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RESEARCH ARTICLE

# The major Italian landraces of lentil (*Lens culinaris* Medik.): Their molecular diversity and possible origin

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**Abstract** The eleven most known landraces from central and southern Italy were analysed using ISSR markers on 15 randomly chosen individuals for each landrace, with the aim of assessing genetic variation within and among landraces and possibly ascertaining their origin and genetic relationships. A total of 164 loci were observed, 128 of which (78.05%) were polymorphic. Gene diversity over all landraces was $I = 0.3759$. The highest within-landrace diversity was observed in samples from the Apennine ridge and for one Sicilian landrace; on the other hand, samples from the small Sicily islands were less variable. Principal Component Analysis and AMOVA allowed the discrimination of groups of landraces with higher similarity. Analyses indicate that the small Sicily islands landraces are very closely related to one another and seem to be derived from the peninsular material; moreover, they help disclose relationships among geographically close landraces. The knowledge so acquired can, therefore, contribute to elaborate strategies for increasing the economical value of élite landraces and to protect producers from frauds.

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## Introduction

Lentil (*Lens culinaris* Medik.), traditionally cultivated in the Mediterranean Basin and in Asia, is one of the oldest crop species known to man. Its importance in the past is testified by the numerous quotations to this legume in the Bible and classic literature; it was a staple food for the Romans as reported by several Latin authors, and its importance was highly relevant through the whole Middle Ages (Newman 2001). Two main types of lentils can be distinguished: the large-seeded one, also called ''macrosperma'', and the small-seeded types, the ''microsperma'' (Barulina 1930).

Until the beginning of the 20th century, Italy was a major producer of lentil, but nowadays the cultivation of this crop has dramatically decreased (ISTAT 2000). A small scale production is still remnant in rural areas of Central, Southern Italy and some small islands, mostly devoted to traditional farming systems (Hammer et al. 1992, 1999; Piergiovanni 2000). Similar to other Old World countries (Erskine 1997; Làzaro et al. 2001), Italy's lentil cultivation is mostly based on landraces, local varieties empirically selected by farmers over time and well adapted to the

agro-environment in which they have been grown for decades. Most landraces survive *on farm*, in marginal areas and exposed to a strong risk of genetic erosion and/or extinction (Negri 2003).

The best known Italian lentil landrace, ''lenticchia di Castelluccio di Norcia'' (''Castelluccio'', from now on), cultivated in the Umbria region (Central Italy), has a good market position and potential since it has been appointed the Protected Geographic Indication (PGI) EU protection mark; presently, it is cultivated over some 250 hectares in mountain valleys at 1500 m asl, and has a yearly production of some 200 tons (http://www.cm-novafeltria.ps.it/prodotti/lenticchia.html). The EU protection mark ensures the farmers a higher income.

Over the last 30 years, teams of the Institute of Plant Genetics (IGV) have collected lentil landraces all over Italy and recorded the cultivation conditions in the collection areas. Most landraces are grow over small, marginal areas, generally for self- or local consumption. Their maintenance is often in the care of older farmers and are therefore highly endangered of extinction (Hammer et al. 1999; Negri et al. 2000). Some others are appreciated as niche products, since they are prevailingly cultivated under low input agricultural conditions thus increasing their market potential consequently reducing the threat of extinction. Nevertheless, the survival of this germplasm relies on the possibility of developing convenient support protocols that include their precise identification in order to prevent frauds. Morphological traits are not sufficient to uniquely identify each lentil landrace, or to describe the level of variation within each of them, therefore the use of molecular markers appears as a necessity (Sonnante and Pignone 2001). Inter simple sequence repeat (ISSR) markers have been used in the recent years to evaluate phylogenetic relationships, the level of genetic diversity at inter- and intra-specific levels, and to distinguish among varieties (Prevost and Wilkinson 1999; Iruela et al. 2002; Raina et al. 2001).

In the present study 11 major Italian lentil landraces, which have good perspectives of increasing their market value, were considered. The goal of this investigation was to identify the possible origin of the landraces, to assess their level of genetic diversity, and possibly to identify specific markers to be used in the certification of these landraces and their protection from frauds.

## Materials and methods

Eleven Italian landraces (Fig. 1) were used in the present study, two of which (Altamura and Villalba) belonging to the ''macrosperma'' morphotype, with large and flat seeds, the remaining being ''microsperma''. Samples were collected directly by teams of the IGV or obtained by exchange from local communities. For each landrace, 15 randomly chosen individuals were analysed. Seeds were grown in a growth chamber, young leaves were collected from plantlets and genomic DNA extracted according to the protocol of the ''GenElute Plant Genomic DNA Miniprep Kit'' (Sigma, USA). After assessing DNA concentration by agarose gel electrophoresis, amplification was performed with 6 ISSR primers based on a 'core' sequence of microsatellites, with 2 or 3 selective nucleotides: $(AC)_8YA$, $BDB(CA)_7$, $(AG)_8YC$, $(GT)_8YC$, $(GA)_8YT$, $(CA)_8RY$. The amplification mixture was as follows: 18 μl of reaction volume contained 20 ng DNA, 50 mM KCl, 10 mM Tris–HCl pH 9.0, 1.5 mM $MgCl_2$, 0.1 mM for each nucleotide (dATP, dCTP, dGTP, dTTP), 0.2 μM primer and 0.8 unit of Taq DNA polymerase. The amplification programme included the following steps: one cycle at 94°C for 2′, followed by 35 cycles at 94°C for 45″, 56°C for 45″, 72°C for 90″, and a final cycle at 72°C for 5′.

Amplification products were separated on a pre-cast polyacrylamide gel (Pharmacia, SE) and stained with silver staining. Gels were dried and scanned using a Pharmacia Labscan system. ISSR bands were scored as present (1) or absent (0). Any weak or smeared bands were excluded. The resulting binary data matrix was used to calculate genetic diversity parameters using the POPGENE 1.31 software (Yeh et al. 1997): observed number of alleles ($n_a$), percentage of polymorphic loci (P%), and Shannon's information index ($I$). Nei's (1972) genetic identities and distances were calculated among landraces and the resulting matrix used to draw a UPGMA dendrogram.


**Fig. 1** The eleven Italian landraces of lentil analysed and their geographical origin

The ISSR band matrix was also used to calculate genetic similarity based on Jaccard similarity index (JS, Jaccard 1908) using NTSYS 2.0 (Rohlf 2000); the resulting matrix was subjected to Principal Coordinate Analysis (PCO). The first three Principal Coordinates were used to produce a three-dimension scatterplot in order to visualize the similarity of the analysed genotypes.

Finally the robustness of the groups formed on the basis of PCO analysis was estimated by analysing the partition of molecular variation among groups and populations using the Analysis of Molecular Variance (AMOVA) implemented in the Arlequin 3.0b package (Excoffier et al. 2005).

## Results

Only clear bands were considered as true loci. The high reproducibility of ISSR patterns was established in a preliminary test conducted on 10 samples not included in the present analysis (Fig. 2). The utilised 6 primers produced a total of 164 loci, 128 of which were polymorphic; the proportion of polymorphic loci was, therefore, P% = 78.05 (Table 1). The size of ISSR fragments varied from 150 to 900 bp. Not all loci were present in all landraces; moreover, some landraces exhibited unique loci not present in the others. For instance, Villalba lacked the fragment identified as $(AC)_8YA\_9$, which was detected, at variable frequency, in all the other landraces, whereas it showed the unique presence of the band $(AG)_8YC\_15$.

The summary of genetic diversity parameters for the landraces analysed is reported in Table 1. The number of alleles at the 164 loci over all landraces was determined in $n_a = 1.7805$ per locus, and Shannon information index $I = 0.3759$. These parameters were also calculated within

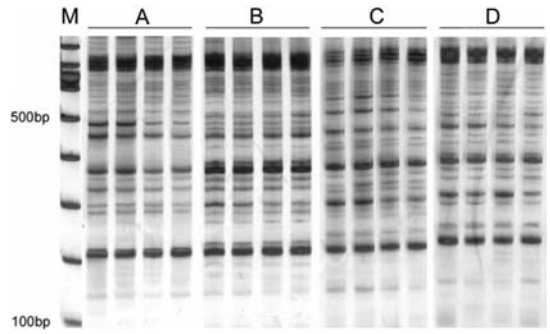


**Fig. 2** Composite plate showing ISSR reproducibility. Each group of lanes (A...D) represents DNA from the same individual amplified with the primer $(GA)_8YT$ four times independently

each landrace separately. The gene diversity parameter *I*, although lower in each landrace, showed some level of variation: the highest values were observed in the landraces Colfiorito, Villalba and S. Stefano (*I* = 0.1346, 0.1090, 0.1029), while the lowest values were detected in Linosa, Ventotene, and Pantelleria landraces (*I* = 0.0252, 0.0456, 0.0514).

The pairwise genetic identity between landraces (Table 2) was quite variable, ranging from 0.645 (Villalba–Castelluccio) to 0.975 (Linosa–Pantelleria). The landraces from the small Sicily islands (Ustica, Linosa and Pantelleria) showed a high degree of genetic identity, with relative identities all above 0.926; the Onano and Colfiorito landraces also showed a high level of genetic identity (0.962). Nei's pairwise genetic distances were used to construct a UPGMA dendrogram (Fig. 3). Three main branches were identified, one of which includes only the Villalba landrace. Of the remaining two, one groups the Castelluccio, S. Stefano, Colfiorito, and Onano landraces, and the other is further subdivided into two subbranches: Capracotta and Altamura on one side, and the landraces from small Italian islands on the other. Association between genetic identity and geographical position of the landraces was not always possible. As a matter of example, the landraces Castelluccio and Colfiorito are grown in very close areas but their genetic identity value is 0.767, while the landraces Colfiorito and Onano, separated by a distance some ten-fold higher than Castelluccio and Colfiorito show a genetic identity value of 0.962. At the same time landraces from small Sicily islands (Ustica, Linosa and Pantelleria) display high levels of genetic identity, and altogether show a certain degree of similarity to the Ventotene landrace; in this frame, the landraces from these islands share a quite high level of identity (Table 2).

The PCO analysis considered the differences in band constitution of the single individual analysed in each landrace. Each band is treated as a variable and, based on the degree of their correlation, all variables are condensed into a lower number of independent variables called principal coordinates (PCs). The first three PCs in our study account for 53.3% of the whole variation observed in the dataset and the fourth explains an additional 10%. Principal coordinates can be used to device clusters where the individual differences are at a minimum; moreover they can be plotted in a graph that visualizes the relationships. We considered to draw a scatterplot for the first three PCs (Fig. 4), using, for the sake of simplicity, only the

**Table 1** Genetic diversity indices calculated for each landrace and overall landraces

| Landrace | $n_a$ | *I* | P% |
|---|---|---|---|
| Capracotta | 1.1707 | 0.0910 | 17.07 |
| Pantelleria | 1.1098 | 0.0514 | 10.98 |
| Linosa | 1.0732 | 0.0252 | 7.32 |
| Ustica | 1.1341 | 0.0614 | 13.41 |
| Castelluccio | 1.1585 | 0.0827 | 15.85 |
| Ventotene | 1.0976 | 0.0456 | 9.76 |
| S.Stefano | 1.2073 | 0.1029 | 20.73 |
| Altamura | 1.1220 | 0.0533 | 12.20 |
| Villalba | 1.1951 | 0.1090 | 19.51 |
| Colfiorito | 1.2561 | 0.1346 | 25.61 |
| Onano | 1.1585 | 0.0769 | 15.85 |
| Overall | 1.7805 | 0.3759 | 78.05 |

$n_a$ = Observed number of alleles; *I* = Shannon Information index; P% = percentage of polymorphic loci.

**Table 2** Pairwise comparison of Nei's genetic identity (above diagonal) and distance (below diagonal) between major Italian landraces of lentil

| Landrace | Caprac. | Pantel. | Linosa | Ustica | Castell. | Ventot. | S. Stef. | Altam. | Villalba | Colfior. | Onano |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Capracotta | – | 0.824 | 0.829 | 0.840 | 0.844 | 0.834 | 0.813 | 0.826 | 0.665 | 0.805 | 0.779 |
| Pantelleria | 0.194 | – | 0.975 | 0.930 | 0.778 | 0.838 | 0.753 | 0.715 | 0.680 | 0.727 | 0.666 |
| Linosa | 0.187 | 0.026 | – | 0.926 | 0.781 | 0.847 | 0.755 | 0.734 | 0.668 | 0.729 | 0.672 |
| Ustica | 0.174 | 0.072 | 0.077 | – | 0.814 | 0.827 | 0.771 | 0.764 | 0.691 | 0.728 | 0.687 |
| Castelluccio | 0.170 | 0.252 | 0.247 | 0.206 | – | 0.774 | 0.823 | 0.719 | 0.645 | 0.767 | 0.699 |
| Ventotene | 0.182 | 0.177 | 0.167 | 0.190 | 0.256 | – | 0.742 | 0.769 | 0.666 | 0.698 | 0.658 |
| S. Stefano | 0.208 | 0.284 | 0.282 | 0.260 | 0.195 | 0.298 | – | 0.789 | 0.768 | 0.869 | 0.822 |
| Altamura | 0.191 | 0.336 | 0.310 | 0.269 | 0.330 | 0.263 | 0.237 | – | 0.698 | 0.822 | 0.813 |
| Villalba | 0.408 | 0.385 | 0.404 | 0.369 | 0.439 | 0.407 | 0.265 | 0.359 | – | 0.741 | 0.703 |
| Colfiorito | 0.217 | 0.319 | 0.316 | 0.318 | 0.265 | 0.360 | 0.141 | 0.196 | 0.300 | – | 0.962 |
| Onano | 0.250 | 0.406 | 0.397 | 0.376 | 0.358 | 0.419 | 0.196 | 0.207 | 0.353 | 0.039 | – |

diverse genotypes within each landrace. In this plot, based on PCO analysis, individuals belonging to the same landrace form definite groups, with the exception of Colfiorito–Onano on one side and Linosa–Pantelleria on the other, which overlap; the Villalba landrace occupies a well distinct spot, and the remaining landraces are placed in definite positions, although revealing different levels of relatedness with one another. For instance, the landraces from small Italian islands (Linosa, Pantelleria, Ustica, and Ventotene) seem rather close to each other, thus supporting the inference based on Nei's genetic distances. Altogether, three or five main groups of landraces can be hypothesised, respectively:

(a) Villalba; Linosa–Ustica–Pantelleria–Ventotene; Apennine landraces, or
(b) Villaba; Linosa–Ustica–Pantelleria–Ventotene; Colfiorito–Onano; Castelluccio–S.Stefano; Capracotta–Altamura.

In order to estimate the strength of grouping, different landrace combinations within a varying number of groups were evaluated using

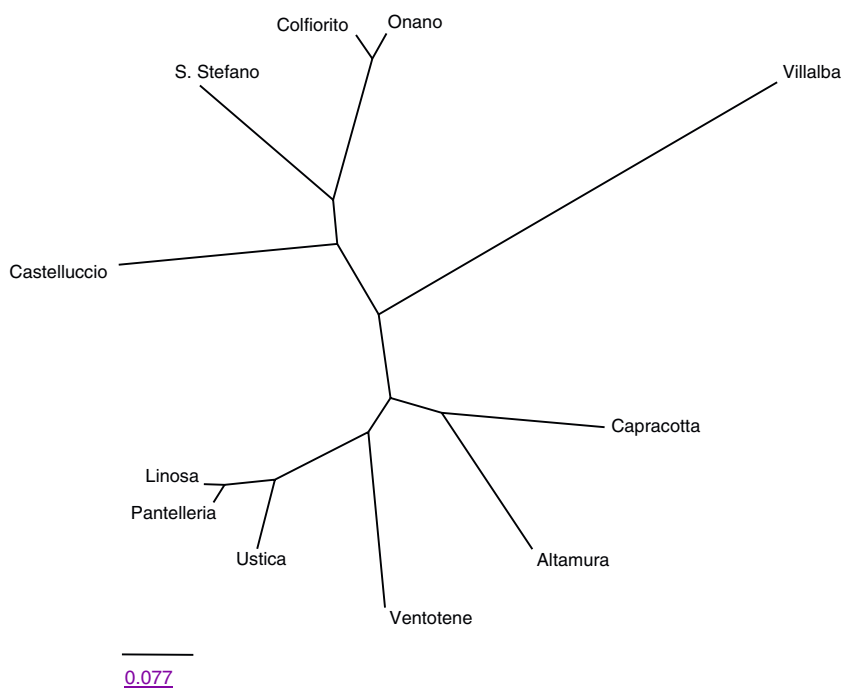


**Fig. 3** UPGMA tree based on Nei's genetic distances

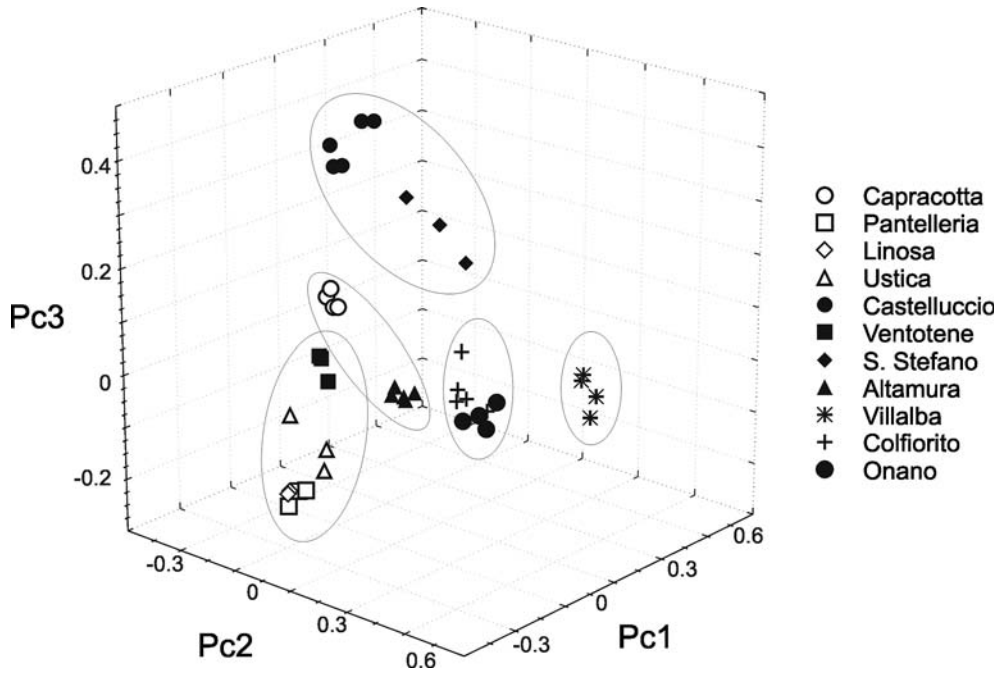


**Fig. 4** Principal Coordinate Analysis scatterplot

AMOVA. The molecular variance within all landraces accounted only for some 15% of the total variation. The groups tested were the two above reported ones, plus (c) all Apennine landraces; all islands landraces.

The grouping that reduced to a minimum the component of variance within population of the same group was (b). All the other combinations tested, plus other not shown in the Table 3, showed a lower level of group homogeneity.

## Discussion

Landraces are a particularly good system to study the diffusion and domestication of a crop plant, since they are the result of a non scientific selection operated by farmers over the centuries, which has reduced but not eliminated the genetic variation within the landrace itself (Beebe et al. 2001). Within this frame, the whole selection process of existing landraces reflects both natural causes and preferences in the uses to be made of the crop; it can be assimilated to natural evolution when considering that the evolutionary forces in action, guided by human selection as opposed to natural selection, mostly act only on few loci (Doebley et al. 1997; Grandillo et al. 1999). This phenomenon is generally known as the ''domestication syndrome'' (Harlan 1992).

For these reasons, morphological divergence is not always parallel to genetic divergence as measured on the basis of markers that are more copious and presumably have a wide genome distribution. Bejiga et al. (1996) reported a high morphological differentiation within Ethiopian lentil germplasm, mostly at the level of 100-seed weight and time to flower, few quantitative traits strongly affected by human preference, with a low adaptive value. Despite an appreciable level of morphological variation, DNA markers revealed quite a low level of genetic variation in cultivated lentil (Havey and Muehlbauer 1989; Abo-elwafa et al. 1995; Sharma et al. 1995, 1996; Sonnante and Pignone 2001), but, in turn, this variation is representative of larger proportions of the genome. As a consequence, morphological characters are not always sufficient to reveal the variation present in this crop species or to uncover the dissemination pathway throughout its cultivation area.

In this study, ISSR primers generated a high number of alleles in lentil, the most of which were

**Table 3** AMOVA analysis of different population groupings

| Grouping | Source of variation | Variance % |
|---|---|---|
| a | Among landraces | 37.24 |
| | Among landraces within groups | 49.31 |
| | Within groups | 13.45 |
| b | Among landraces | 49.14 |
| | Among landraces within groups | 36.42 |
| | Within groups | 14.44 |
| c | Among landraces | 24.67 |
| | Among landraces within groups | 61.40 |
| | Within groups | 13.93 |
| | Among all landraces | 85.31 |
| | Within all landraces | 14.69 |

polymorphic, as previously suggested (Sonnante and Pignone 2001). The analyses, conducted on the 11 landraces considered, demonstrate that 85% of the genetic differentiation is among landraces, while 15% resides within them. This is not unexpected due to the strict inbreeding behaviour of lentil. In a previous study based on 105 lentil germplasm accessions using isozyme loci as markers, an overall outcrossing rate of 3.7%, ranging from 2.2% to 6.6% was apprised, an estimate higher than previous reports (Erskine and Muehlbauer 1991). It is worth noting that not all landraces show a similar distribution of the genetic variation; in fact, some of them reveal a high degree of variation, as testified by higher values of the gene diversity parameter *I* associated to a higher proportion of polymorphic loci, while others exhibit lower levels for both these parameters. The highest levels of genetic diversity are observed in some landraces from the Apennine ridge (Colfiorito, S. Stefano, and Capracotta) and mainland Sicily (Villalba). Unfortunately, at present the Villalba landrace is the only one available to the authors from mainland Sicily, thus no comparison could be made with other materials coming from the same area.

Lower levels of genetic diversity are found in landraces from the small Sicily islands Linosa and Pantelleria. In fact, especially in the Linosa landrace, this value is much lower than the value of *I* over all landraces; a low value is also recorded for the proportion of polymorphic alleles. During migration of a landrace, especially towards isolated areas, often reduction of genetic diversity occurs as a result of a bottleneck effect (Sonnante et al. 1994; Laghetti et al. 2005). In this respect, isolated areas showing low genetic variation may well be the end point of the crop migration. On the other end, the noticeable level of diversity found in the landraces from the Apennine ridge might indicate that they are older than the others, or that they represent a remnant of fewer, older, and more widely distributed landraces.

It is clear that lentils were introduced into small islands by human populations from mainland Italy; therefore it can be assumed that the low value of the *I* index is attributable to a founder effect. It can positively be assumed that small seed samples were in the beginning introduced into the islands, which gave rise to new landraces by adaptation to different environments and/or selection. The combined effect of a small original population and adaptation likely yielded landraces with lower internal variation as compared to the landraces from the mainland.

It is also worth noticing that the three landraces from Pantelleria, Linosa, and Ustica show a high level of genetic similarity. This might imply that the original material spread to these islands had the same origin and the differences nowadays observable can be attributed to the component of genetic drift caused by the adaptation to different agro-climatic environments and human preference. The closer landrace to this group is that from Ventotene, another island of the Tyrrhenian sea. This observation does not imply necessarily that the landraces from small Sicily islands derived from the Ventotene landrace, but might indicate that they share a common origin. Some historical records appear to confirm this hypothesis: Racheli (1987) indicated that Ventotene

populations came from the close mainland around the XVIII century, and Laghetti et al. (2001) quote older reports indicating that in the Sicilian small islands legumes were introduced from the Naples area.

When considering the analyses of genetic distances, it appears clear that some landraces tend to form definite clusters of more similar entities. Although association between genetic similarity and geographic distance among landraces is not always clear, a pattern emerges that distinguishes the Villalba landrace from one side and the small islands entities from the other. In the material from Apennines three groups, overlapping to three different areas of the Apennine, may be hypothesised, which, although, show less clear differentiation: central-western (Colfiorito–Onano), central-eastern (Castelluccio–S.Stefano), and southern (Capracotta–Altamura). Based on genetic distances, the latter two groups appear to share a higher level of similarity. It is also interesting to notice that the Colfiorito and Castelluccio landraces, although geographically close, show a comparatively low level of genetic identity.

Some landraces appear to retain a high level of genetic identity, as in the case of Colfiorito and Onano. This might be due to migration of seeds through the transhumance route from central Apennine to the Tyrrhenian coast. Periodic migrations of herds might also explain the fact that the groups Castelluccio–S. Stefano and Capracotta–Altamura pertain to clusters with a lesser level of differentiation; in fact, shepherds from both these areas used to follow routes heading towards close destinations on the Adriatic coast (Piccioni 1993; Gambino and Romano 2003).

On the basis of genetic identity parameters, some landraces show a clear differentiation from the others, as in the case of Villalba, Altamura, and, to a minor extent, Capracotta. This difference is further supported by the observation of landrace-specific alleles. These findings altogether give a solid scientific base to trade development expectations of local communities for some landraces, while spoiling the prospects of others. For instance, the high similitude between Colfiorito and Onano does not allow actions based on genetic identity to promote these landraces separately; a different market strategy needs to be devised. On the contrary, the specific genetic traits of Altamura and Villalba support actions based on the genetic uniqueness of these landraces. Moreover, the presence of specific alleles might allow a sort of bar-coding, enabling the exact identification of these landraces, thus providing a powerful tool for the prevention of frauds.

Some of the considered landraces, although well adapted to the local agro-climatic conditions, might need some breeding action in order to optimise their agronomic performance and quality. Nevertheless, it should be considered that landraces are the result of empirical selection for local adaptation and therefore breeding programmes must be designed in order to keep the genetic integrity and cultural identity of the landraces themselves (Erskine 1997).

In conclusion, the present study has demonstrated that the major Italian lentil landraces have among them a level of genetic diversity that supports some of the expectations of appreciation, and it has helped to elucidate the origin and diffusion history of some landraces. Although some minor landraces have not been considered in this study, taken altogether, the data presented here provide a clear picture of the level of diversity present in this group of Italian lentil germplasm.

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