative stability and shelf life of their oils.

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References

Amaral, J. S.; Casal, S.; Pereira, J. A.; Seabra, R. M. & Oliveira, B. P. P. (2003), 'Determination of Sterol and Fatty Acid Compositions, Oxidative Stability, and Nutritional Value of Six Walnut (*Juglans regia* L.)

Cultivars Grown in Portugal', *J. Agric. Food Chem* **51**, 7698-7702.

ARPA FVG – OSMER (2015). Schede climatiche del Friuli Venezia Giulia. http://www.clima.fvg.it (In Italian; accessed 05.09.16).

Baldini, M.; Ferfuia, C. & Vischi, M. (2014), 'Improvement of the oil quality of the main oil crops', *CAB Reviews* **9**(20), 1-16.

Byfield, G. E. & Upchurch, R. G. (2007), 'Effect of Temperature on Delta-9 Stearoyl-ACP and Microsomal Omega-6 Desaturase Gene Expression and Fatty Acid Content in Developing Soybean Seeds', *Crop Science* **47**, 1698-1704.

Caglarirmak, N. (2003), 'Biochemical and physical properties of some walnut genotypes (Juglans regia L)', *Nahrung/Food* **47**, 28–32.

Canvin, D. (1965), 'The effect of temperature on the oil content and fatty acid composition of the oils from several seed crops.', *Can. J. Bot.* **43**, 63–69.

Christopoulos, M. V. & Tsantili, E. (2014), 'Oil composition in stored walnut cultivars—quality and nutritional value', *Eur. J. Lipid Sci. Technol.* **116**, 1-10.

Dogan, M. & Akgul, A. (2005), 'Fatty acid composition of some walnut (*Juglans regia* L) cultivars from east Anatolia.', *Grasas Aceites* **56**, 328-331.

FAOSTAT (2016), Database on Agriculture [http://faostat.fao.org/]. Rome: Italy. Food and Agriculture Organization of the United Nations; 2016 Available from: (accessed 12/15/16)

FGIS-USDA, 2009. Nuclear Magnetic Resonance (NMR) Handbook, FGIS-USDA, United States

Department of Agriculture, Grain Inspection, Packers and Stockyards Administration.

Harris, H.; Mc William, R. & Mason, W. (1978), 'Influence of temperature on oil content and composition of sunflower seed.', *Aust. J. Agric. Res.* **29**, 1203–1212.

Hayes, D.; Angove, M. J.; Tucci, J. & Dennis, C. (2016), 'Walnuts (Juglans regia) Chemical Composition and Research in Human Health', *Critical Reviews in Food Science and Nutrition* **56**, 1231-1241.

Izquierdo, N. & Aguirreza´bal, L. (2008), 'Genetic variability in the response of fatty acid composition to minimum night temperature during grain filling in sunflower', *Field Crops Research* **106**, 116–125.

Izquierdo, N.; Aguirreza´bal, L.; Andrade, F. & Pereyra, V. (2002), 'Night temperature affects fatty acid composition in sunflower oil depending on the hybrid and the phenological stage', *Field Crops Research* **77**, 115-126.

Izquierdo, N. G.; Aguirrezàbal, L. A.; Andrade, F. H. & Cantarero, M. G. (2006), 'Modeling the Response of Fatty Acid Composition to Temperature in a Traditional Sunflower Hybrid', *Agron. J.* **98**, 451-461.

Kader, A. A. & Thompson, J. E.Kader, A., ed. (2002), *Postharvest Technology of Horticultural Crops*, Vol. pp. 399–406., University of California Division of Agriculture and Natural Resources, Oakland (USA).

Malvolti, M. E.; Pollegioni, P.; Bertani, A.; Mapelli, S. & Cannata, F. (2010), '*Juglans regia* Provenance Research by Molecular, Morphological and Biochemical Markers: A Case Study in Italy', *Bioremediation, Biodiversity and Bioavailability* **4**, 84-92.

Martìnez, M. L.; Labuckas, D. O.; Lamarque, A. L. & Maestri, D. M. (2010), 'Walnut (*Juglans regia* L.): genetic resources, chemistry, by-products', *J Sci Food Agric* **90**, 1959-1967.

Miller, J. & Fick, G. N. (1997), The genetics of sunflower'Sunflower Technology and Production', ASA; CSSA; SSSA, .

Minihanea, A. & Harlanda, J. (2007), 'Impact of oil used by the frying industry on population fat intake.', *Critical Reviews in Food Science and Nutrition* **47**, 287-289.

Nagao, A. & Yamazaki., M. (1983), 'Lipid of sunflower seeds produced in Japan', *J. Am. Oil Chem. Soc.* **60**, 1654–1658.

Ozkan, G. & Koyuncu, M. (2005), 'Physical andchemical compositionof some walnut (*Juglans regia* L) genotypes grown in Turkey.', *Grasas Aceites* **56**, 141-146.

Pereira, J. A.; Oliveira, I.; Sousa, A.; Ferreira, I. C.; Bento, A. & Estevinho, L. (2008), 'Bioactive properties and chemical composition of six walnut (*Juglans regia* L.) cultivars', *Food and Chemical Toxicology* **46**, 2103-2111.

Pham, A.-T.; Lee, J.-D.; Shannon, J. G. & Bilyeu, K. D. (2011), 'A novel FAD2-1 A allele in a soybean plant introduction offers an alternate means to produce soybean seed oil with 85% oleic acid content', *Theor Appl Genet* **123**, 793–802.

Pham, A.-T.; Shannon, J. & Bilyeu, K. (2012), 'Combinations of mutant FAD2 and FAD3 genes to produce high oleic acid and low linolenic acid soybean oil', *Theoretical and Applied Genetics* **125**(3), 503-515.

Savage, G.; Dutta, P. & McNeil, D. (1999), 'Fatty Acid and Tocopherol Contents and Oxidative Stability of Walnut Oils', *JAOCS* **76**(9), 1059-1063.

Savage, G. P. (2001), 'Chemical composition of walnuts (*Juglans regia* L.) grown in New Zealand.', *Plant Foods Hum. Nutr.* **56**, 75-82.

Sena-Moreno, E.; Pardo, J. E.; Pardo-GimÃ©nez, A.; GÃ³mez, R. & Alvarez-OrtÃ, M. (2016), 'Differences in Oils from Nuts Extracted by Means of Two Pressure Systems', *International Journal of Food Properties* **19**(12), 2750-2760.

Sobrino, E.; Tarquis, A. & Dìaz., M. C. (2003), 'Modeling the oleic acid content in sunflower oil', *Agron. J.* **95**, 329-334.

Yerlikaya, C.; Yucel, S.; Ümran Erturk & Korukluo'lu, M. (2012), 'Proximate Composition, Minerals and Fatty Acid Composition of *Juglans Regia* L. Genotypes and Cultivars Grown in Turkey', *Braz. Arch. Biol. Technol* **55**(5), 677-683.

Vischi M., Chiabà C., Raranciuc S., Poggetti L., Messina R., Ermacora P., Cipriani G., Paffetti D., Vettori C., Testolin R. (2017) 'Genetic Diversity of Walnut (*Juglans Regia* L.) in the Eastern Italian Alps', *Forests* **8** (3), 81-94.

Zeneli, G.; Kola, H. & Dida, M. (2005), 'Phenotypic variation in native walnut populations of Northern Albania', *Scientia Horticulturae* **105**, 91-100.

Zwarts, L.; Savage, G. & McNeil, D. (1999), 'Fatty acid content of New Zealand-grown walnut (*Juglans regia* L.)', *International Journal of Food Sciences and Nutrition* **50**, 189-194.

Figures legend

Fig. 1a) Geographic location of walnut collection sites; b) Identification of 6 zones for the 35 genotypes sampled in both years.

Fig.2 Relationship between oleic acid content and PUFAs (Sum Linoleic+Linolenic acid) in nut oil.

Figure.3 a) Oleic acid concentration as linear function of daily minimum temperature during fruit development in walnut (n=10; *p*-value < 0.01); b) Linolenic acid concentration as linear function of daily minimum temperature during fruit development in walnut (n=10; *p*-value < 0.01).

Fig.4 Relationship between oil content and altitude (m a.s.l.) of sampled accessions

Table 1. Altitude, annual rainfall and mean temperature during the seed development at locations where nuts samples were collected in both years.

Location	Elevation (m a.s.l.)	Annual Mean temperature (1991-2014) (°C)	Annual Raifall (1991-2014) (mm)	2013 Mean temperature during seed development (°C)	2014 Mean Temperature during seed development (°C)	Accessions collected (n)
Carnia Alps	693	4.0-11.0	1681	19.5	14.7	9
Pre-alpine belt	468	4.4-12.7	2347	14.2	17.6	10
Friuli plain	50	13.0-13.2	1450	23.4	22.0	7
Foothills	322	13.0-13.2	2193	22.7	20.9	6
Tarvisio Alps	718	6.8-7.4	1743	17.4	15.9	2
Canal del Ferro valley	568	9.4	1952	19.4	17.6	1

Elevation = it represent the average m a.s.l. where sampled walnut trees grew.

Annual mean temperature = average of daily mean temperatures recorded during year. There are several meteorological stations for each location so reported temperatures are range of variations, excepted for Canal del Ferro Valley where there was a meteorological station alone.

Annual rainfall = is the long-term average (1991-2014) of annual precipitation.

Mean temperature during seed development= starting from the end of walnut flowering and ending to physiological maturity (See text for details).

Table 2. Oil content and main fatty acids of walnut accessions and controls represented by five commercial cultivars. Values are means ± standard deviation, and between bracket in the line below the range of variation.

Year (no. accessions)	Oil content (% w/w)	Main Fatty Acids (%)				
		Palmitic C16:0	Stearic C18:0	Oleic C18:1	Linoleic C18:2	Linolenic C18:3
2013 (58)	65.3 ± 3.2 [56.2-71.6]	8.18 ± 0.98 [6.11-11.37]	2.85 ± 0.80 [1.54-5.23]	16.20 ± 2.82 [11.26-25.09]	60.54 ± 2.46 [53.51-66.53]	12.00 ± 1.96 [6.89-16.13]
2014 (166)	66.3 ± 3.1 [54.2-72.2]	7.24 ± 0.82 [3.90-9.59]	2.21 ± 0.71 [1.07-4.42]	16.49 ± 2.72 [10.05-24.90]	61.28 ± 2.94 [46.91-68.62]	12.17 ± 2.02 [7.37-17.57]
Controls	70.2 ±1.62 [68.5 –72.9]	7.92 ± 0.98 [6.95 – 8.74]	3.29 ± 0.70 [2.82 – 3.83]	15.63 ± 2.99 [12.21 –19.72]	58.01 ± 0.87 [56.94 – 59.03]	14.79 ± 2.64 [11.86 – 18.65]

Table 3. Kernel oil content and main fatty acids composition in the 35 accessions sampled in both years.

Accession	Kernel oil content (%)		Palmitic C16:0 (%)		Stearic C18:0 (%)		Oleic C18:1 (%)		Linoleic C18:2 (%)		Linolenic C18:3 (%)	
	2013	*2014*	*2013*	*2014*	*2013*	*2014*	*2013*	*2014*	*2013*	*2014*	*2013*	*2014*
W005	63.5	66.9	8.07	7.98	2.24	2.58	15.44	14.23	60.78	57.86	13.19	16.18
W012	66.6	70.0	8.39	8.16	2.80	3.97	13.72	15.35	61.96	58.77	13.14	12.68
W027	68.1	66.3	7.81	3.90	2.71	1.87	17.88	15.15	58.96	64.57	12.49	14.11
W028	67.5	70.1	6.86	6.28	2.23	1.73	15.92	16.23	61.44	60.28	13.40	14.10
W034	65.9	63.0	6.11	7.01	5.23	1.41	19.17	16.91	58.82	61.56	10.67	12.41
W037	61.2	62.5	6.65	6.77	2.36	2.52	25.09	23.32	56.20	57.12	9.19	9.84
W042	60.4	61.5	6.31	6.03	2.81	1.66	17.25	15.97	61.74	64.51	11.89	11.69
W045	62.9	65.5	8.32	8.69	2.99	4.42	16.26	14.22	60.64	61.79	11.23	10.86
W046	63.6	65.9	6.97	6.36	2.59	2.29	21.20	20.08	58.26	60.27	10.51	10.54
W051	63.7	64.9	8.11	7.68	2.72	2.56	16.30	12.76	59.60	62.43	13.27	14.43
W079	70.0	70.2	9.30	7.29	2.98	2.13	13.19	16.87	60.48	58.16	13.84	15.37
W087	65.3	64.0	9.16	7.72	2.71	3.38	11.26	11.05	64.87	60.78	11.99	15.60
W089	67.8	65.7	8.45	7.01	3.51	1.47	20.67	17.32	56.01	63.05	11.07	10.91
W097	67.1	62.2	8.53	7.47	3.59	2.27	16.70	14.71	61.87	65.91	9.31	9.55
W100	63.3	68.9	8.12	7.43	3.50	2.74	16.83	21.15	64.42	59.52	6.89	8.11

W102	65.6	67.6	7.96	7.86	3.05	3.58	13.85	15.74	61.50	59.37	13.59	13.01
W104	68.6	68.8	9.11	7.54	2.37	2.70	13.68	12.02	60.72	61.44	13.89	15.99
W136	70.3	68.9	10.81	9.59	5.11	4.19	13.66	17.71	58.10	56.11	12.31	12.39
W140	65.0	66.3	7.72	7.09	2.56	2.87	18.99	13.98	58.22	60.77	12.49	14.22
W141	63.3	66.6	8.05	6.14	2.33	2.48	16.18	17.98	60.80	60.91	12.55	12.17
W142	71.0	62.9	7.29	6.00	ND	1.60	17.45	13.31	63.30	68.35	11.87	10.55
W144	69.7	68.5	7.59	6.71	2.66	3.02	17.04	15.46	61.51	61.34	11.07	12.98
W145	66.8	69.4	8.54	6.87	4.58	1.73	17.91	18.49	57.57	60.25	11.23	12.37
W148	70.1	68.7	11.37	8.07	4.47	3.95	12.20	16.45	58.08	61.03	13.88	10.03
W150	60.0	68.3	7.07	7.63	3.01	2.68	19.18	14.57	59.59	59.49	10.99	14.41
W153	63.9	64.1	9.37	6.45	2.39	1.66	14.93	15.41	60.22	62.22	13.09	13.88
W160	56.2	54.2	8.82	7.59	2.29	2.99	14.47	10.05	65.73	68.62	8.43	9.89
W162	65.2	68.9	7.56	7.08	1.97	1.40	20.24	17.86	59.99	62.69	10.10	10.49
W179	63.5	63.1	8.60	6.81	1.54	2.55	13.63	15.16	61.46	59.41	14.74	15.50
W180	62.2	59.6	9.05	7.25	2.09	1.55	14.49	11.97	60.43	65.71	13.85	13.37
W201	66.6	67.9	8.97	7.49	2.92	2.34	19.33	21.67	58.23	57.94	10.28	9.69
W204	64.7	65.8	8.42	6.70	2.29	1.91	13.90	14.31	62.53	62.86	12.86	13.74
W206	65.1	65.3	7.98	7.73	2.71	3.34	11.42	12.68	66.53	64.44	10.87	11.34
W212	66.3	66.9	8.92	9.18	2.61	2.59	14.43	13.08	58.91	59.19	15.12	15.85
W215	65.6	65.8	8.18	7.08	4.41	3.51	15.92	17.05	61.44	63.62	9.49	7.98

ND. Not determined

Table 4. Correlation coefficients between seed oil content and concentration of fatty acids in the walnut genotypes tested (n=35).

	Palmitic	Stearic	Oleic	Linoleic	Linolenic	PUFAs
Palmitic	1					
Stearic	**0.45****	1				
Oleic	-0.29	-0.08	1			
Linoleic	-0.26	-0.28	**-0.55****	1		
Linolenic	0.08	-0.19	**-0.49****	-0.21	1	
PUFAs	-0.17	**-0.41***	**-0.83*****	**0.74*****	**0.50****	1

Notes: *, ** and *** Significant at the $p< 0.05$, 0.01 and 0.001 levels, respectively.

Table 5. Bootstrap Analysis of linear relationship, coefficient of determination (R^2), for daily mean temperature (T°med) and daily minimum temperature (T°min).

	Bootstrap samples	Parameter	Mean R^2	Standard Deviation	Median R^2	Min R^2	Max R^2
Oleic	10,000	Tmin	0.55	0.05	0.54	0.12	0.88
	10,000	Tmed	0.24	0.10	0.22	0.01	0.56
Linolenic	10,000	Tmin	0.45	0.07	0.46	0.14	0.68
	10,000	Tmed	0.25	0.11	0.25	0.03	0.55

Fig. 1.a) Geographic location of collection sites for the studied walnut genotype

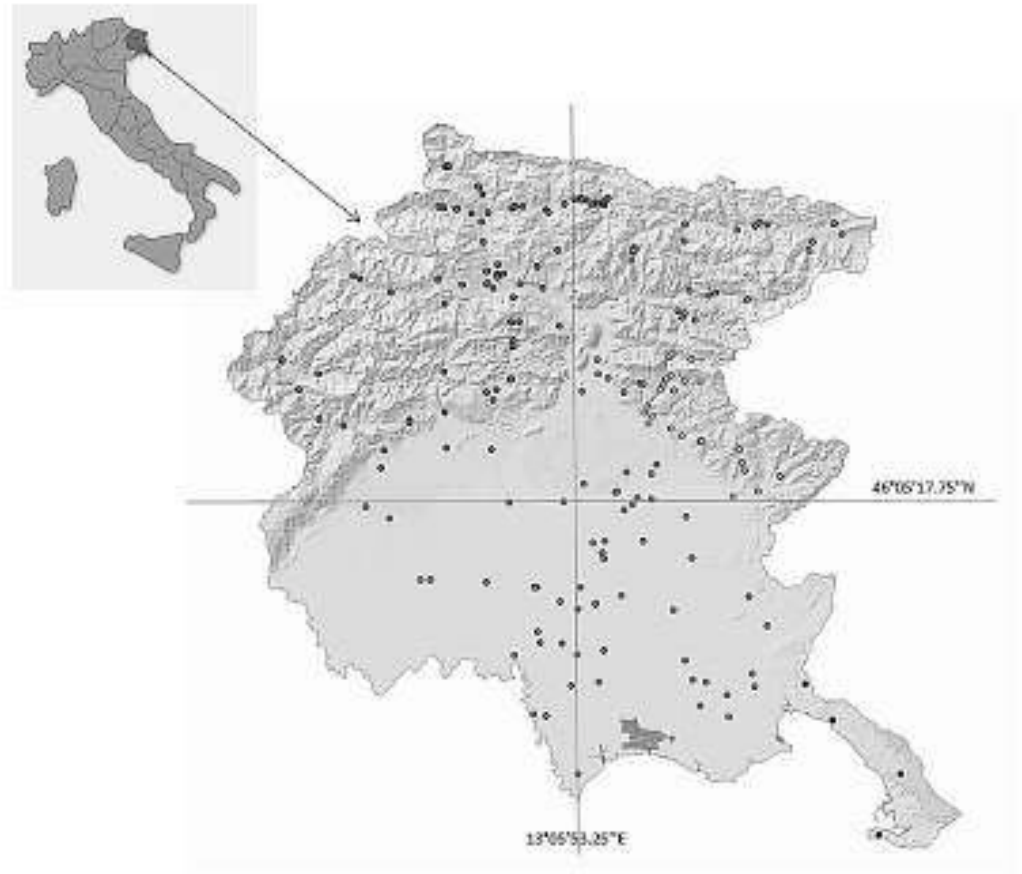


Fig. 1.b) Identification of 6 zones for the 35 genotypes sampled in both years.

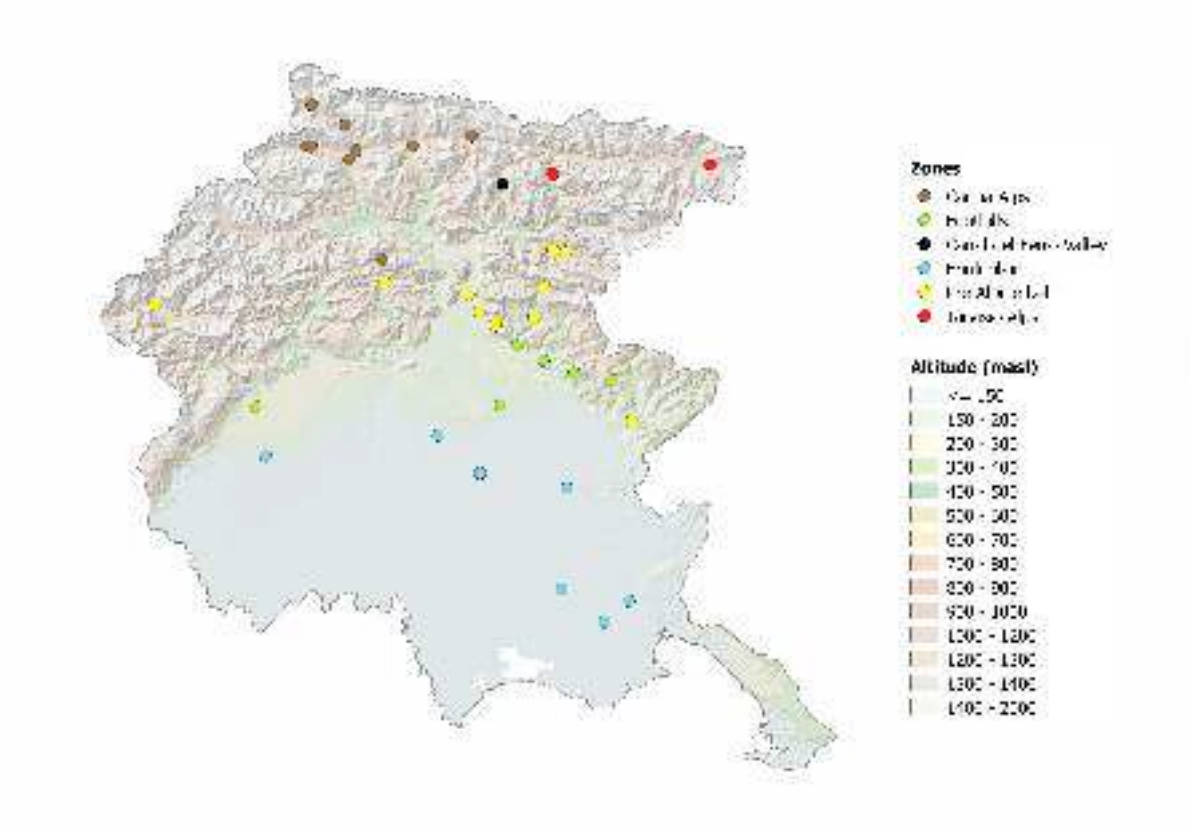


Fig.2 Relationship between oleic acid content and PUFAs (Sum Linoleic+Linolenic acid) in nut oil.

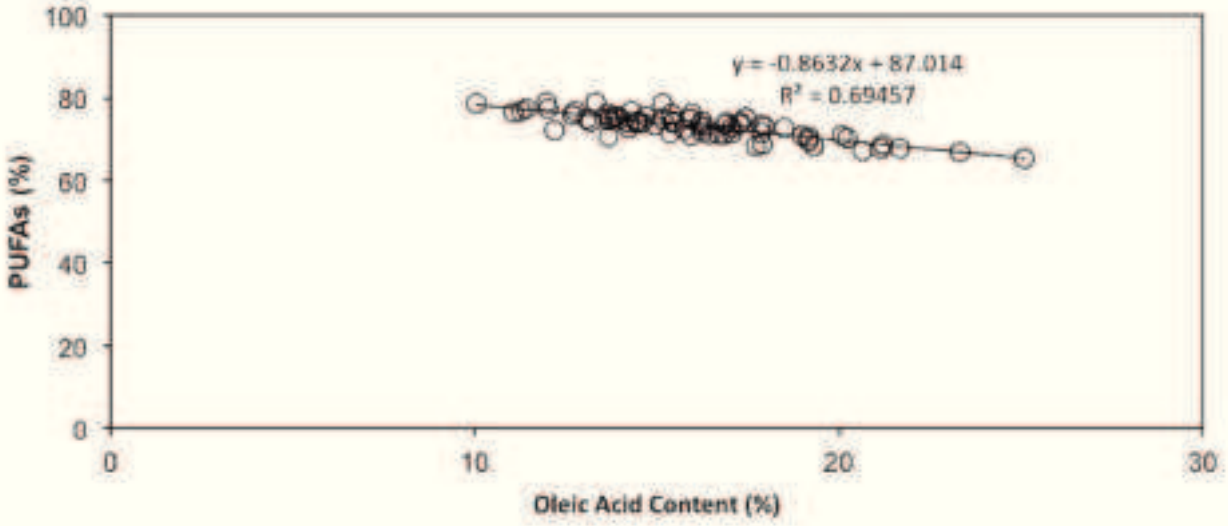
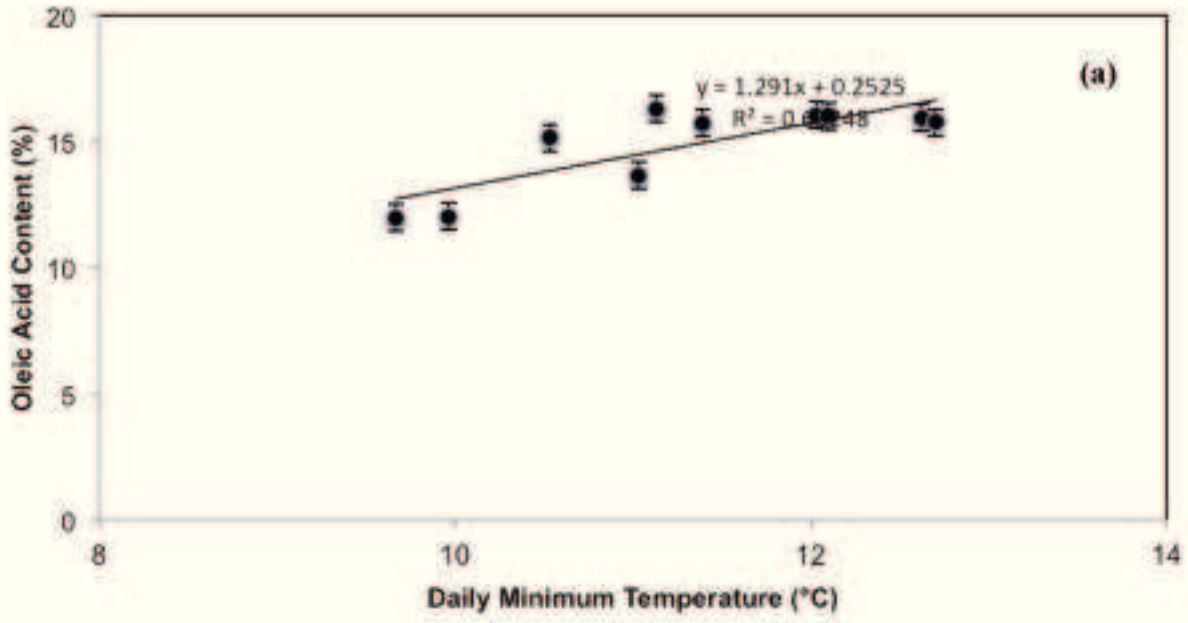


Figure 3. a) Oleic acid concentration as linear function of daily minimum temperature during fruit development in walnut (n=10; p-value < 0.01).

Figure 3. b) Linolenic acid concentration as linear function of daily minimum temperature during fruit development in walnut (n=10; p-value < 0.01).



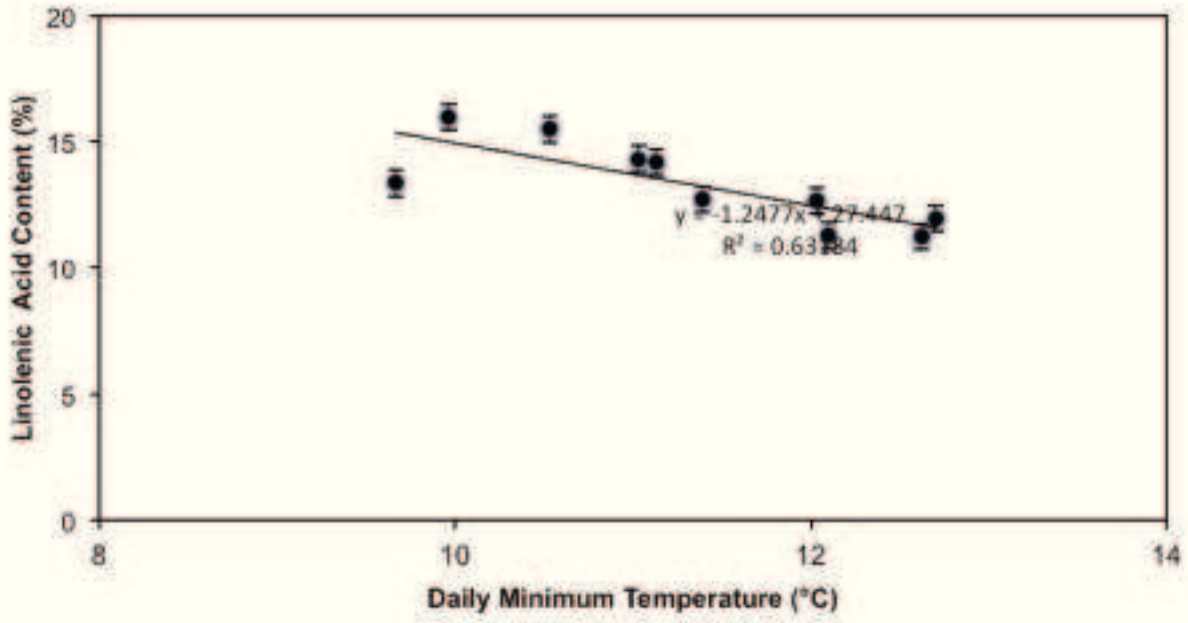
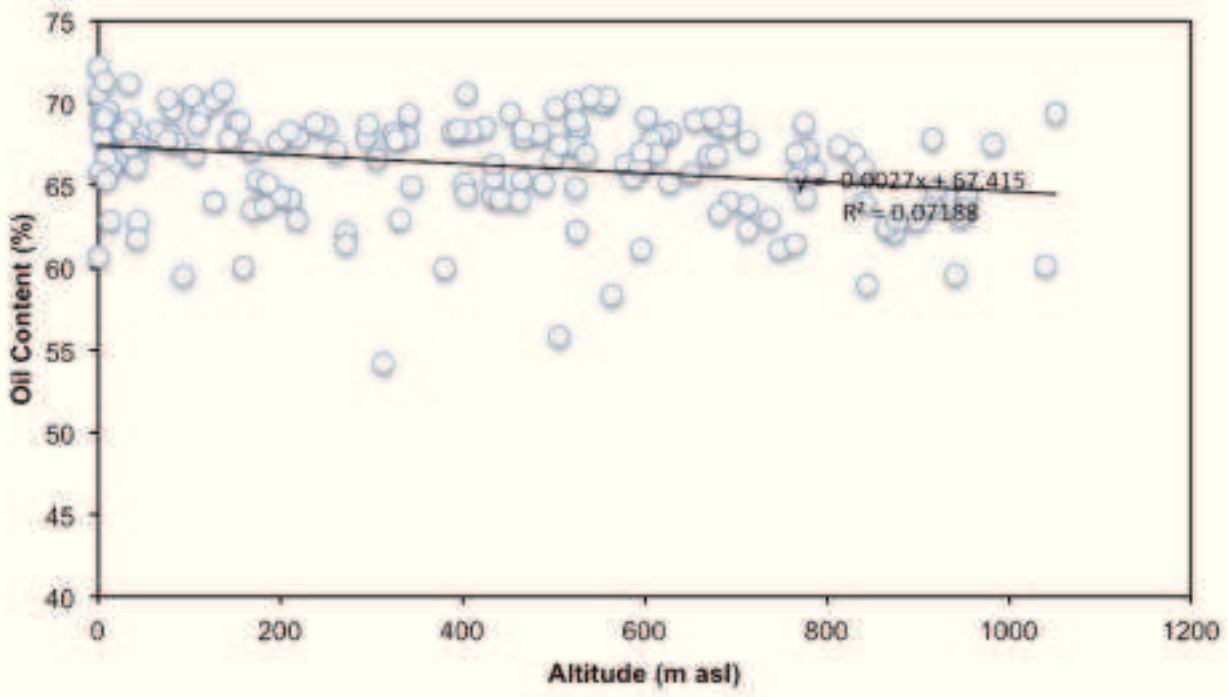


Fig.4 Relationship between oil content and altitude (m a.s.l.) of sampled accessions



Supplementary Materials

Analytic non-destructive techniques are fundamental in breeding activity. Indeed, seeds will be used for obtaining a new plant generation. Total fat content in seed is usually determined by Soxhlet apparatus, which is a destructive and time consuming technique. On the other hand, Nuclear Magnetic Resonance (NMR) is a non-destructive and fast technique. It is usually used in oil crops breeding to detect superior seeds with high oil content. No information was found about the use of NMR in Walnut breeding, so a preliminary comparative trial was carried out comparing NMR and Soxhlet.

Briefly, the walnuts were shelled and then ground in a coffee mill for 30 seconds. Immediately after grinding, the walnuts were extracted for 12 h with Petroleum Ether (b.p. 40-60°C) using a Soxhlet apparatus. Each sample was extracted in triplicate. Whole seeds (shelled; 10 g) were analyzed for oil content by Nuclear Magnetic Resonance (NMR Oxford Instruments 4000). Pure walnut oil was used as standard in NMR calibration. Each sample was extracted in triplicate. Means were calculated for oil content in the three walnut-type and compared using the t-test (independent t-test; two tailed).

Oil content was classified in low, medium and high oil content in the seed. Using Sohxlet methods, total fat was highest than NMR in each of the analyzed walnut type. NMR and Soxhlet method gave a comparable oil content in walnut seeds (t-Test= 2.85, $p>0.05$). NMR is a suitable method to detect oil content in walnut seed.

Table S.1. Comparison between NMR and Soxhlet method. Values are means ± SD.

Sample	NMR	Sohxlet	Δ
Low oil content	62.58 ± 0.03	64.35 ± 0.28	+1.77
Medium oil content	67.99 ± 0.11	68.09 ± 0.66	+0.10
High oil content	72.60 ± 0.01	73.17 ± 0.22	+0.58

DISTRIBUTION AND DAMAGE OF TWO NON-NATIVE INSECT SPECIES (*COPTODISCA LUCIFLUELLA* AND *RHAGOLETIS COMPLETA*) ASSOCIATED TO COMMON WALNUT (*JUGLANS REGIA* L.) IN FRIULI VENEZIA GIULIA (NORTH-EASTERN ITALY)

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ABSTRACT

Coptodisca lucifluella (Lepidoptera, Heliozelidae) and *Rhagoletis completa* (Diptera, Tephritidae) are two walnut insect pests originating from America. Both species are reported for Italy. The first species, a leafminer, is not yet listed for Friuli Venezia Giulia, while the second one, a carpophagous pest, is present in this region for at least 25 years.

During 2015 samples of leaves and fruits were collected from common walnut trees (*Juglans regia*) in 219 sites at different altitude in the Friuli Venezia Giulia region to detect the distribution and the damage of both species. Samples of moth larvae were subjected to DNA analysis. Also observations on other pests and beneficial species were carried out.

In the investigated area *C. lucifluella* has been found in 55 out of the 219 considered sites. This is the first report of the species for the region. The species has been identified not only by typical leaf symptoms, but also by means of mitochondrial DNA barcode. The level of pest infestation in Friuli Venezia Giulia resulted lower than in other Italian areas and its diffusion is negatively correlated with the altitude. The species has not been observed in sites above 600 m a.s.l. The low infestation observed could be associated with the activity of native natural enemies, as parasitoids of the genus *Chrysocharis* (Hymenoptera, Encyrtidae) that were observed into the mines.

R. completa has been found in 89 out of the 185 walnut trees with fruits. In many sites, especially in lowland localities, the infestation level was very high, as to suggest treatments with insecticides. The level of infestation decreased with altitude: at sites above 700 m a.s.l. either there was no infestation or the infestation was very low. No correlation has been found between the average weight of the fruits and the level of infestation.

The presence of other leaf pests, as *Aceria tristriata* (12 sites out 219) and *Aceria erinea* (41 sites out 219) (Acarina, Eriophyidae), has been observed.

Keywords: leafminer, carpofagous sp, altitude, Nord-East Italy, control

1. Introduction

In different Italian regions, several species of insect and mite pests infest the common walnut (*Juglans regia* L.) and in several situations they can cause severe damage and compromise the walnut yield (Pollini, 1998). Among them, carpophagous species as the codling moth *Cydia pomonella* L. (Lepidoptera, Tortricidae), phytomyzous species as the large walnut aphid *Panaphis juglandis* (Goeze) (Homoptera, Aphididae), the small walnut aphid *Chromaphis juglandicola* (Kaltenbach) (Homoptera,

Aphididae) and xylophagous species as the carpenter moth *Cossus cossus* L. and the leopard moth *Zeuzera pyrina* L. (Lepidoptera, Cossidae) can be mentioned.

Several cases of severe decline of walnut in young plantations were also observed. The most evident disease symptoms were dieback and sprouts originating from the stem near the ground level. Walnut tree decline was associated with *Xylosandrus germanus* (Blandford) (Coleoptera, Scolytidae) and fungal pathogens of the genus *Fusarium* (Stergulc et al., 1999).

In addition to native species of insects and mites, the damage to plants due to non-native pests and the consequent need of insecticide treatments to control them should be considered. Among them, the leafminer *Coptodisca lucifluella* (Clemens) (Lepidoptera, Heliozelidae) and the walnut husk fly *Rhagoletis completa* Cresson (Diptera, Tephritidae) are of particular interest.

The leafminer *C. lucifluella* is native of North America and recently has invaded some European countries. This phytophagous species is associated to black walnut (*Juglans nigra* L.) and common walnut.

In September 2010, the characteristic leaf mines of *Coptodisca* sp. on black walnut and common walnut were observed in Italy (Campania and Lazio) (first report for Europe) (Bernardo et al., 2011; Bernardo et al., 2012). The found specimens were rather similar to *Coptodisca juglandella* (Chambers), a species already known as walnuts pest. Later, the taxon has been detected in other Italian regions (Basilicata, Tuscany and Veneto) (Bernardo et al., 2015). On the basis of morphological (forewing pattern) and molecular (cytochrome oxidase c subunit I sequence) evidences, the taxon was recently identified as *C. lucifluella* (Bernardo et al., 2015).

C. lucifluella is a species of potential economic interest. The leafminer completes three to four generations per year and the mature larvae of the last generation start to overwinter in September-October (Bernardo et al., 2011; Bernardo et al., 2015).

The impact of the pest was substantial in the Campania region, with all walnut leaves infested at the end of the last generation (Bernardo et al., 2015).

The walnut husk fly is native to North America (Midwestern US and north-eastern Mexico) and has invaded several European countries in the past decades.

R. completa specimens were observed in Italy (and in Europe) for the first time in the summer of 1991 in the Veneto and Friuli Venezia Giulia regions (Duso, 1991). Then the species has rapidly expanded its range in other regions of northern Italy (Ciampolini & Trematerra, 1992), reaching other neighbouring countries such as Slovenia (Seljak & Žežlina, 1999), Croatia (Bjeliš, 2008), Switzerland (Mani et al., 1994). Currently it is widespread in many European countries (CABI, 2014).

R. completa is a species of high economic interest. The species completes one generation per year and overwinters as pupa in the soil under the canopy of the infested plants (Duso & Dal Lago, 2006). The larvae, feeding within the husk, cause staining of nutshells. Moreover, the husks remain attached to the shells and are difficult to remove. Early infestation of *R. completa* may be the cause of walnut fruits reduced size and kernel shrivelling, further the walnuts may fall before ripening. Already a moderate infestation can significantly reduce the walnut quality .

The aim of this study is to acquire information on the distribution and damage in Friuli Venezia Giulia of two non-native insect species (*C. lucifluella* and *R. completa*) that could affect the vegetative development and fruit production of walnut, taking into account their impact in different areas of the region in which walnut orchards could be developed to give positive perspectives to national and international marketing.

2. Materials and Methods

2.1.Studied Walnut trees

Samples of walnut leaves and fruits were collected from common walnut trees in 219 sites of the Friuli Venezia Giulia region (Fig. 1). The sites are grouped into five geographically homogeneous areas, represented by 1) the eastern Alpine highlands ('Julian Alps'), 2) the western Alpine highlands ('Carnic Alps'), 3) the 'Friuli plains', 4) the 'Torre and Natisone valleys', and 5) the 'Trieste Karst'. In each site leaves and fruits were sampled from the oldest walnut tree in the area (possibly > 40 years old). Each sampled walnut tree was georeferenced and the altitude of the site noted. The studied walnut trees have never been treated with insecticides or fungicides.

2.2. Study of the leafminer species of the genus Coptodisca

2.2.1. Field samplings

The samplings were carried out in August 2014 on walnut trees in the 219 sites. For each tree, 15 compound leaves were randomly collected, inserted into paper bags and transferred to the laboratory.

2.2.2. Laboratory studies

Within one month from leaf collection, the leaves were observed in the laboratory under a stereomicroscope and on each leaf (consisting of several leaflets) the characteristic mines of the leafminer counted.

On the leaves, the presence of galls ("erinea") caused by eriophyid mites, as well as the occurrence of pupae of hymenopteran parasitoids into the mines were noted.

2.2.3. Molecular analysis to establish the identity of the species

During examination of the walnut leaves under the stereomicroscope, some samples of leafminer viable larvae present inside the mines were collected and inserted in a test tube with alcohol to determine if they belong to the non-native species *C. lucifluella*, recently identified (even at molecular level) on walnut in some Italian regions (Bernard et al., 2015). Molecular identification (Tab. 1) was carried out with the Cytochrome c Oxidase Subunit I (COI) Nucleotide Sequence, a widely used mitochondrial DNA barcode suitable for species identification (Hebert et al., 2004)

2.2.4. DNA estraction

DNA was extracted from fresh larvae stored in alcohol using a cetyltrimethyl-ammonium bromide (CTAB)-based protocol as in Doyle and Doyle (1990). The DNA quality was checked on 1% agarose gel and concentration quantified by absorbance measurement at 260 nm using NanoDrop ND-1000 (Thermo Fisher Scientific Inc.) using 1 µl of sample. The PCR reaction was used to amplify the mitochondrial cytochrome oxidase c subunit I (COI) gene using the primers LCO1490 and HCO2198 (Former et al., 1994). The PCR was carried out in 25 µl of a solution containing 10 ng genomic DNA (1 µl), 2.5 µl 10 x Mg-free PCR buffer solution, 1.5 µl MgCl2 25 mM, 2 µl dNTPs 2.5 mM, 1 µl forward primer 10 µM (LCO1490), 1 µl reverse primer 10 µM (HCO2198), 0.125 µl AmpliTaq Gold DNA polymerase (5u/µl; Applied Biosystems) and 15.875 µl dH2O.

Amplification was performed in a GeneAmp 9700 Thermal Cycler (Applied Biosystems) as follows: 8 min. At 94°C followed by 40 cycles of: 1 min at 94°C, 1 min at 49 °C, 1 min at 72°C and a final extension

stage of 5 min at 72°C .

Loci were amplified on the thermocycler GeneAmp® PCR System 9700 (Applied Biosystem). The amplification products were purified and sequenced in both directions using a BigDye Terminator Cycle Sequencing Kit v.2.0 (Applied Biosystems, USA) on a 3730 automatic sequencer (Applied Biosystems, USA).

Electropherograms obtained after sequencing were base-called with Phred (Ewing & Green, 1998). Forward and reverse sequences were edited, trimmed and assembled with the Staden package software (Staden, 1996). The full-length sequences were aligned with Clustal W (Thompson et al., 1994). Finally, eight sequences were suitable for the submission to GenBank (Table 2).

Sequence similarity search was performed using BOLD (Ratnasingham and Herbert 2007) animal identification engine (http://www.boldsystems.org) (Fig. 9).

2.3.Study of the carpophagous walnut husk fly Rhagoletis completa

Field studies were carried out from August 25 to September 5, 2015. On the ground under the canopy of each walnut tree up to 60 fruits with husks were collected. Sometimes (N. 34) no fruits were found. In these cases the sites/trees were not used in the statistical test. When a number of walnut slower than 10 there was examined, the data were not used in the statistical analysis.

In the laboratory, the fruits were singly observed, and the occurrence of symptoms related to the activity of *R. completa* larvae, such as the presence of galleries in the husk and black spots on the shell, were detected and noted.

2.4.Statistical analysis

To investigate the relationships between variables, regression analysis was used. In particular, the linear regression analysis was applied between:

a) the altitude of all walnut trees (N. 219) and the number of *C. lucifluella* mines per 15 leaves;

b) the altitude of the only walnut trees infested by *C. lucifluella* (N. 56) and the number of mines per 15 leaves;

c) the altitude of walnut trees of the "Torre e Natisone valleys" area (N. 25) and the number of *C. lucifluella* mines per 15 leaves;

d) the altitude of all walnut trees with fruits (N. 185) and the percentage of walnut fruits infested by *R. completa*.

To estimate the contribution of two indipendent variables to the constant variable, the Multiple Regression Analysis was applied:

a) the percentage of walnut fruits infested by *R. completa* (constant variable); a1) only the data related to all sites where fruits were present (infested or not infested) (N. 185); a2) only the data related to sites where fruits were infested (N. 89);

b) the altitude of walnut trees with fruit presence (infested or not infested by *R. completa*);

c) the average weight of walnut fruits of different trees; this information was derived from the study of all pomological accessions.

To compare groups, the Kuskal-Wallis test (a non-parametric test) was used because the data did not pass normal test:

a) the number of mines of *C. lucifluella* per 15 leaves of walnut trees (N. 219) included in the five considered geographical areas;

b) the percentage of fruits infested by *R. completa* of walnut trees with examined fruits (N. 153) included in the five considered geographical areas).

When the Kruskal-Wallis test indicated a significant difference between groups, the *post hoc* Dunn's Multiple Comparison Test was used.

3. Results and Discussion

3.1. *The leafminer Coptodisca lucifluella*

3.1.1. Identification of the species

Figure 1 shows the result of Specimen Identification Request to the BOLD system for one sample of COI sequence. All the submitted sequences of 10 larvae, collected in 10 different sites of Friuli plains and Trieste Karst, matched to *Coptodisca lucifluella* with a similarity of 100%. The specimens of *C. lucifluella* (collected in different localities) carried the same two haplotypes s reported by Bernardo et al. (2015) Also the morphology of leaf mines and holes caused by the larvae lead to the same result. Therefore, it is the same non-native leafminer species recently observed in other Italian regions (Bernardo et al., 2015).

3.2.1. Distribution

The species was found in 55 sites (out of 219 sites sampled) of several areas and therefore it resulted widespread in the Friuli Venezia Giulia region (Fig. 2). The spread in the region of *C. lucifluella* resulted to be related to the altitude of all sites where the sampled walnut trees grow. The linear regression between the altitude of the sites and the number of mines per 15 leaves was highly significant ($P < 0.001$) (Fig. 3). In particular, above 400 m a.s.l. the infestation was rare, while above 600 m the infestation was absent. However, it is possible that the species has not colonized the alpine areas yet. The linear regression between altitude of the sites included in "Torre and Natisone valleys" area (where the leafminer species probably has been able to invade all sites) and number of mines per 15 leaves was just below the significance level ($P < 0.0557$) (Fig. 4). Also in this case the regression line indicated absence of infestation above 600 m a.s.l..

3.2.2. Damage to leaves

In the five considered regional areas the infestation of *C. lucifluella* was different (Fig. 5). In particular, in the two Alpine areas ("Carnic Alps" and "Julian Alps") the infestation resulted significantly lower than in the other three areas. The leaf infestations were low (maximum value observed: 88 mines per 15 leaves in a plain site) compared to those observed in Campania (up to 80 mines per leaf; percentage of infested leaves approached 100%) (Bernardo et al., 2011; Bernardo et al., 2012). This could indicate that the colonization of *C. lucifluella* in Friuli Venezia Giulia is recent and/or that some biotic or abiotic factors could control the density of population of the species.

However, the level of foliar infestation observed in this study does not appear to reach harmfulness thresholds. Moreover, during the laboratory study on the leaf mines, in some of them blackish pupae

of leafminer larval parasitoids were observed. Later, adults of hymenopteran Encyrtidae belonging to the genus *Chrysocharis* emerged from the pupae. These wasps were the most frequent parasitoids of *C. lucifluella* detected in Campania and Lazio (Bernardo et al., 2012).

3.2.The walnut husk fly Rhagoletis completa

3.2.1. Distribution

Damaged fruits by walnut husk fly were found in 89 sites (out of 219 ones sampled) of several areas and therefore this fly resulted widespread in the Friuli Venezia Giulia region (Fig. 6). The spread in the region of *R. completa* appeared to be related to the altitude of sites where the walnut trees were sampled. The linear regression between altitude of the sites where walnuts fruits were found and the percentage of walnut fruits infested was highly significant ($P < 0.0001$) (Fig. 7). In particular, up to about 450 m a.s.l. the infestation interested 100% of walnut fruits, while above 700 m the infestation was absent. These data are in agreement with those from a study carried out in Switzerland, where the species was found to be firmly established mainly in low altitude areas (Aluja et al., 2011). In the region the species has been there for more than 25 years (Duso, 1991), being therefore able to expand its distribution area to the most suitable sites. Thus, the absence of the species in the mountain areas above 700 m a.s.l. indicates that at these altitudes the conditions are too adverse. In this regard, it is known that the incidence of *R. completa* in Switzerland is closely related to meteorological mean spring temperature patterns, but not to winter temperatures. In particular, the areas in which the fly is absent correspond to localities where the mean spring temperatures fall below 7°C (Aluja et al., 2011).

3.2.2. Damage to fruits

In the five considered regional areas the infestation of *C. lucifluella* was different (Fig. 8). In general, in the two Alpine areas ("Carnic Alps" and ""Julian Alps") the infestation resulted to be significantly lower than in the "Friuli plains" area, with the other two areas in intermediate positions, even if more similar to "Friuli plains". The walnut fruit infestation was up to 100% in several sites. Only in the areas higher than 700 m a.s.l. the infestation was low or absent. The level of walnut fruit infestation observed in this study appeared to reach harmfulness thresholds and therefore insecticide treatments are necessary to control this pest.

3.2.3. Relationships between the level of infestation, altitude of the sites and fruit size

The first multiple regression analysis applied to three variables (a. the percentage of walnut fruits infested or not – all sites with observed fruits –, b. the altitude of sites/walnut trees and c. the average weight of walnut fruits) revealed that the considered variables had highly significant effects (R^2 = 46.31%; $P < 0.0001$). However, the regression analysis shown that the altitude of sites contributed highly and negatively to the pest infestation ($P < 0.0001$), while the average weight of the fruits resulted not to be significant ($P = 0.8090$).

When considering only the infested sites, the same multiple regression was always highly significant (R^2 = 19.71%; $P < 0.0077$), albeit in this case not only the contribution of altitiude is significant ($P = 0.0163$) but also the contribution of fruit was close to significance ($P = 0.0784$).

The results of the multiple regression are in agreement with those of the linear regressions, that indicate the importance of the altitude factor in the *R. completa* infestation level. Instead, the walnut size (average weight) turned out not to be particularly important, even if it is close to the statistical

significance. This latter occurrence would be in agreement with the results of Guillén et al., (2011), that shown that the larger fruits are more attractive for *R. completa* females as oviposition site.

3.3.*Other observed pests*

During the study of the walnut leaves, on the basis of leaf symptoms (galls), two species of eriophyid mites were detected: *Aceria erinea* (Nalepa) (= *Eriophyes erineus* Nalepa) and *Aceria tristriata* (Nalepa) (= *Eriophyes tristriatus* Nalepa). They are known as "walnut leaf gall mites" or "walnut blister mites".

The first species causes characteristic leaf galls: large yellowish blisters (1-2 cm) on the upper surface of the walnut leaves with corresponding hollows on the underside, which are lined with an erineum of whitish or pale brown hairs. Galls of *A. erinea* were observed in 41 sites.

Instead, *A. tristriata* induces rounded pustules up to 2-3 mm across on the upper leaf surface. The galls usually develop on or close to the veins. Young galls are green, later they turn yellowish and than brown. Galls of *A. tristriata* were observed in 12 sites.

The walnut eriophyid mites are generally not considered as serious walnut pest and their impact is mostly aesthetic. However, they can transmit the bacterium *Xanthomonas juglandis*, agent of walnut blight, which can cause significant yield loss.

4. Concluding remarks

This study shown the wide spread in the Friuli Venezia Giulia region of two non-native species, whose extent appeared to be limited by the altitude where the host plant grew.

C. lucifluella had not been reported yet for in the Friuli Venezia Giulia region. Since the species is widespread in many localities of Friuli Venezia Giulia, probably its presence in other Italian regions is still uninvestigated. The damage caused by the leafminer appears to be slight, indicating an active biological control by natural enemies of the species.

Also *R. completa* is widespread in the region. Unlike the first species, the walnut husk fly appears to be very harmful especially in lowland localities. Therefore, the walnut trees must be protected from this carpophagous species (but also from *Cydia pomonella*) by means of insecticide treatments (Ciampolini & Trematerra, 1992; Duso & Dal Lago, 2006).

In the context of Integrated Pest Management (IPM) strategies, it will be interesting to study the occurrence in Friuli Venezia Giulia of walnut accessions characterized by resistance or tolerance to walnut husk fly. In fact, some walnut cultivars with different susceptibility to this pest are known (Shelton & Anderson, 1990; Guillén et al., 2011). In particular, in some studies the cultivars 'Howard' and especially 'Chandler' appeared to be more tolerant to the walnut husky fly, while the cultivars 'Payne', 'Serr', 'Pedro' and 'Hartley' were instead found to be highly susceptible (Coates, 2004).

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5. References

Aluja M., Guillén L., Rull J., Höhn H., Frey J., Graf B., Samietz J., 2011 – Is the alpine divide becoming more permeable to biological invasions? - Insights on the invasion and establishment of the Walnut Husk Fly, *Rhagoletis completa* (Diptera: Tephritidae) in Switzerland. Bulletin of Entomological Research, 101 (4): 451-465.

Bernardo U., Sasso R., Gebiola M., Viggiani G., 2011 – Minatrice fogliare segnalata in Italia su noce. L'Informatore Agrario, 67 (46): 64-65.

Bernardo U., Sasso R., Gebiola M., Viggiani G., 2012 – First record of a walnut shield bearer *Coptodisca* (Lepidoptera: Heliozelidae) in Europe. Journal of Applied Entomology, 136 (8): 638-640.

Bernardo U., van Nieukerken E.J., Sasso R., Gebiola M., Gualtieri L., Viggiani G., 2015 – Characterization, distribution, biology and impact on Italian walnut orchards of the invasive North-American leafminer *Coptodisca lucifluella* (Lepidoptera: Heliozelidae). Bulletin of Entomological Research, 105 (2): 210-224.

Bjeliš, 2008 – Fruit flies from the family *Rhagoletis* (Tephritidae) in Croatia [Voćne muhe iz roda]. Glasilo Biljne Zaštite, 8 (1): 25-28.

CABI, 2014 – *Rhagoletis completa*. Distribution Maps of Plant Pests 2014, December, Map 337 (2nd revision), CABI, Wallingford (UK).

Ciampolini M., Trematerra P., 1992 – Diffusa presenza di mosca delle noci (*Rhagoletis completa* Cresson) nel nord Italia. L'Informatore Agrario, 48 (48): 52-56.

Coates W.W., 2004 – Walnut husky fly: varietal susceptibility and quality observations. Walnut Research Report, University of California, Berkeley (CA)

Doyle J. J. e Doyle J. L. Isolation of plant DNA from fresh tissue. Focus, 12 (1), 13-15 (1990).

Duso C., 1991 – Sulla comparsa in Italia di un Tefritide Neartico del noce: *Rhagoletis completa* Cresson (Diptera, Tephritidae). Bollettino di Zoologia Agraria e di Bachicoltura, 23 (2): 203-209.

Duso C., Dal Lago G., 2006 – Life cycle, phenology and economic importance of the walnut husk fly *Rhagoletis completa* Cresson (Diptera: Tephritidae) in northern Italy. Annales de la Société Entomologique de France, 42 (2): 245-254.

Ewing B., Hillier L.D., Wendl M.C.,and Green P., 1998-Base-Calling of Automated Sequencer Traces Using Phred. I. Accuracy Assessment. Genome Res. (1998) 8: 175-185

Folmer O., Black M., Hoeh W., Lutz R., and Vrijenhoek R., 1994 – DNA primers for amplification of mi - tochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology (1994) 3(5): 294-299

Guillén, L., Aluja M., Rull J., Höhn H., Schwizer T.;, Samietz J., 2011 – Influence of walnut cultivar on infestation by *Rhagoletis completa*: behavioural and management implications. Entomologia Experimentalis et Applicata, 140 (3): 207-217.

Hebert P.D.N., Penton E.H., Burns, J.M., Janzen D.H., Hallwachs W., 2004 – Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. Proceeding of National Academy of Sciences of the United States of America, 101, 14812-14817.

Mani E., Merz B., Brunetti R., Schaub L., Jermini M., Schwaller F., 1994 – Zum Auftreten der beiden amerikanischen Fruchtfliegenarten. Mitteilungen der Schweizerischen Entomologischen Gesellschaft, 67 (1-2): 177-182.

Pollini A., 1998 – Manuale di Entomologia applicata. Edagricole, Bologna: ix + 1462 pp.

Ratnasingham S. and Herbert P.D.N., 2007- Bold: The Barcode of Life Data System (http://www.barcodinglife.org). Molecular Ecology Resources, (2007) 7 (3): 355–364 Seljak G., Žežlina I., 1999 – Pojav in razširjenost orehove muhe (*Rhagoletis completa* Cresson) in Slovenia. Zbornik predavanj in referatov 4. Slovenskega Posvetovanja o Varstvu Rastlin v Portorožu od 3. do 4. Marca 1999: 231-238.

Shelton M.D., Anderson J.L., 1990 - Walnut cultivars: evidence for differential susceptibility to insect pests. Fruit Varieties Journal, 44 (4): 179-182.

Staden R., 1996- The staden sequence analysis package. Molecular Biotechnology, (1996) 5:233

Stergulc F., Frigimelica G., Zandigiacomo P., Battisti A., 1999 – Gravi deperimenti del noce comune in giovani impianti da legno in Friuli-Venezia Giulia. Sherwood – Foreste ed Alberi Oggi, 5 (4): 27-31.

Thompson J.D., Higgins D.G., Gibson T.J., 1994- CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research, (1994) 22 (22): 4673-4680

Fig. 1 – Distribution in Friuli Venezia Giulia of the sites with the occurrence of one old common walnut tree sampled in the present study.

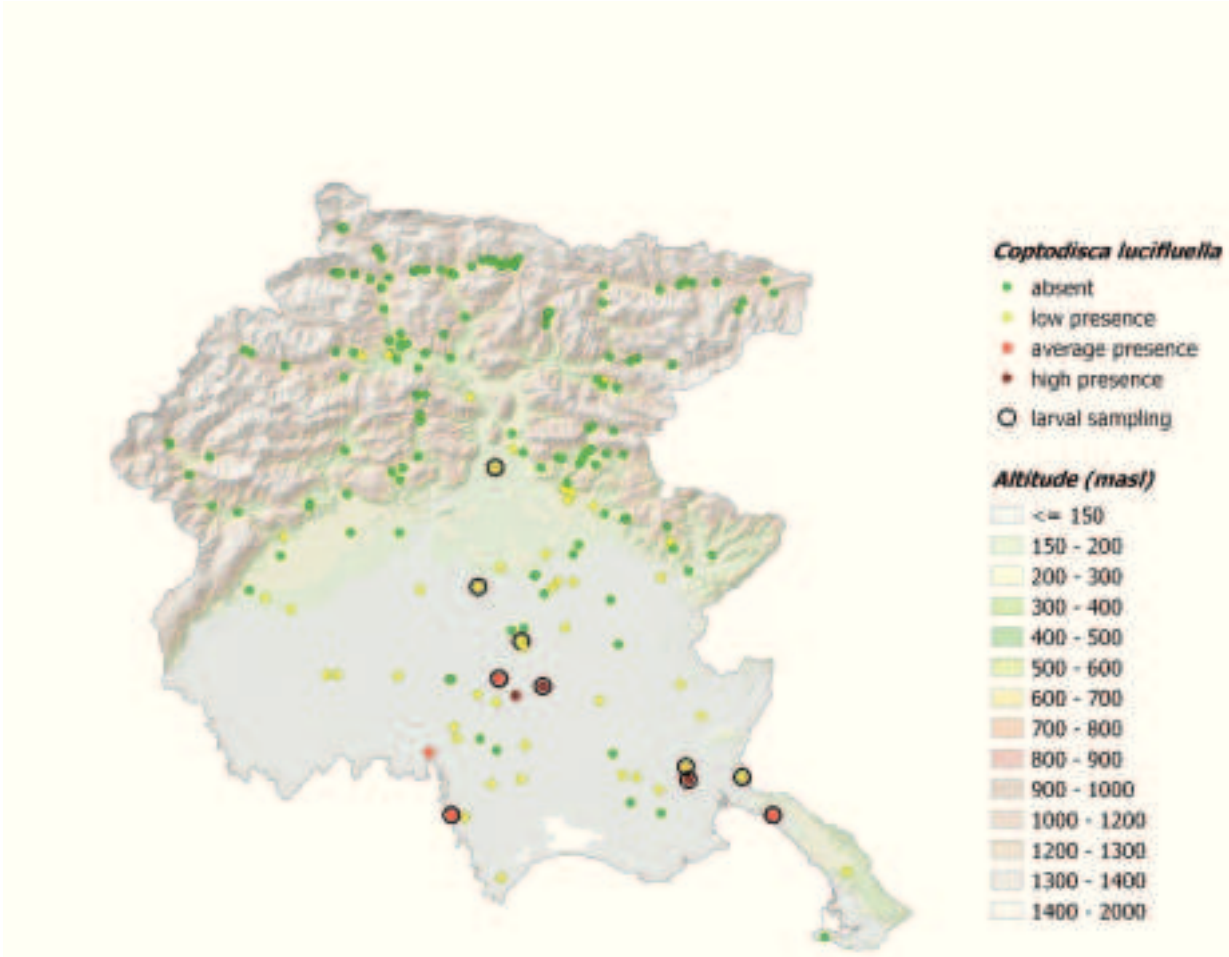

Fig. 2 – Distribution in Friuli Venezia Giulia of common walnut trees with the presence or absence of *Coptodisca lucifluella* leaf infestation. The following markers were used: green circle = absence of leaf infestation; yellow circle = up to 5 mines per 15 leaves; orange circle = 6 to 15 mines per 15 leaves; red circle = more than 16 mines per 15 leaves.

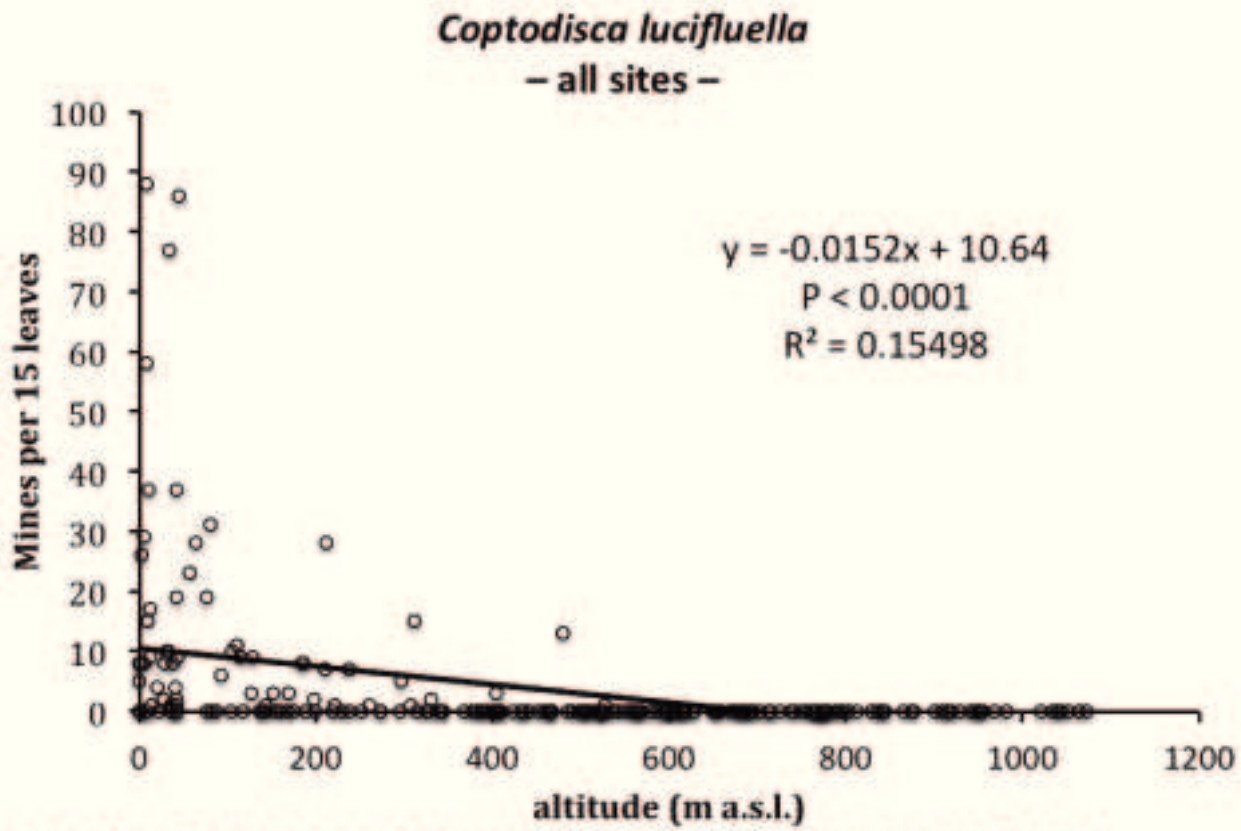

Fig. 3 – Linear regression between the altitude (m a.s.l.) of all sites and the number of mines of *Coptodisca lucifluella* per 15 leaves.

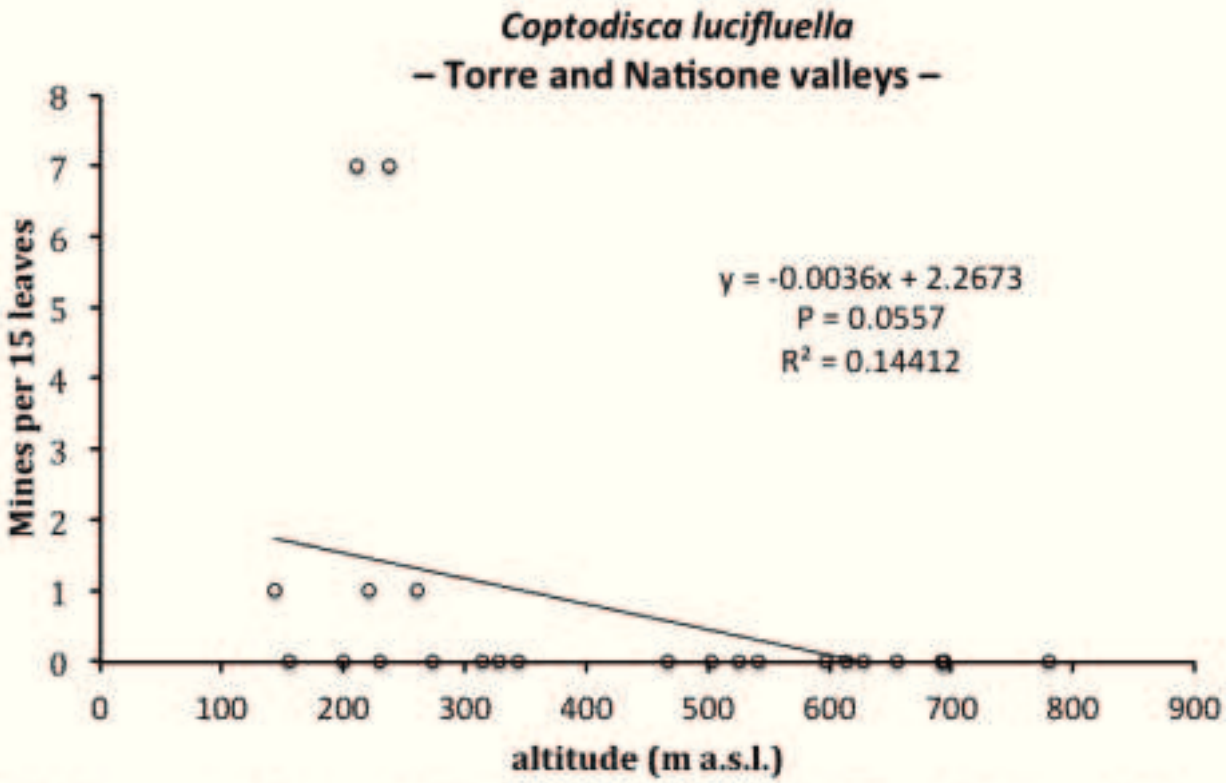

Fig. 4 – Linear regression between the altitude (m a.s.l.) of the sites in the "Torre and Natisone valleys" area and the number of mines of *Coptodisca lucifluella* per 15 leaves.

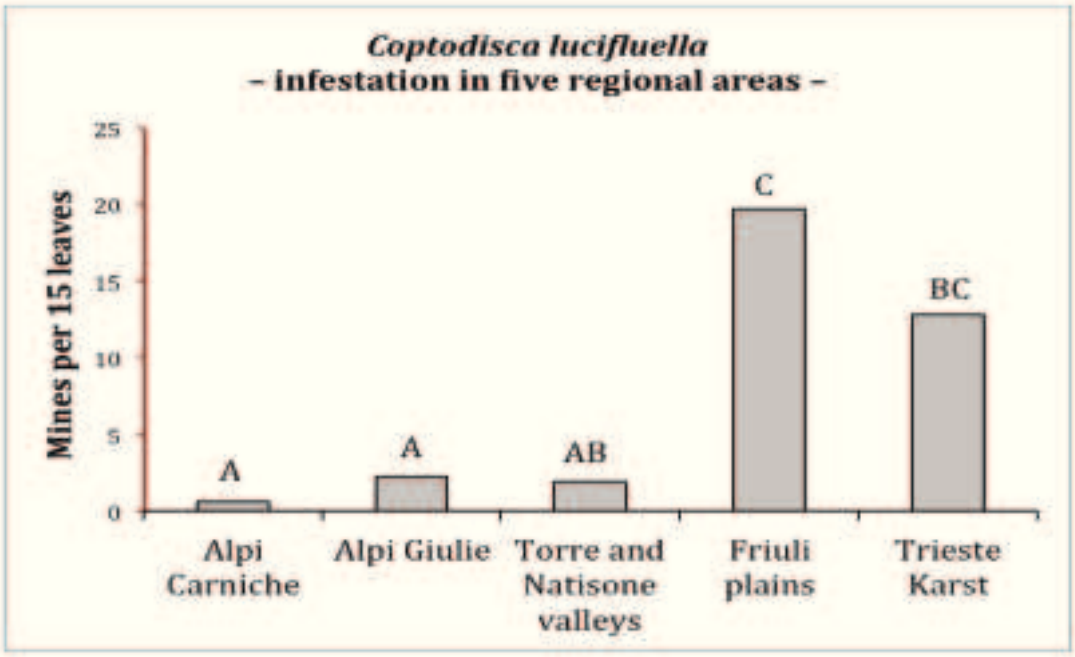

Fig. 5 – Level of infestation of *Coptodisca lucifluella* observed in the five regional areas. Different capital letters among areas indicate significant differences at 0.01 level (Dunn's Multiple Comparison Test).

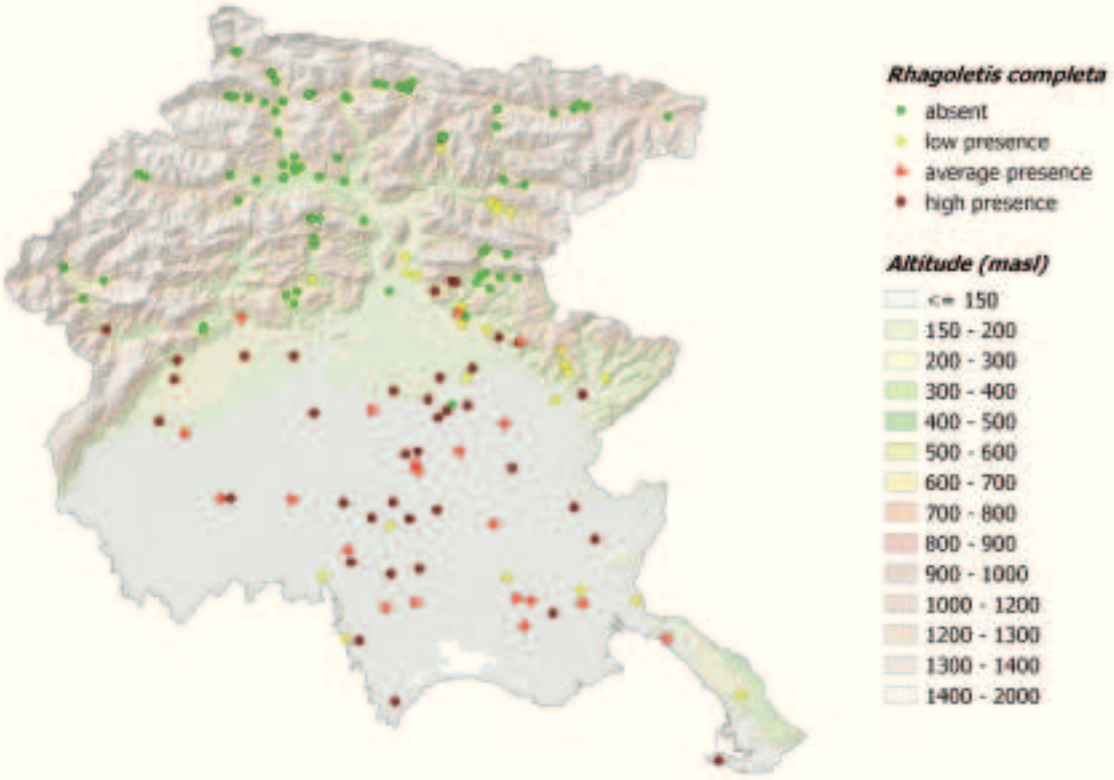

Fig. 6 – Distribution in Friuli Venezia Giulia of common walnut trees with the presence or absence of *Rhagoletis completa* walnut infestation. The following markers were used: white circle: no data (absence of fruits found on the ground under the canopy of walnuts; green circle = absence of walnut infestation; yellow circle = walnut infested up to 20%; orange circle = walnut infested 21 to 50%; red circle = walnut infested more than 51%.

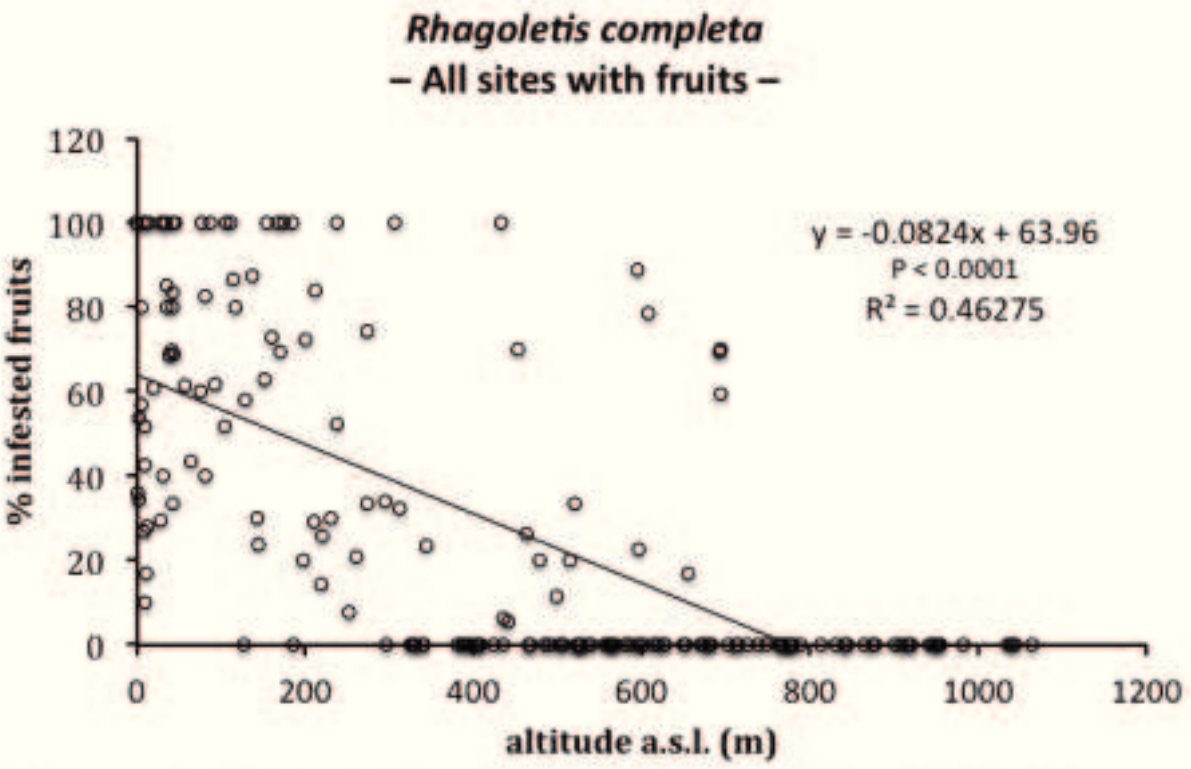

Fig. 7 – Linear regression between the altitude (m a.s.l.) of sites where fruits were found on the ground under the canopy of walnuts and percentage of walnut fruits infested by *Rhagoletis completa*.

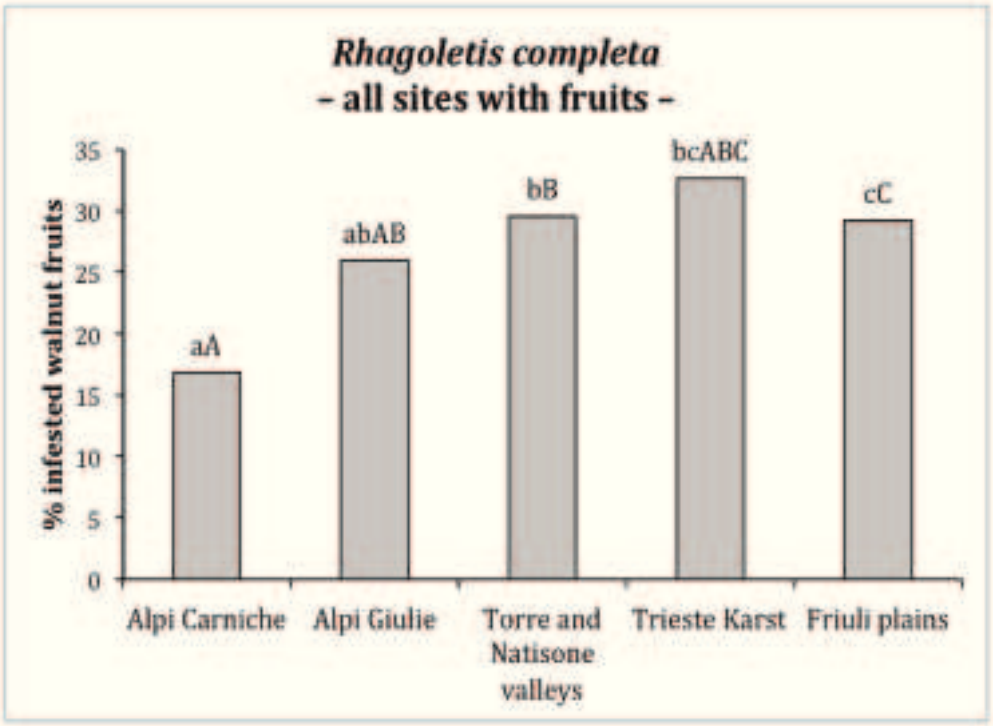

Fig. 8 – Level of infestation of *Rhagoletis completa* observed in the five regional areas. Different small letters and different capital letters among areas indicate respectively significant differences at 0.05 and 0.01 level (Dunn's Multiple Comparison Test).

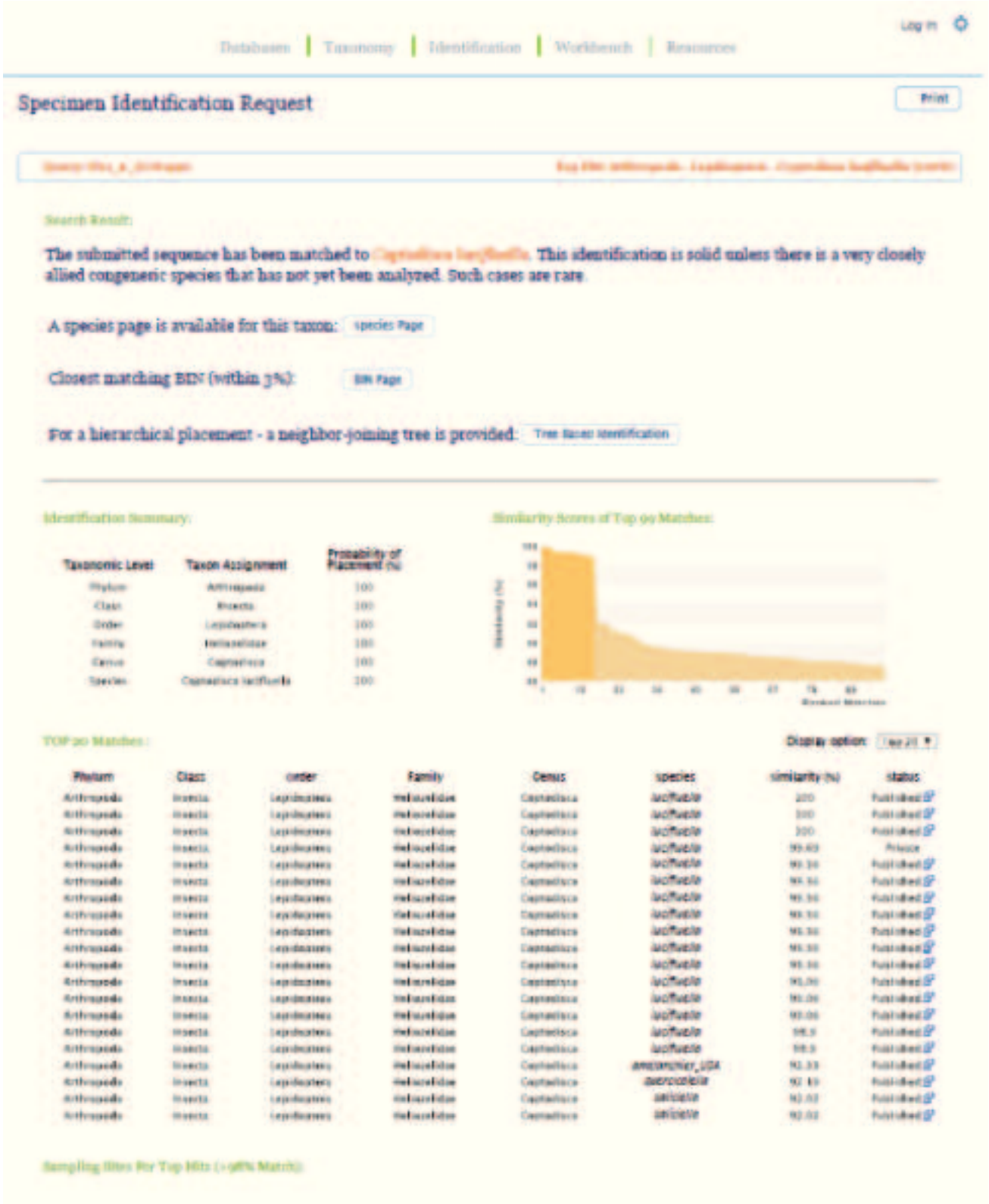

Fig. 9 - BOLD specimen identification request

Table 1 – Number and origin of the Coptodisca lucifluella larvae submitted to the molecular study.

acronym of the accession	regional area	municipality (locality)	altitude (m a.s.l.)	N. of studied larvae
W087	Friuli plains	San Vito di Fagagna (Silvella)	128	1
W114	Friuli plains	Campoformido	64	5
W116	Friuli plains	Bertiolo (Pozzecco)	43	3
W121	Friuli plains	Latisana	9	2
W128	Friuli plains	Mortegliano	45	3
W198	Friuli plains	Osoppo	186	2
W205	Friuli plains	Ronchi dei Legionari	9	1
W206	Friuli plains	San Pier d'Isonzo (San Zanut)	12	1
W075	Trieste Karst	Duino Aurisina (Sistiana)	81	2
W076	Trieste Karst	Doberdò del Lago	43	1

Table 2 – Gen bank codes of the submitted sequences.

Sequence ID	Collected by	Country	Identified by	Isolate	Lat Lon	Specimen voucher	Gen Bank	snp
seq1_LCO	Luca Poggetti	Italy	Pietro Zandigiacomo	W087		W-Di4A-01	KY937892	GTG
seq3_LCO	Luca Poggetti	Italy	Pietro Zandigiacomo	W114		W-Di4A-03	KY937893	GTG
seq6_LCO	Luca Poggetti	Italy	Pietro Zandigiacomo	W116		W-Di4A-06	KY937894	ACA
seq7_LCO	Luca Poggetti	Italy	Pietro Zandigiacomo	W121		W-Di4A-07	KY937895	ACA
seq11_LCO	Luca Poggetti	Italy	Pietro Zandigiacomo	W128		W-Di4A-11	KY937896	ACA
seq13_LCO	Luca Poggetti	Italy	Pietro Zandigiacomo	W198		W-Di4A-13	KY937897	ACA
seq17_LCO	Luca Poggetti	Italy	Pietro Zandigiacomo	W205		W-Di4A-17	KY937898	GTG
seq15_HCO	Luca Poggetti	Italy	Pietro Zandigiacomo	W076		W-Di4A-15	KY937899	ACA

7. Conclusion and outlook

The present work is the cornerstone of all the activities carried out within the "Progetto noce FVG" (www.progettonocifvg.com). The project has two main goals. On one side it aims to conduct both a census and a characterization of the local walnut germplasm, on the other side the purpose is to increase the cultivation of walnut in the region and to advertise nuts both as fresh product and as characteristic ingredient for traditional food.

The goal of characterizing the walnut population in Friuli-Venezia Giulia has been reached. The results of the study can be considered representative of the population since the investigation was extended to a relevant number of old trees and was carried out all over the regional area where the species is spread. Moreover, the seedling origin of these trees was verified and grafted plants or plants belonging to improved cultivars were never found. Historical data regarding how walnuts and their fruits were employed are very interesting starting points for further investigations related to different fields of expertise.

The first work demonstrated that the genetic variability within the species is moderate. Besides the analysis already provided in the conclusion section of chapter 1, further considerations can be made. In the investigated areas most of the plants that were suitable for getting wood were cut down after the second postwar period. This happened since the main cultivation areas, consisting of marginal zones, were getting depopulated and therefore the species was losing its importance as food source. Another reason was the high request from the wood industry and the notable surplus value of walnut wood. In the plains the species, together with the other arboreal plants, was substituted with cereal cultivations. The nowadays walnut population has thus developed from a limited number of genotypes, indirectly selected for their productive attitude. From the results it is clear that the human influence had a huge impact on the population dynamics. Moreover, the nuts commercialization from the mountain areas to the plains caused a spread of the genetic material from the regions where the species was cultivated to those where the occurrence of plants was very low or completely absent. The human influence was shown also for areas in which the present walnut trees were believed to be local (Beer *et al.* 2008). The investigations performed directly on the territory confirmed what shown in two main points of the genetic analysis. First of all, the local population was not influenced by improved varieties. Secondly, an isolated population, different than the local one, was found that comes from imported nuts (in the particular case from Russia, as came up interviewing the farmer).

The goal of the second work was to identify genotypes with promising traits concerning cultivation and breeding. Also in this case the aim has been achieved, iden-

tifying interesting material both for antracnose tolerance and nuts traits. Regarding the latter aspect, the results revealed that the larger nuts show a kernel ratio that is lower if compared with literature data (Cosmulescu and Botu 2012, Khadivi et al 2015 and Rouskas and Zakynthinos 2001). Despite that, the local genotypes are anyway of interest if compared with improved varieties. In the future the best genotypes should be propagated and kept in germplasm field collections. Furthermore, an agronomic comparison in homogeneous conditions of cultivation should be performed. Setting up germplasm field collections will be necessary to be able to carry out phenological observations, not practicable now due to the diversity of the environments were the trees are located.

The purpose of the third work has been reached, too. Genotypes were identified that show higher oil yield than the improved cultivars. What is important in this case is that genotypes with very different acidic spectra were found. This leads to suppose that it will be possible to select varieties with different acidic profile depending on the oil purpose. A particularly interesting outcome is the identification of some genotypes for which the ratio of the unsaturated fatty acids contained in the oil is influenced by an environmental factor (see chapters 2 and 3).

Also the goal of the fourth work has been reached. The investigation involved two allochthonous pests. Regarding the leafminer belonging to the genus *Coptodisca* the species and the actual diffusion area were identified. Of interest are the hypothesis that would explain the lower level of diffusion and infestation with respect to other italian areas (Bernardo et al. 2015) (see conclusion section of chapter 4). The investigation set the basis for future monitoring of the species diffusion, to be able to understand whether it is expanding or its diffusion is limited by some factors, like for instance the identified parassitoid. Regarding *Rhagoletis completa*, its occurrence has been established for more than 20 years, leaving no doubts about its capability of settlement. It might be interesting to suggest the walnut cultivation in the areas the insect has not reached yet, in order to avoid the employment of chemicals.

To sum up, the investigation carried out demonstrated the occurrence of an important phenotypic variability within the Friuli Venezia Giulia Walnut local population and set the basis for its conservation and use in the future. This is particularly important also considering that currently the walnut cultivation is experiencing a notable expansion in the North East area of Italy.

References

Beer R., Kaiser F., Schmidt K., Ammann B., Carraro G., Grisa E., Tinner W., 2008. Vegetation history of the walnut forests in Kyrgyzstan (Central Asia): natural or anthropogenic origin? *Quaternary Science Reviews*, 27: 621-632.

Bernardo U., van Nieukerken E.J., Sasso R., Gebiola M., Gualtieri L., Viggiani G., 2015. Characterization, distribution, biology and impact on Italian walnut orchards of the invasive North-American leafminer *Coptodisca lucifluella* (Lepidoptera: Heliozeli-

dae). *Bulletin of Entomological Research*, 105 (2): 210-224.
Cosmulescu, M. Botu 2012. Walnut biodiversity in south-western Romania-resource for perspective cultivars. *Pakistan Journal of Botany*, 44(1): 307-311.
Khadivi-Khub A, Aziz Ebrahimi, Fatemeh Sheibani, Alireza Esmaeili, 2015. Phenological and pomological characterization of Persian walnut to select promising trees. *Euphytica*, 205: 557–567S.
Rouskas, D. and G. Zakynthinos. 2001. Preliminary evaluation of seventy walnut (*Juglans regia* L.) seedlings selections in Greece. *Acta Hort.* (ISHS), 544: 61-72.