# Molecular Characterization of Swiss Chestnut Cultivars (*Castanea Sativa* Mill.) Using RAPD, AFLP and ISSR Markers

## Abdelhamid, S.[1*], Omri, A.[1], Araouiki, A.[1], Sghair A.[1].

[1] Institut de l'Olivier, Laboratoire de Biologie Moléculaire, Cité Taffala, 4000-Sousse, Tunisia

**Abstract:** *Domestic chestnut is one of the most widespread tree species in south Switzerland. It covers more than 26.000 ha. In the past, chestnut fruits used to be of vital importance in the diet of people and livestock.*

*Three different kinds of molecular markers: Random Amplified Polymorphic DNAs (RAPDs), Amplified Fragment Length Polymorphism DNAs (AFLPs) and Inter Simple Sequence Repeats (ISSRs) have been used for studying the genetic variability in 52 Swiss accessions of cultivated and wild Chestnut (Castanea sativa Mill.). Variability within and between cultivars is examined. 98 RAPD, 222 AFLP and 35 ISSR polymorphic markers were amplified using 12, 4 and 5 primers respectively.*

*Clustering analysis performed with the three sets of markers group the 52 studied accessions according to their similarity coefficients separated the genotypes into clear groups with some differences. It is interesting to note that AFLP generated the highest number of polymorphic bands and clustered fairly chestnut cultivars. These results allowed us to resolve problems generated by synonyms and homonyms in different chestnut accessions.*

*Molecular markers results are comparable. The similarity matrices based on the three sets of data give a high significant correlation between RAPD and AFLP, RAPD and ISSR data (r = 0.78 and r = 0.75 respectively).*

**Keywords:** *Castanea sativa, molecular markers, genetic diversity, RAPD, AFLP, ISSR.*

## 1. INTRODUCTION

Chestnut was cultivated since the Roman Empire. Cultivated chestnut has colonized many European environments. It is a typical nut crop in many mountainous regions of the European area and one of the most common forest trees in the southern alps of Switzerland since nearly 2000 years (Conedera *et al.,* 1997). Swiss Alps accounts approximately 26 000 ha of chestnut forests which was wide spread into 250 town ships. A total of 94 names of chestnut varieties are known. In Southern Alps, chestnut was considered as an important source of food. Moreover, chestnut is widely known for its double usefulness. Its fruit was used for nutrition (man and animal) and its wood is a means for providing either fire or stokes. Numerous cases of homonymy and synonymy could occur in different traditional chestnut areas in Switzerland (Gobbin *et al.,* 2007). Despite the great number of existing varieties, the need for a powerful identification and characterization method in wild and cultivated varieties is crucial.

Typically, the characterisation of Swiss chestnut cultivars was based on morphological traits such as fruit and leaves has been used to verify the identity of uncertain cultivars. Moreover, this approach yielded considerable redundant number of different chestnut cultivars. However, because of most discriminating characters is related to fruit and flower, morphological identification has to be completed by a molecular approach.

Some authors applied isozymes techniques to identify and to study the genetic variability of European chestnut (Pereira *et al.,* 1999). The use of isozyme markers to characterize and to study the genetic variation between and within cultivars, depend on several factors (environmental, limited in number) that limit the wider adoption of this technique (Pereira-Lorenzo *et al.,* 1996).

Recently, molecular markers were used to identify chestnut clones and cultivars (Angela Martin *et al.,* 2010). DNA molecular markers techniques based on *in-vitro* enzymatic amplification of specific fragments of DNA via PCR (polymerase chain reaction) are of greater suitability in genetic diversity estimations and identification of chestnut genotypes (Casasoli *et al.,* 2006; Mellano *et al.,* 2012;

McCleary *et al.,* 2013). Previous studies have assessed the genetic variability of chestnut using Random Amplified Polymorphic DNA (RAPDs) (Oraguzie *et al.,* 1999), Amplified Fragment Length Polymorphism (AFLPs) (Yamamoto *et al.,* 1998), Inter Simple Sequence Repeats (ISSRs) (Goulão *et al.,* 2001; Casasoli *et al.,* 2001) and Simple Sequence Repeats (SSRs or Microsatellites) (Pereira-Lorenzo *et al.,* 2011).

The objective of this study were (i) to assess the relative efficiencies of different molecular markers and (ii) to compare different molecular marker systems for genetic diversity analysis and to assess the extent of genetic variability available within and among cultivated cultivars and natural chestnut of coppice shoots.

## 2. MATERIAL AND METHODS

### 2.1. Plant Material and DNA Extraction

In the present study, 52 accessions were collected from morphologically different varieties of different areas in southern Switzerland. The origin of studied accessions of Swiss varieties and coppice shoots are listed in Tab. 1.

**Table1.** *Studied accessions of Swiss chestnut genotypes and their origin*

| Accession order | Genotype | Origin/Locality |
|---|---|---|
| 1 | Verdanesa | Calonico 01. Switzerland |
| 2 | Verdanesa | Calonico 04. Switzerland |
| 3 | Verdanesa | Giornico 03. Switzerland |
| 4 | Verdanesa | Giornico 06. Switzerland |
| 5 | Verdanesa | Giornico 04. Switzerland |
| 6 | Verdanesa | Chironico 02. Switzerland |
| 7 | Verdanesa | Chironico 06. Switzerland |
| 8 | Verdanesa | Chironico 10. Switzerland |
| 9 | Verdanesa | Chironico 12.  Switzerland |
| 10 | Verdanesa | Chironico 13. Switzerland |
| 11 | Verdanesa | Lodrino 04. Switzerland |
| 12 | Verdanesa | Torricella 10. Switzerland |
| 13 | Verdanesa | Torricella 13. Switzerland |
| 14 | Lüina | Calonico 02. Switzerland |
| 15 | Lüina | Calonico 07. Switzerland |
| 16 | Lüina | Giornico 01. Switzerland |
| 17 | Lüina | Giornico 02. Switzerland |
| 18 | Lüina | Chironico 01. Switzerland |
| 19 | Lüina | Chironico 05. Switzerland |
| 20 | Lüina | Chironico 08. Switzerland |
| 21 | Lüina | Chironico 14. Switzerland |
| 22 | Lüina | Lodrino 03. Switzerland |
| 23 | Lüina | Lodrino 05. Switzerland |
| 24 | Lüina | Lodrino 14. Switzerland |
| 25 | Lüina | Torricella 08. Switzerland |
| 26 | Lüina | Torricella 09. Switzerland |
| 27 | Lüina | Torricella 17. Switzerland |
| 28 | Bonè negro | Calonico 03. Switzerland |
| 29 | Bonè negro | Calonico 05. Switzerland |
| 30 | Bonè negro | Calonico 06. Switzerland |
| 31 | Bonè negro | Calonico 08. Switzerland |
| 32 | Bonè negro | Chironico 03. Switzerland |
| 33 | Bonè negro | Chironico 04. Switzerland |
| 34 | Bonè negro | Lodrino 02. Switzerland |
| 35 | Bonè negro | Lodrino 11. Switzerland |
| 36 | Bonè negro | Lodrino 12. Switzerland |

| 37 | Bonè negro | Lodrino 13. Switzerland |
|----|------------|-------------------------|
| 38 | Berögna | Lodrino 07. Switzerland |
| 39 | Berögna | Lodrino 08. Switzerland |
| 40 | Berögna | Prosita 07. Switzerland |
| 41 | Pinca | Vezio 21. Switzerland |
| 42 | Pinca | Vezio 22. Switzerland |
| 43 | Pinca | Vezio 31. Switzerland |
| 44 | *C. sativa*: Coppice | East Switzerland |
| 45 | *C. sativa*: Coppice | East Switzerland |
| 46 | *C. sativa*: Coppice | East Switzerland |
| 47 | *C. sativa*: Coppice | East Switzerland |
| 48 | *C. sativa*: Coppice | East Switzerland |
| 49 | *C. sativa*: Coppice | East Switzerland |
| 50 | *C. sativa*: Coppice | East Switzerland |
| 51 | *C. sativa*: Coppice | East Switzerland |
| 52 | *C. sativa*: Coppice | East Switzerland |

Total DNA was extracted from small leaves using hexadecyltrimethyl ammonium bromide (CTAB) according to the method described by Porebski *et al.* (1997). DNA was resuspended in TE (pH 8) solution and stored at $-20$ °C.

## 2.2. DNA Amplification

### RAPD

Using RAPD method, 12 primers from set OPA 02, 04, 07,10 and 15, OPB 08, OPD 20, OPE 01, 04, 16 and 19 and OPX 17 (Operon Technologies, Alameda, Calif.), were able to amplify specific markers.

PCR were performed in a total volume of 25-µl. The amplification reaction contained 1 x PCR buffer, 1.4 mM MgCl2, 0.2 mM dNTP, 0.4 µM primer, 1U/µl Taq polymerase (Eurobiotaq) and 20 ng/µl template DNA. PCR was performed in a Hybaid PCR express thermal cycler (HBP x 220) with following cycling profile: an initial denaturation at 94 °C for 4 min, followed by 38 cycles of 1 min at 93 °C, 1min at 45 °C and 1 min at 72°C with a final extension at 72 °C for 5 mn. The DNA fragments were then visualized under UV light.

### AFLP

For AFLP, 4 sets of selective primer combinations were used (E-AGG/M-CTT, E-AAC/M-CTT, E-AGT/M-CAT and E-AAC/M-CAT) from the GIBCO BRL AFLP$^{TM}$ Core Reagent Kit to generate AFLP fragments.

Digestion was done with 5 U of *Eco*RI and 5 U of *Mse*I (Biolabs) in T4-buffer. For the ligation reaction, the following mixture was added to the restriction-reaction mixture; *Eco*RI adapter (40 p mol/µl), *Mse*I adapter (40 p mol/µl), 1 U T4 DNA ligase, 1 x T4 DNA ligase buffer and incubated for 3 h at 37 °C.

Preamplification was carried out in volumes of 20-µl. Reactions contained 1 x PCR buffer, 1.5 mM MgCl$_2$, 1 mM dNTP, 10 pmol/µl for each preselective primer *Eco*RI-A and *Mse*I-C, 1 U *Taq* polymerase (Qiagen AG, Basel) and 100 ng/µl template DNA. Preamplification was performed with the cycling following profile: 2 min DNA denaturation step at 94 °C, followed by 28 cycles of 45 sec at 94 °C, 45 sec at 56 °C and 1min at 72 °C. A final elongation step was done at 72 °C for 10 min.

Selective Amplification reactions were performed in 20-µl volume contained 1 x PCR buffer,

0.75 mM MgCl$_2$, 1 mM dNTP, 0.25 µM of each selective primers *Eco*RI-ANN and *Mse*I-CNN, 1 U *Taq* polymerase (Qiagen AG, Basel). PCR was performed for 36 cycles with the following cycle profile: a 30 s DNA denaturation step at 94 °C, a 30 s annealing step and a 1 min extension step at 72

°C. Samples were loaded and run on the ABI-310 automated DNA sequencer (capillary electrophoresis).

## ISSR

According to ISSRs method, 5 primers were selected UBC 810, UBC 834, UBC 836, UBC 841 and UBC 890 (obtained from UBC primer set 100/9, University of British Columbia, Canada) based on their capacity to amplify polymorphic fragments.

Amplification reactions were carried out in volumes of 25-μl. Reaction contained 1 x PCR buffer, 1.4 mM MgCl$_2$, 0.2 mM dNTP, 0.4 μM of primer, 1U/μl *Taq* polymerase (Eurobiotaq) and 30 ng/μl template DNA. PCR reactions were performed with the following conditions: 4 min at 94°C for initial denaturation, 35 cycles of 35 s at 93 °C (denaturation), 45 s at optimal temperature ranging from 52 °C to 55 °C (annealing) and 90 sec at 72 °C (extension). A final extension step at 72 °C for 5 min followed. PCR products were separated on denaturing polyacrylamide gels multiphor II (Pharmacia Biotech) DNA silver staining kit (Pharmacia Biotech).

### 2.3. Data Scoring and Analysis

Polymorphic DNA fragments for the three types of markers were scored as present (1) or absent (0). Dendrograms were constructed by UPGMA (Unweighted Pair-Group Method with Arithmetic Averages) cluster analysis according to Jaccard's coefficient (Sneath and Sokal 1973) using the cluster (http:www.biology.ualberta.ca/jbrzusto) analysis software, and then visualized with TREEVIEW program (Page 1998). The coefficient of similarity was calculated (Sneath and Sokal 1973) and then similarity matrices were calculated to perform Principal Coordinates Analysis (PCoA).

The correlation between RAPD, AFLP and ISSR methods was investigated by the Mantel test of matrix correspondence (Mantel 1967). Mantel's tests were performed with the R4 (Beta version) package (Philippe Casgrain & Pierre Legendre, Dép. Des sciences biologiques, Université de Montréal) and statistical significance was determined by random permutation (999 permutations).

## 3. RESULTS AND DISCUSSION

The three methods used in this study were able to uniquely fingerprint each of the 52 cultivated chestnut accessions and were efficient in the characterization of chestnut cultivars. The 12 RAPD primers used on the whole set of plants produced a total of 98 polymorphic bands. For all the genotypes, the highest number of polymorphic and scorable bands was obtained by primer OPE-01 (10 fragments). For AFLP, the four primer combinations (E-AGG/M-CTT, E-AAC/M-CTT, E-AGT/M-CAT and E-AAC/M-CAT) yielded 222 polymorphic fragments with an average of 55 polymorphic bands per reaction, which ranged from 50 to 350 bp. For ISSR, five primers amplified bands, which are visible in polyacrylamide gels. The 5 ISSR primers detected a total of 35 polymorphic fragments with an average of 7 polymorphic bands per reaction. The highest number of polymorphic bands was obtained by primer UBC-841. Our present study is largely in accordance and comparable with previous work (Abdelhamid *et al.,* 2014) using molecular markers genetic analysis method to assess the genetic variability of *Castanea* species.

In all dendrograms generated by the three methods, the species of *C. sativa* was clustered into two groups: the first cluster is composed of Swiss cultivars and the second is composed of coppice shoots Fig. (1-4). Swiss cultivars are used for nut production, whereas coppice shoots are basically used for wood production. The different uses for special ends relating to such group explain in a way the division formerly showed by dendrograms. The grouping of Swiss cultivars and coppice shoots into two different clusters by all the dendrograms may suggest that grafting methods, generally practised by farmers, for chestnut propagation, could have transformed natural forests into clonal or polyclonal fruit orchard.
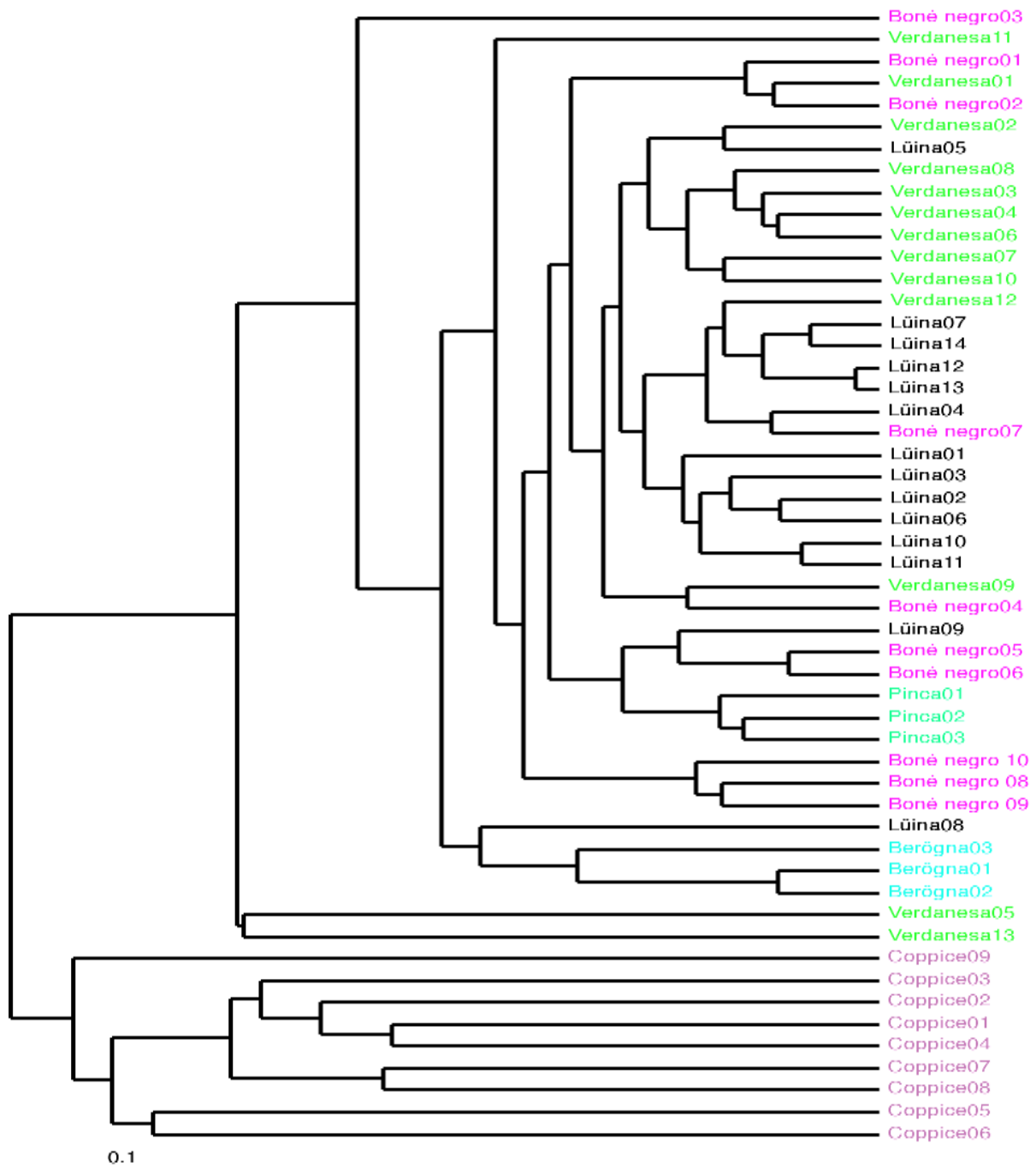
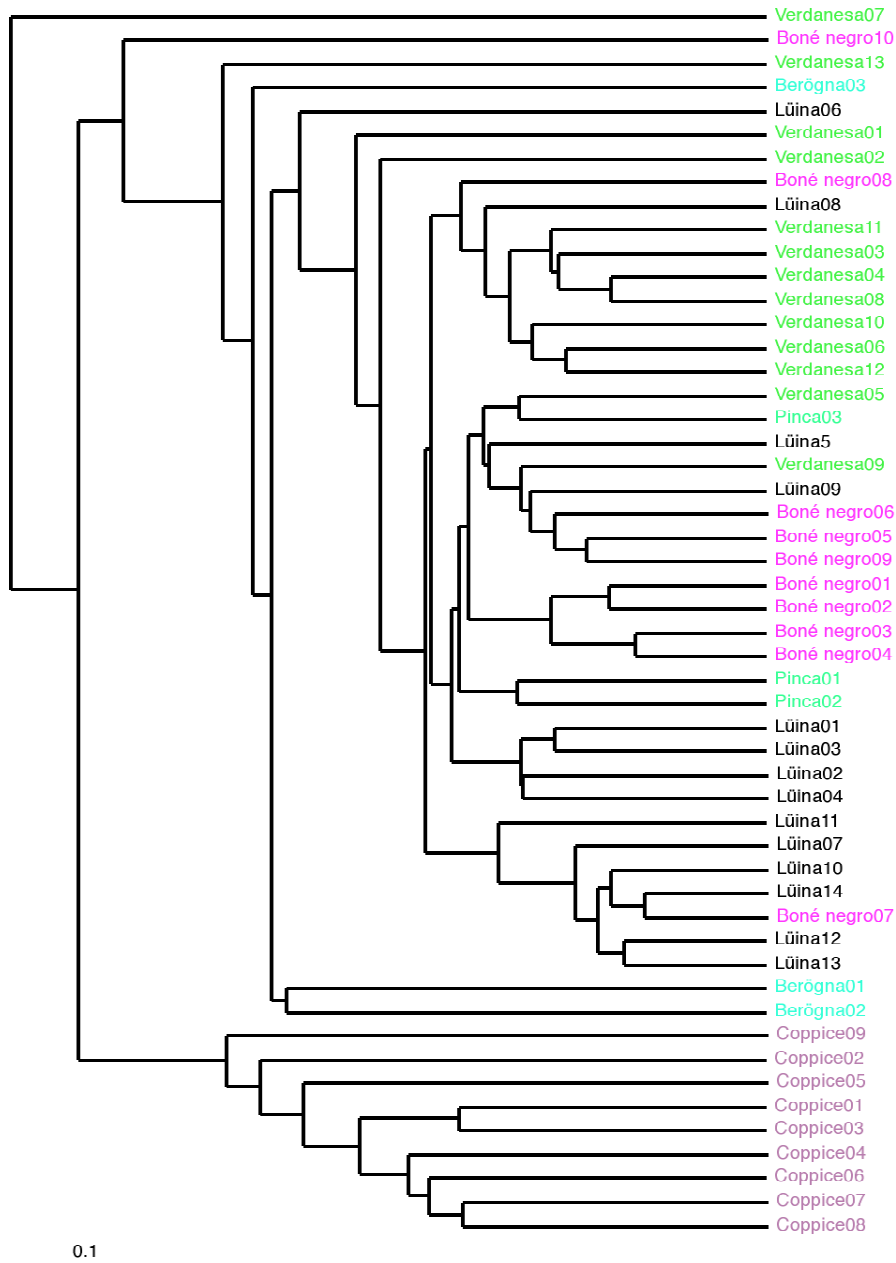**Fig1.** *UPGMA dendrogram obtained using RAPD analysis*.

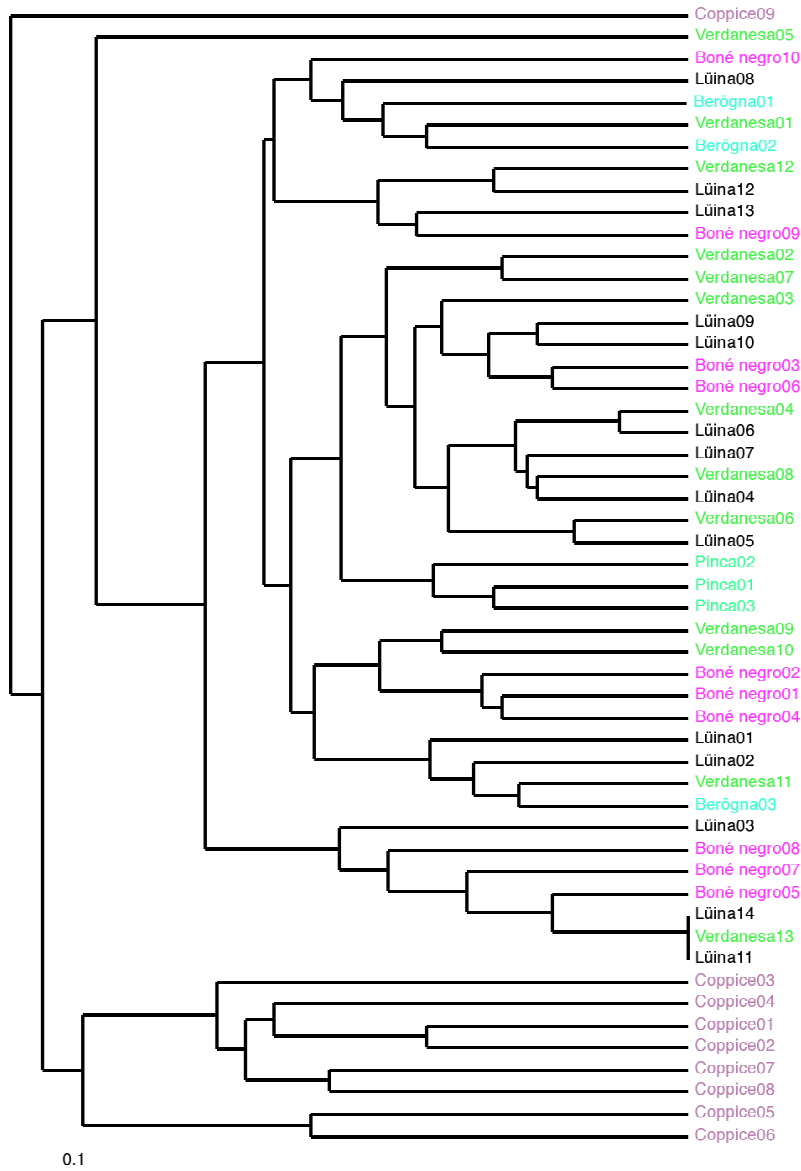**Fig2.** UPGMA *dendrogram obtained using AFLP analysis.*

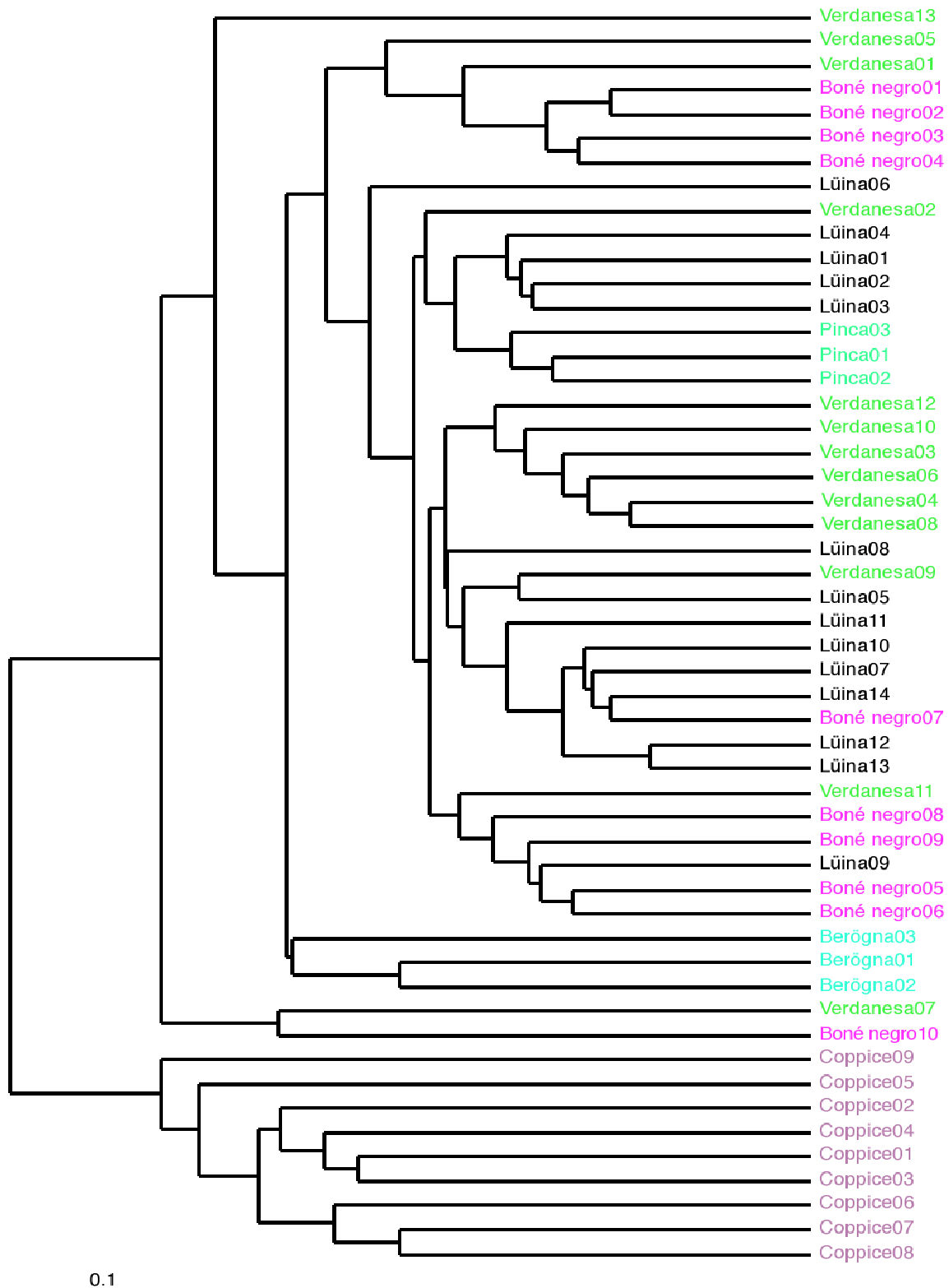**Fig3.** *UPGMA dendrogram obtained using ISSR analysis.*

Fig.4. *UPGMA dendrogram obtained using combined data analysis.*

The three methods mentioned above show that Swiss cultivars are genetically closely related, our results agreed with the results based on morphological traits (Martín *et al.,* 2007), this may be explained by the fact that southern Switzerland is a rich assortment of polyclonal varieties, which are the results of a clonal selection process, generally done by farmers in terms of their needs or in terms

of their aspiration to superior quality of chestnut fruit. The agro-ecological adaptation area of cultivars (not wide geographical distribution), a possible exchange of plant material and seed among the different regions of southern Switzerland and the old cultural traditions may explain the homogeneity among cultivars.

RAPDs and combined data ordered Pinca and Berögna, as expected, according to the supposed affiliation to a variety. Also, ISSRs cluster Pinca in a clear distinct group.

Discrepancies in forming clear groups within Lüina, Verdanesa and Boné negro varieties were observed in dendrograms produced by all the kind of markers.

Furthermore, RAPD, AFLP and combined data clustered clearly Lüina, Verdanesa and Boné negro, though with exceptions, into separate groups. ISSR did not separate accessions of cultivars clearly. It seems interesting to mention that several small groups of accessions have been formed that made identification very difficult. This was the case for the three cultivars: Verdanesa, Lüina and Boné negro.

The molecular markers data did not discriminate clearly Swiss cultivars, while combined data show a clear discrimination of cultivars that appear to be more clearly clustered in separated groups in the plot.

RAPDs, AFLPs and combined data provided fairly a clear separation of cultivars which were positioned within varieties. Boné negro 07 and Verdanesa 12 were closely related to Lüina, Lüina 05 was closely related to Verdanesa and Lüina 09 was related to Boné negro as shown by RAPD. For AFLP, Lüina 08, Boné negro 08 were clustered with Verdanesa and Boné negro 07 was related to Lüina cultivars.

RAPD and combined data methods could separate two cultivars (Pinca and Berögna) in a clear distinct group showing some similarity in clustering cultivars. Also, ISSR cluster accessions of Pinca in one distinct group.

Considering the Verdanesa related accessions, the topology of each tree revealed with RAPD, AFLP and combined data sets is unique with some similarity. There was not very good discrimination of Verdanesa cultivars by ISSR.

Lüina variety was mainly constituted by Lüina 01, 02, 03, 04, 07, 10, 11, 12, 13, 14 accessions and was consistently reported by RAPD, AFLP and combined data, with some exceptions. A not clear discrimination among accessions showed by ISSR dendrogram.

Four accessions of Boné negro cultivars (Boné negro 01, 02, 05 and 06) were clustered together by RAPD, AFLP and combined data and formed a clear group. Dendrogram generated by ISSR could not clearly distinguish accessions of Boné negro.

The results obtained with the three methods indicate that Verdanesa, Lüina, pinca, Berögna and verdanesa are genetically related. This result could be explained by the common origin of European chestnut trees. As reported by some previous studies (Fineshi *et al.,* 2000), shown that European chestnuts were coming from the east and were widely diffused in Europe by human activities. Other data related to Italian (Pigliucci *et al.,* 1990; Villani *et al.,*1991; Mancuso *et al.,* 1999), Portuguese (Seabra *et al.,* 2001) and on Spanish chestnut (Pereira-Lorenzo *et al.,* 1996) (studies based on isozyme traits) showed a close relationship among varieties as well.

High genetic similarities between and within Swiss cultivars were shown by the three used markers analysis. Similar results are found by (Gobbin *et al.,* 2007) and show that Swiss varieties appear morphologically more uniform. The same authors confirm this genetic uniformity among cultivars by isozymes traits. The same findings were confirmed by Conedera *et al.* (1993), who showed that those varieties are morphologically homogenous and have a similar architecture of the crown. Also, the asexual propagation through grafting and crossing between trees affects the closeness of the genetic relationships among cultivars.

As for the intracultivar polymorphism, dendrograms obtained from RAPD and combined data separated Pinca and Berögna into distinct groups. This clustering may be explained by the low number of accessions per variety (3 individuals); moreover, those varieties are regionally distributed:

individuals from the former have the same centre of origin (Lodrino), so as individuals from the later have been sampled from the same area of origin (Vezio).

Mantel test indicated a high correlation between matrices based on AFLPs and RAPDs (r = 0.78). The high correlation confirmed the similarity of clustering cultivars by these types of markers. Also, a high Mantel's test correlation was found between RAPDs and ISSRs data (r = 0.75) (Tab. 2).

**Table2.** *(Mantel's r) Correlation coefficients of a distance matrix created by RAPDs, AFLPs and ISSRs markers. Correlations were highly significant (\*\*: P<0.01).*

| **AFLP** | | | |
|---|---|---|---|
| **ISSR** | 0.50** | | |
| **RAPD** | 0.78** | 0.75** | |
| **Combined data** | 0.48* | 0.26 | 0.36 |
| | **AFLP** | **ISSR** | **RAPD** |

The complementarity between the two methods in the identification of cultivars was confirmed by a high correlation between the two types of markers (r = 0.78). RAPD and AFLP markers also allowed us to solve problems generated by synonyms and homonyms in different accessions. Those methods allowed us to prove that some accessions with different names, which could have been considered as different cultivars, were actually closely related, showing a very similar or identical genotype, such as for example, in the case of Boné négro 07 that was suspected to be similar to Lüina cultivars. This result was confirmed by the dendrogram revealed by combined data. Method based on ISSR's discriminate not clearly accessions of cultivated Swiss cultivars. These results may be explained by the fact that AFLP and RAPD yielded a large number of polymorphic bands (222 and 98 respectively); while a low number of markers was produced by ISSR (35). This result was confirmed by the high correlation between combined data and AFLP (0.48) and between RAPD and AFLP data (0.78). Both are dominant markers but AFLP can generate a larger number of polymorphic bands per primer combination (222 vs. 98) in our study.

Moreover, the use of additional primers may contribute to better distinction among accessions (Cervera *et al.,* 1996).

## REFERENCES

[1] Abdelhamid, S., Công-Linh, L., Conedera, M., Kûpfer, Ph. (2014). The assessment of genetic diversity of *Castanea* species by RAPD, AFLP, ISSR and SSR markers. Turk J Bot, **38**, 835-850.

[2] Angela Martin, M., Mattioni, C., Cherubini, M., Taurchini, D., Villani, F. (2010). Genetic diversity in European chestnut populations by means of genomic and genic microsatellite markers. Tree Genet Genom, **6**, 735–744.DOI 10.1007/s11295-010-0287-9.

[3] Casasoli, M., Mattioni, C., Cherubini, M., Villani, F. (2001). A genetic linkage map of European chestnut (*Castanea sativa* Mill.) based on RAPD, ISSR and isozyme markers. Theor Appl Genet, **102**, 1190-1199.

[4] Casasoli, M., Derory, J., Morera-Dutrey, C., Brendel, O., Porth, I., Guehl, J.M., Villani, F., Kremer, A. (2006). Comparison of Quantitative Trait Loci for Adaptive Traits Between Oak and Chestnut Based on an Expressed Sequence Tag Consensus Map. Genetics, **172**, 533-546.

[5] Cervera, M.T., Gusmao, J., Steenackers, M., Van Gysel, A. (1996). Application of AFLP $^{TM}$ – based molecular markers to breeding of *Populus* spp. Plant growth Regulation, **20**, 47-52.

[6] Martín, M.A., Moral, A., Martín, L.M., Alvarez, J.B. (2007). The genetic resources of European sweet chestnut (*Castanea sativa* Miller) in Andalusia, Spain. Genet Resour Crop Evol, **54**, 379–387.

[7] Conedera, M., Jermini, M., Sassella, A. (1997). Nouvelles perspectives pour la culture du châtaignier au sud des Alpes. Revue Suisse Vitic Arboric Hortic. **29**, 01-07.

[8] Fineschi, S. , Taurchini, D., Villani, F., Vendramin, G.G. (2000). Choroplast DNA polymorphism reveals little geographical structure in Castanea sativa Mill. (Fagaceae) throughout southern European countries. Mol Ecol, 10, 1495-1504.

[9] Gobbin, D., Hohl, L., Conza, L., Jermini, M., Gessler, C., Conedera, M. (2007). Microsatellite-based characterization of the *Castanea sativa* cultivar heritage of southern Switzerland. Genome, **50**, 1089-1103.

[10] Goulão, L., Valdiviesso, T., Santana, C., Moniz Oliveira, C. (2001). Comparison between phenetic characterisation using RAPD and ISSR markers and phenotypic data of cultivated chestnut (*Castanea sativa* Mill.). Genet Resour Crop Evol, **48,** 329-338.

[11] Mancuso, S., Ferrini, F., Nicese, F.P. (1999). Chestnut (*Castanea sativa* Mill.) genotype identification: An artificial neural network approach. J Hortic Sci Biotech, **74**, 777-784.

[12] Mantel, N. (1967). The detection of disease clustering and a generalized regression approach. Cancer Res, **27**, 209-220.

[13] Mellano, M.G., Beccaro, G.L., Donno, D., Torello Marinoni, D., Boccacci, P., Canterino, S., Cerutti, A.K., Bounous, G. (2012). *Castanea* spp. biodiversity conservation: collection and characterization of the genetic diversity of an endangered species. Genet Resour Crop Evol, **59**, 1727–1741. DOI 10.1007/s10722-012-9794-x.

[14] McCleary, T., McAllister, M., Coggeshall, M., Romero-Severson, J. (2013). EST-SSR markers reveal synonymies, homonymies and relationships inconsistent with putative pedigrees in chestnut cultivars. Genet Resour Crop Evol, 60, 1209–1222. DOI 0.1007/s10722-012-9912-9.

[15] Oraguzie, N.C., Paterson, A.M., McNeil, D.L. (1999). Origin and relationships of New Zealand chestnut (*Castanea sp. Fagaceae*) selections reflect patterns of graft failure. Plant Syst Evol, **218**, 193-204.

[16] Page, R.D.M. (1998). Tree drawing software for Apple Macintosh and Microsoft Windows, Division of Environmental and Evolutionary Biology, Institute of Biomedical and Life Science, University of Glasgow.

[17] Pereira-Lorenzo, S., Fernandez-Lopez, J., Moreno-Gonzalez, j. (1996). Variability and grouping of Northwestern Spanish Chestnut cultivars.II. Isoenzyme traits. J Am Soc Hortic Sci, **121**, 190-197.

[18] Pereira-Lorenzo, S., Costa, R.M.L., Ramos-Cabrer, A.M., Ciordia-Ara, M., Ribeiro C.A.M., Borges, O., Barreneche, T. (2011). Chestnut cultivar diversification process in the Iberian Peninsula, Canary Islands, and Azores. Genome, **54**, 301–315. doi:10.1139/G10-122.

[19] Pereira, M.J., Castro, L.F., Torrea-Pereira, J.M., Lorenzo, S.P. (1999). Isozyme polymorphisms in Portuguese chestnut cultivars. Acta Hortic, **494**, 283-286.

[20] Pigliucci, M., Benedetelli, S., Villani, F. (1990). Spatial patterns of genetic variability in Italian chestnut ( *Castanea sativa*). Canadian J Bot, **68**, 1962-1967.

[21] Porebski, S., Bailey, G., Baum, R.B. (1997). Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. Plant Mol Biol Rep, **15**, 08-15.

[22] Seabra, C.R., Simöes, A.M., Baeta, J., Pais, M.S. (2001). Evaluation of Portuguese chestnut stands by RAPDs. For Snow Landsc Res, **76**, 435-438.

[23] Sneath, PH.A., Sokal, R.R. (1973). Numerical taxonomy. The principales and practice of numerical classification. W.H. Freeman and Co, San Francisco.

[24] Villani, F., Pigliucci, M., Benedettelli, S., Cherubini, M. (1991). Genetic differentiation among Turkish chestnut (*Castanea sativa* Mill.) populations. Heredity, **66,** 131-136.

[25] Yamamoto, T., Shimada, T., Kotobuki, K., Morimoto, Y., Yoshida, M. (1998). Genetic characterization of Asian chestnut varieties assessed by AFLP. Breeding Sci, 48, 359-363.

## AUTHORS' BIOGRAPHY

**S. Abdelhamid** is an associate professor at Olive Institute (Tunisia), he works on the genetic diversity of olive and chestnut by using molecular markers.

**A. Omri** is PhD student, she interested on olive oil authenticity by the use of molecular markers.

**A. Araouki** is PhD student, she interested on waste water olive studies.

**A. Sghair** is PhD student, she interested on the domestication of Tunisian olive varieties.