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RESEARCH ARTICLE

Analysis of the molecular diversity of *Olea europaea* in the Mediterranean Island of Malta

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Abstract The island of Malta is a small densely populated land mass located in the middle of the Mediterranean Sea. Genetic material from local olive tree varieties on the island was amplified using RAPD, 36 loci were subsequently generated and a dendrogram based on Jaccard's similarity coefficient constructed. Analysis of clustering patterns indicated a high degree of genetic diversity (0.18–0.68), an early separation between the majority of the native varieties and more recently introduced varieties, supporting the idea of a history of limited genetic exchange and indicating a separate clustering for the majority of the 'Malti' and 'Bidni' varieties. Principal Component Analysis of banding patterns confirmed these clustering patterns and analysis of the incidence of bands for primers OPA-17, OPC-19, OPI-18 (vector correlation significance <0.01) as well as for OPAG-13, OPAN-15 (vector correlation between 0.01 and 0.05) showed strong correlation. Native Maltese varieties were characterised by a higher number of bands arising from the former group of primers indicating their use as a possible means to distinguish between local and imported varieties.

Keywords Genetic diversity · *Olea europaea* · RAPD

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Introduction

The Maltese Archipelago offers an ideal opportunity for the study of the migratory paths of plant germplasm in the Mediterranean as it is separated from the European and African mainlands by a distance of over 200 km (Hughes 1999). Like many island refuges it may retain evidence of past germplasm movements and shed light on distinct evolutionary paths. Studies such as that by Laghetti et al. (2004) have focussed on the investigation of the genetic resources of crop plants in the Maltese Archipelago and indeed recorded the presence of distinct crop varieties.

As is the case for countries of the Mediterranean region, the Maltese Archipelago offers particularly favourable conditions to the growth of the olive tree. Typical of the Southern Mediterranean, the climate is hot and dry in summer and mild in winter. The soil is calcareous, ranging from mild to moderately alkaline.

There is also considerable historical evidence that shows ancient olive presence on the island. Traces of olive pollen were detected in a Bronze Age Pit (Godwin 1961) and then again repeatedly in deep sediments dated to Neolithic and Punic periods derived from an alluvial plain (Fenech 2009). Evidence exists showing that olive cultivation was widespread during the Roman colonization Period, with the remains of around seventeen country houses and several oil presses having been unearthed (Bonnano 1977). There is also substantial archaeological evidence that the island shared close maritime links with the major ancient colonizing and trading powers associated with the Westward migration of the olive and that Maltese harbours were used by Alexandrian grain ships (indicative of a link with the African continent; Gambin 2012). In subsequent centuries and then rapidly from the nineteenth century onwards, the olive industry fell into a period of decline. This appears to have been brought about by the demand to produce rapid—growing cash crops to feed increased population numbers, as well as the need to utilise and optimise space for anthropogenic use (Fig. 1).

Despite these significant pressures which still act today, pools of olive tree varieties still survive. These can be loosely grouped into three types. First there are cultivated varieties of *Olea europaea* Linnaeus such as the 'Bidni' and 'Malti', which are believed to be among the most ancient forms on the island (Borg 1922). Second there are forms of the oleaster or wild-type olive *O. europaea* var. *sylvestris* Brot. These undergo sexual reproduction spontaneously and therefore many diverse forms should be expected. Within this group, the possibility that local varieties escaped from cultivation and regressed to the wild-type form cannot be overlooked. Third, there are olive trees consisting of known foreign varieties that have been introduced to the island. This process appears to have been an ongoing one (Borg 1922) but appears to have accelerated over the last 30 years with Spanish and Italian olive varieties, such as 'Carolea', being most common (Galea 2011).

This research was therefore concerned with the genetic characterisation of local cultivated olive varieties using DNA molecular markers. Malta's geographical isolation, pedo-climatic conditions, history and ultimately the micro-scale of crossing events make

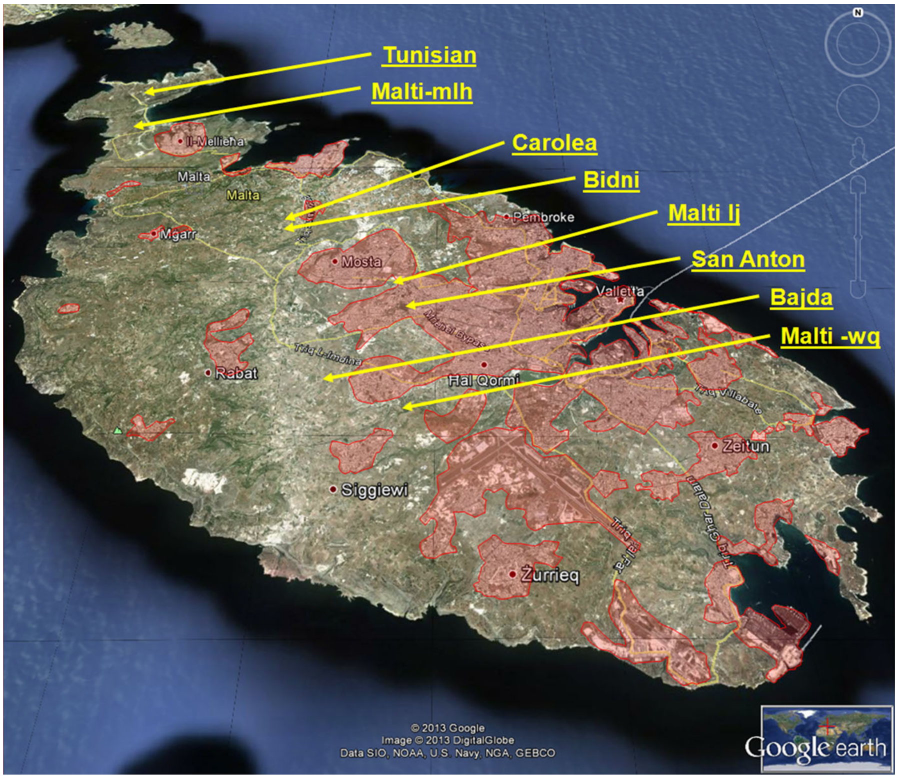

Fig. 1 Map showing the island of Malta. The *red shaded* areas represent built-up areas; individual houses and numerous small hamlets have been omitted. Locations marked in *bold white* print represent the sites where varieties were collected. (Color figure online)

it ideal to search for surviving traces of ancient germplasm that may shed new light on the debate concerning the migratory paths of *Olea*. Despite the potential of such a study, surprisingly no molecular studies have been carried out on the genetic heritage of olive trees from the Maltese Islands.

Materials and methods

As a research strategy, it was decided to focus on the analysis of local trees that had a long record of cultivation on the islands, using the records compiled by Borg (1922). Trees were identified using standard morphometric qualities. In the majority of cases, leaves from centennial native trees were specifically selected for the study. Wherever possible leaves from at least four specimens of each of the varieties studied were included in the study. Leaf specimens were placed on ice, transported rapidly to the laboratory and stored at −80 °C till used. Samples were collected from the varieties shown in Table 1.

2.5 g of each leaf tissue was placed in a mortar and crushed using liquid nitrogen. DNA was then extracted using the CTAB method developed by Doyle and Doyle (1987). The DNA samples were analysed quantitatively and qualitatively on a Thermo Scientific NanoDrop™ 2000. All the samples were then treated with ribonuclease A. The RAPD amplification steps were performed in a 25 µL aqueous volume containing 50 ng of template DNA, 1× PCR buffer B, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 1.0 U DNA polymerase, and 1 µM of the single primer.

A total of 14 decamer oligonucleotides [Operon Technologies Inc.] were tested to develop the required DNA electrophoretic patterns. The primers used included OPA-17, OPAN-15, OPA-07, OPAQ-15, OPB-18, OPA-16, OPBC-05, OPC-19, OPA-09, OPAG-13, OPA-04, OPA-03, OPA-05 and OPI-18. Negative controls were also set up, containing all the reaction components but lacking template DNA. The PCR amplifications were performed in an Eppendorf® Mastercycler Personal using the following conditions: an initial denaturation of 94 °C for 5 min, followed by 45 cycles of 45 s at 94 °C, 1 min at 34 °C and 45 s at 72 °C, and a final elongation step at 72 °C for 5 min. Rigorous selection criteria were adoped. For each variety amplification was repeated using genomic DNA isolated from a minimum of four different specimens (where available). Each separate amplification reaction was repeated three times and only reproducible bands were considered for analysis. Any amplified bands that arose in the negative control were omitted from subsequent analysis. Each of the molecular markers selected was then assigned a code according to the primer used to produce the marker and the estimated size in base pairs. The distances moved by the PCR products were measured using UVP Doc-It LS Image Analysis Software. The same software was then used to measure the distance travelled by the reference fragments of the 100 bp ladder.

Results

Eight primers were ultimately selected from the 14 primers tested. These were chosen on the basis of the

Table 1 Summary of the characteristics of the different varieties used in the study

Variety	Provisional age of trees (years)	Size of drupe	Colour of drupe	Recorded use
Bidni	>1,000	Small	Black	Oil
Bajda 'white'[a]	~>300	Large	White	Oil/Table
Malti (lj)	~>300–400	Medium	Green	Oil/Table
Malti (mlh)	~>400	Medium	Green	Oil/Table
Malti (mwq)	~>500	Small	Black	Oil/Table
Maltese 'San Anton variety'	~300–400	Small	N/A	Ornamental
Tunisian 'Chemlali'	50	Small–Medium	Green	Oil
Carolea	NA	Large	Green	Table/Oil
Sicilian (Cetrala)	NA	Medium–Large		Table/Oil

The original ancient Bidni variety is protected by law and therefore leaves from grafts of the original trees were used

[a] During the study only one specimen of Bajda variety was available

presence, quality and the reproducibility of the banding patterns obtained. A total of 36 loci were obtained using these eight core primers. The largest number of polymorphic bands generated varied from eight using OPB-18, followed closely by six bands for primer OPAN-15 and OPAG13. The lowest number of loci was generated using OPBC-05, which generated three loci. The average number of loci obtained per primer using the total cultivars reported in this paper was 4.5, a value which is close to that reported by Sesli and Yegenoglu (2009).

The electrophoretic banding patterns were analysed and used to construct a table in which the presence or absence of each band (locus) was recorded. The data was analysed using the PAST programme and a dendrogram was constructed using Jaccard's genetic distances (Fig. 2). The dendrogram was characterised by a fairly wide range of genetic distances among genotypes with similarity coefficients ranging from 0.17 to 0.68. The lowest similarity, that is the greatest separation, was seen at circa 0.17 between local trees and introduced foreign cultivars, while the greatest similarity was observed between trees derived from the same variety within the same geographical location ('Malti'-Lija). This early separation between local and foreign varieties was also noted by Diaz et al. (2011) in their study of centennial olive trees in Spain. The trend that native varieties tend to cluster together according to their geographical origin was also noted by Gonzalo-Claros et al. (2000) in their study of olive trees in Malaga. Indeed, the latter suggested that this type of separation is consistent with a hypothesis of autochthonous origin. No strong clustering according to their final use, for consumption or pressing, by size or by colour of the drupe could be noted.

The varieties that are thought of as being native to the islands, that is the 'Malti' and 'Bidni' varieties co-separated into a single cluster. For this cluster, the greatest similarity was observed between trees of the

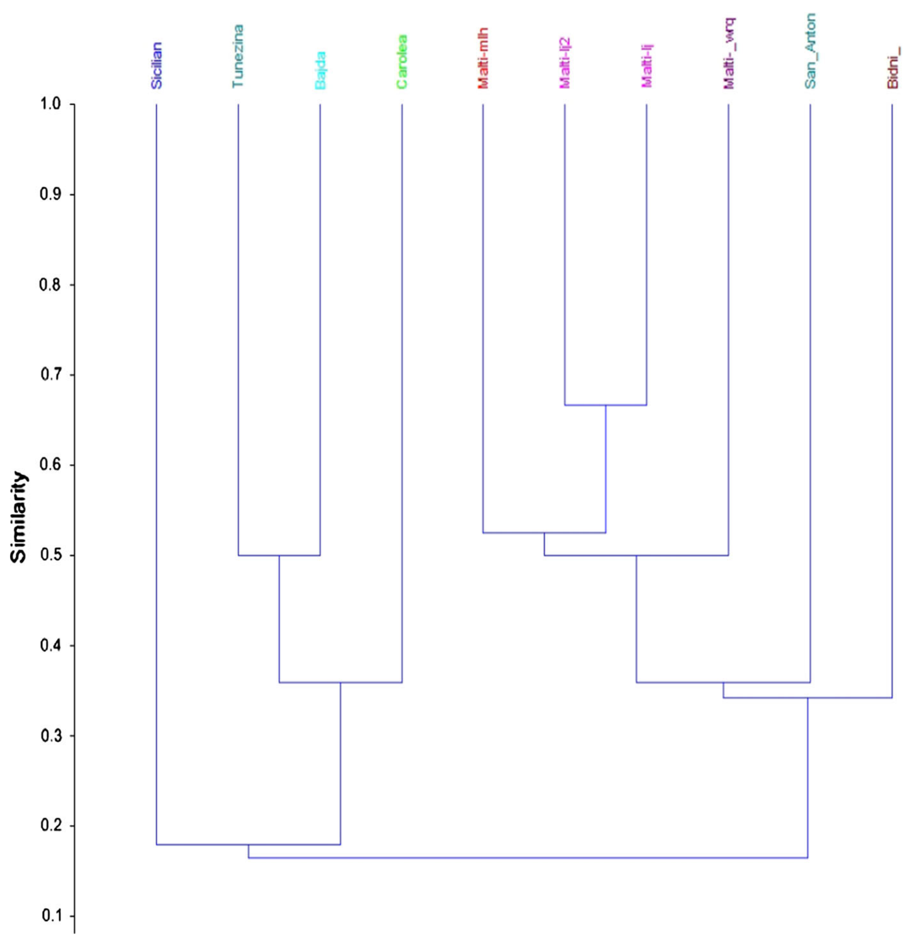

Fig. 2 Hierarchal dendrogram of olive varieties based on Jaccard's coefficients

'Malti' variety, with more genetic similarity being seen with each other than with the 'Bidni' variety.

It is interesting that the San Anton variety, consisting of ancient gigantic trees, which Borg (1922) suggested were of a wild-type oleaster variety, appeared to share genetic similarity with the Maltese varieties. This may possibly imply a link between 'Malti' and wild-type varieties and could possibly be evidence of some selection from native wild-types genotypes, although further studies on ancient oleasters[1] would be needed to confirm this.

The second cluster consisted of most of the other foreign varieties tested (with the exception of 'Bajda'), implying that they shared more similarity between themselves than with the native trees. The presence of these distinct olive trees appears to reflect waves of introductions of genetically distinct foreign varieties. Within this second cluster, the 'Bajda' or 'white' variety is an example of a variety exhibiting Leucocarpa. Although it is often referred to as a Maltese variety, the fact that it separated within the second cluster of foreign varieties offers credence to the idea that this tree may be a variant that had been introduced to the island. This hypothesis was originally proposed by Borg in 1922 before the advent of molecular techniques and it is interesting that modern genetic tools appear to reinforce this proposition.

The 'Bidni' varieties tested were grafts, hence clones, of an original ancient 'Bidni' variety currently limited to a secluded grove of 26 massive trees, presumed to be over 1,500 years old. This provisional dating is supported by their location which borders the remains of an ancient Roman Villa where equipment used in the oil-industry, including a stone tapetum, was found. Other circumstantial evidence such as the enormous girth of the trunks as well as their planting distance of 9 m, which was in use by the Romans and more than twice the more conventional 4 m used today (Cato, as cited in Buhagiar 2012), are also thought to support the idea of age.

To establish whether the hierarchal genetic link between the 'Malti' and 'Bidni' varieties was also observed with other methods, and to highlight any other possible genetic links, the total number of bands using the different primers in each variety was analysed using Principal Component Analysis (PCA). The results obtained in the PCA are similar to those seen in the cluster analysis (Fig. 3). A high degree of genetic similarity existed between the 'Malti' varieties designated 'Malti'-lj and 'Malti'-wq. The San Anton and 'Bidni' varieties exhibited distinct clusters, while the Tunezina and 'Bajda' varieties were in a third, separate, cluster, which unexpectedly also included the Sicilian 'Cetrala' variety.

The PCA additionally revealed some interesting information. The incidences of bands arising from the primers OPA-17, OPC-19 and OPI-18 were strongly correlated (vector correlation significances <0.01), whilst those arising from OPAG-13 were also well correlated with those of OPAN-15 and OPBC-05 (vector correlation significances between 0.01 and 0.05). The cluster composed of the three 'Malti' varieties indeed appeared to be characterised by a higher number of bands arising from these two groups of primers, whilst the cluster composed primarily of imported varieties was characterised by a lower number of bands from these two groups of primers. It appears that there is evidence to support the use of the OPA-17, OPC-19 and OPI-18 primers as a means of distinguishing between local and imported varieties of olive trees.

Discussion

The olive industry in the Maltese islands is currently undergoing a rapid revival and there is great interest in establishing whether varieties associated in folklore as being native are indeed unique to the island. The data in this study does offer some evidence that 'Bidni' and 'Malti' varieties form a separate group from the other catalogued varieties studied. Mazzitelli (2013) postulates that this separate clustering pattern may reflect a history of limited genetic exchange with other varieties during their cultivation history. Mazzitelli (2013) also suggests that limited cross-pollination may have been maintained due to processes of temporal or physical isolation. For example, the original ancient 'Bidni' variety appears to be limited to a secluded grove of 26 massive trees, and most trees would have been propagated by grafting. To determine whether the disparate nature is the result of an endemic origin (in the case of 'Malti') or a history of limited exchange

[1] Surviving ancient oleasters may be difficult to find, as any wood not having an immediate utilitarian use would in the past have been used as kindling.

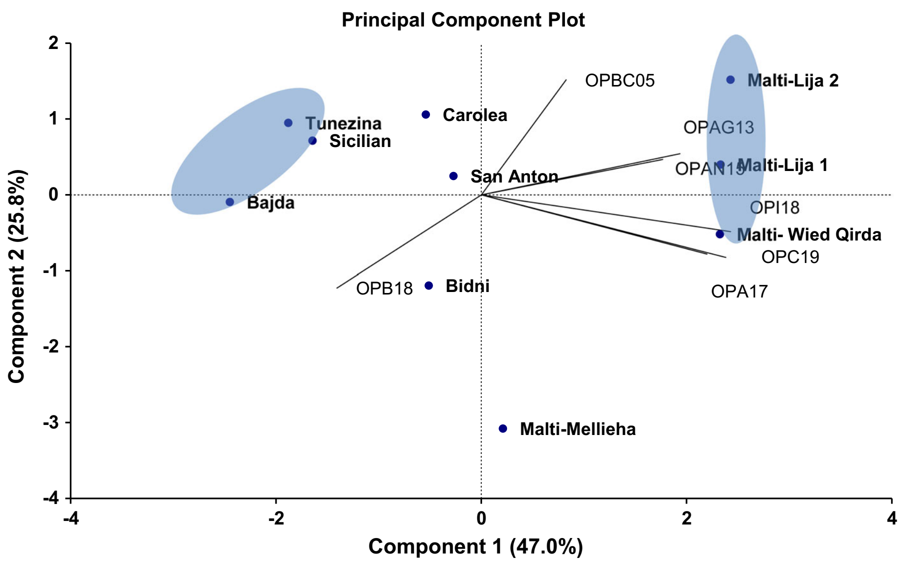

Fig. 3 Principal Component Analysis bands generated by different primers for the olive tree varieties investigated in the study

in more recent times, further studies would be recommended.

The 'Malti' (Maltese) variety is traditionally believed to be the tree that '*constituted the bulk of the olive groves in past times*' (Borg 1922). The variety is today still distributed across the island, although the numbers are under threat. It is interesting that in cultivated areas, a number survive as individual isolated trees in minute pockets of land framing cultivated fields. This appears to be a remnant of a policy where trees were propagated by individual farmers as isolated single trees for the exclusive provision of oil and table use for the farmer's needs, leaving the rest of the available land for cultivation of crops. Given the age, physical location and methods of grafting of these trees, it is very likely that opportunities of genetic exchange with other varieties would have been limited, maintaining the degree of genetic distance seen in Fig. 3. Overall the genetic distance seen in the study implies that certain 'Malti' varieties studied, share a common genetic base, but appear to have collected genetic variation over the years.

The question why the 'Malti' variety was widespread, despite the repeated introduction, of many other cultivars that were deemed more suitable for table use is an interesting one (Borg 1922; Table 1). It is postulated here that this hardy tree with its low needs, high yields, high drought tolerance and known resistance to the olive fruit-fly *Bactrocera* (*Daculus*) *oleae* (Gmelin) may have been agronomically attractive to farmers. Alternatively, its distribution may simply reflect remnants of widespread distribution of a native tree. Indeed, the link with the wild-type variety (San Anton) appears to point towards an autochthonous or native origin, although further studies on oleasters or traces of historical pollen or ancient olive wood would be needed to confirm this.

With respect to conservation, it may be argued that the data generated in Fig. 2 indicates that there has been little repercussion on the local gene-pool by the rapid introduction of large numbers of foreign varieties such as 'Carolea' that has taken place over recent years. However it would be recommended that additional studies are repeated, focussing on larger number of oleasters as well as the drupes of native varieties collected from regions close to where foreign varieties are concentrated, to confirm whether this is in fact so.

In this study RAPD amplification was adopted as the molecular method of choice. This decision was taken as it has a number of advantages, including that it makes use of primers that are short in length, universal, and show complementarity with genomic sequences of an unknown nature. However we are aware of its limitation in that it is overly sensitive to small variations in experimental conditions including factors that may have influenced the results in this study, such as DNA concentration, type of enzyme and operator. There is also awareness that the absence of an allele, or indeed the presence of an allele, may not necessarily be evidence of a shared genetic link. As a method, it is less conducive to compare with data in the public domain, making determination of genetic links between the varieties studied here and other catalogued varieties from the European and African mainlands difficult. Certainly, in recent years, amplification of microsatellite regions using the method known as SSR has been shown to be more specific and reliable. However, a high degree of complementarity between the genomic DNA (regions neighbouring the microsatellites) and the primers is required, and in the case of the Maltese varieties, selected primers of the DCA and GAPU families failed to offer informative distinctions between the native and foreign varieties.

Conclusions and future work

In this study a high genetic diversity was noted among cultivars on the island and analysis using statistical methods indicated a dichotomy between the local varieties and more recently introduced catalogued varieties. This work indicates that local varieties are distinct, worthy of further study in terms of the information they may yield on genetic lineage and their unique agricultural properties, and for 'Malti' which is often overlooked in favour of foreign varieties, deserving of additional measures for their conservation. The complex and wide genetic hierarchal arrangement shown offers evidence that introductions of foreign varieties and propagation of local genotypes were not mutually exclusive processes but both appear to have been occuring simultaneously.

Future work will be concentrated on identifying a suitable range of SSR primers that are informative on all the native varieties. This would facilitate determination of genetic links with olive stocks on the mainlands.

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