ASSESSMENT OF GENETIC VARIATION OF *TILIA TOMENTOSA* BY RAPD MARKERS

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Abstract: *Tilia tomentosa* varieties were analyzed using Random Amplified Polymorphic DNA (RAPD) primers to determine genetic diversity. In this study, six samples provided by the Botanical Garden "Anastasie Fătu" of Iasi, Romania were compared to a variety collected from an ecosystem in Giessen, Germany. A total of 91 bands were obtained from nine RAPD primers, 79 (86.8%) of which were polymorphic. Marker data was used for a principal component analysis that showed clear differentiation among Romanian and German samples. Furthermore, Romanian genotypes were clustered together in the principal component analysis. PCA analysis was followed by an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis which revealed two major groups. Samples from the Romanian ecosystem showed high genetic similarity. Generally, samples did not separate from each other in the UPGMA analysis, excepting the German sample.

Keywords: DNA, genetic diversity, principal component analysis, silver lime tree, UPGMA cluster analysis.

Introduction

Trees of the genus *Tilia* L. (*Malvaceae*) can be found in urban and forest ecosystems especially in the northern hemisphere in Europe, but also in Asia and Northeastern and Central America. Due to their polymorphism as well as a large number of transitional forms and hybrids, more than 60 species of *Tilia* are reported [WERYSZKO-CHMIELEWSKA & al. 2019]. The Plant List website reports more than 45 accepted *Tillia* species and a number of 477 lines and hybrids. This genus grows in temperate, subtropical and tropical climates and occurs from moist to dry regions [MUIR, 1984]. Linden trees could act as noise filters and purify the air through capturing the pollutants originating from traffic and different industrial activities. They reduce flooding and damage to urban property during stormwater runoff and also urban heat intensity [SJÖMAN & al. 2012]. As a result, representatives of these genera became increasingly important for local Romanian landscapes, in municipal parks in large cities or as avenue trees [SUKOPP & WURZEL, 2000].

In many European cities, growing damages of *Tillia tomentosa* Moench trees are caused by abiotic stress factors like salt and drought [Helsinki: TERHO & HALLAKSELA (2005), Warsaw: CHMIELEWSKI & al. (1998), Budapest: SINKO (2005)]. For this reason, in Europe since the 1960s *T. cordata* Mill. and *T. platyphyllos* Scop. were increasingly replaced by *T. tomentosa* Moench and *T. × euchlora* K. Koch originating from Southeastern Europe and the Caucasian region. These varieties are better adapted to urban conditions in Central Europe. Nowadays, breeding efforts should focus more on better adapted plant

material to ever-changing climatic challenges as high drought stress resistance, salt-resistance and tolerance to soil compaction for urban areas occur. (KARNOSKY & al. 1982).

Material and methods

Plant material

Leaf material was collected from "Teiul lui Eminescu" ("Eminescu's Linden") – an emblematic 500-year old tree for Romanian culture, being set in Copou Park in Iaşi, Romania (P5 in Table 1), three putative clones of P5 (P1 and P2 – cultivated in the Botanical Garden "Anastasie Fătu" of Iaşi since 1981 – in Table 1), P3 – being a clone of P1, cultivated in the Botanical Garden "Anastasie Fătu" of Iaşi since 2012 and offered by the city officialities to the people of Wien and a *T. cordata* Mill. sample (P4 in Table 1) available at the Botanical Garden of Iaşi, Romania, a *T. tomentosa* Moench samples from USAMV-Iaşi, Romania (P6, in Table 1) and one extra sample of *T.* × *euchlora* K. Koch collected from Giessen, Germany (P7 in Table 1). All samples from Iasi are very close to *T. tomentosa* regarding morphological traits, while P4 was identified previously as *T. cordata* and P7 as *T.* × *euchlora* by morphological traits. *T.* × *euchlora* (Caucasian or Crimean linden) is a hybrid whose parentage is unclear but is considered *T. cordata* and *T. dasystyla*. The different species far distant from the putative relatives of the interesting clones were integrated as outgroups to test the reliability of the analytic methods.

Table 1. Plant material collection of genera *Tilia*, including clones

No.	Species	Abbreviation	Location
1.	*T. tomentosa*	P1 (Clone)	Botanic Garden "Anastasie Fătu", Iaşi, RO
2.	*T. tomentosa*	P2 (Clone - atypical appearance)	Botanic Garden "Anastasie Fătu", Iaşi, RO
3.	*T. tomentosa*	P3 (Clone)	Botanic Garden "Anastasie Fătu", Iaşi, RO
4.	*T. cordata*	P4	Botanic Garden "Anastasie Fătu", Iaşi, RO
5.	*T. tomentosa*	P5 (*Teiul lui Eminescu – Eminescu's Linden*)	Parcul "Mihai Eminescu", Iaşi, RO ("Mihai Eminescu" Park)
6.	*T. tomentosa*	P6	USAMV "Ion Ionescu de la Brad", Iaşi, RO
7.	*T. euchlora*	P7 (*Krimlinde*)	Lonystrasse, Giessen, DE

Random Amplified Polymorphic DNA (RAPD) analysis

Fresh tissues were harvested in September 2017 in Iasi and Giessen. Leaves were frozen in liquid nitrogen and homogenized. DNA was isolated following the Doyle and Doyle DNA extraction protocol [DOYLE & DOYLE, 1990] DNA extraction efficiency was evaluated using the Qubit™ Fluorometer (Thermo Fisher Scientific Inc., USA) and NanoDrop™ 2000 spectrophotometric assays (Thermo Fisher Scientific Inc., USA) for specifically quantitate DNA.

A standard protocol was used to carry out the RAPD analysis. As a preliminary study, 12 arbitrary primers (Operon Technologies Inc., USA) were used for the amplification (Table 2). From these 12 primers, nine were selected for Polymerase Chain Reaction (PCR). PCR was

performed in 20 μl reaction volume containing 10-20 ng template DNA, dNTP mix (200 μM each nucleotide), 0.25 μM primer, reaction buffer (including $MgCl_2$ to a final concentration of 2.5 mM) and 1 Unit *Taq* DNA polymerase (Thermo Fisher Scientific Inc., USA).

The amplification reactions were carried out in a Thermocycler (Bio-Rad Laboratories, Inc., USA) with the following program: Initial denaturation 3 minutes at 94 °C; 35 cycles with 1 min 94 °C denaturation, 1 min 35 °C annealing, 1 min 72 °C elongation; final extension 10 min 72 °C; hold 4 °C.

PCR products were separated in 1% agarose gel with 1×Tris-borate-Ethylenediaminetetraacetic acid (EDTA) (TBE) – Life Technologies buffer. The gels were previously run at 120V for 2 hours and afterward were digitally photographed under UV light. The used marker was a 100-bp DNA ladder (Thermo Fisher Scientific Inc., USA). Replications of DNA extraction and amplification were performed with relevant primers and a subset of samples to confirm reproducibility.

Table 2. Molecular markers used for RAPD analysis of *Tilia* specimens

| No.	Primer code	Sequence (5'–3')
1.	R5	CTG GGG CTG A
2.	R8	GGT GGT GAT G
3.	R9	AAA GTG CGG C
4.	R12	AAT GCG GGA G
5.	R17	TGC TCT GCC C
6.	R18	GGT GAC GCA G
7.	L 12	GGG CGG TAC T
8.	OPA 03	AGT CAG CCA C
9.	OPB 10	CTG CTG GGA C
10.	OPE 20	AAC GGT GAC C
11.	OPG 14	GGA TGA GAC C
12.	OPD 11	AGC GCC ATT G

Data analysis

The RAPD bands were scored as present (1) or absent (0). A matrix of similarities between every pair of individuals was obtained by using the Euclidean distance index [GOWER, 1985]. Principal component analysis (PCA) was performed using the RAPD relationship between individuals using the R-code 3.3.3 (R Core Team, 2018) software and the "prcomp" function [VENABLES & RIPLEY, 2002]. Additionally, a dendrogram was generated based on Euclidean's similarity coefficients using the unweighted pair group method with calculating the arithmetic average (UPGMA) by R-code, "agnes{cluster}".

Results and discussions

A total of 12 RAPD primers were tested and nine of them produced suitable and reproducible banding patterns. We obtained 91 fragments that could be scored and used for further analysis. Out of the 91 fragments, 79 were polymorphic with an average of 8 polymorphic fragments per primer. The highest number of polymorphic bands (12) was obtained with primer L12 and R18, whereas the lowest number of bands was obtained with primers R5 (5) and OPG 14 (6).

Based on UPGMA analysis, two major groups were observed: P4 and P7 clustered together with a major group, whereas the other 6 genotypes were grouped together in another major group (Figure 1). The *T. tomentosa* clonal genotypes (P1, P2, P3 indicated in black color) were clustered together with the P5 ("Teiul lui Eminescu") genotypes in the first major group. Notably, the highest similarity value was found to be between genotype P1 and genotype P5. The dendrogram was in accordance with the geographic distributions of *Tilia* genotypes, by separating sample P4 and P7 from the group from Iași.
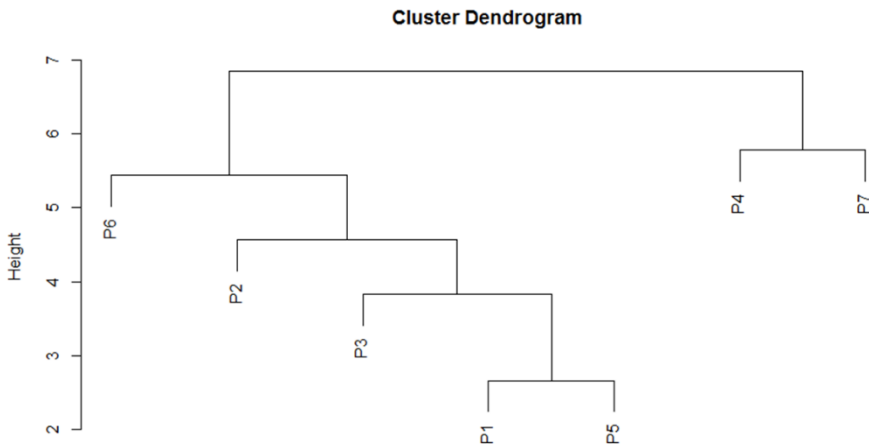
Figure 1. Dendrogram using the unweighted pair group method with calculating the arithmetic average (UPGMA) cluster analysis based on Euclidean similarities of 7 *Tilia* genotypes by using RAPDs (Abbreviations see Table 1).

PCA was performed to visualize the genetic relationship between *Tilia* genotypes (Figure 2). The first two principal components explain a large proportion of genotypic variance present in the data set, namely 46.2% (PC1) and 16.4% (PC2). A wide range distribution profile with two subplots with *Tilia* genotypes was obtained. Two subplots comprised Iasi genotypes (1), whereas the other one (2) comprised the genotypes from Giessen.
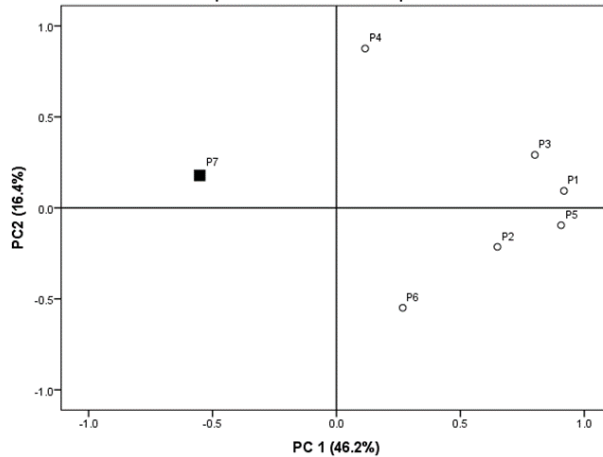
Figure 2. Principal component analysis plot of the RAPDs data among 7 genotypes of *Tilia*

In-plant sciences, biological diversity can be separated into three levels, such as genetic diversity, species diversity and ecosystem diversity [RAO & al. 2002]. Nowadays, tree biodiversity is altered by increased human intervention in natural habitats, fragmentation and loss of fertile soils, the introduction of new species in urban areas, climate change and many more [KOWARIK, 2011]. LOVELESS & HAMRICK (1984) suggested that genetic variations in plant populations are considered to be shaped by location and time. Trees resistance is supported by its lifespan, phenotypic plasticity, and gene diversity among populations. Up to date, the genetic studies realized with Tilia species have been limited [LIESEBACH & SINKO, 2008; FILIZ & al. 2015]. Thus, in this study, the genetic variations of *T. tomentosa* genotypes were investigated by using RAPD markers.

In this study, polymorphic bands and the average population diversity were found to be high in *T. tomentosa* by using nine different RAPD primers. In a similar study, conducted with *T. rubra* populations from the north of Iran, polymorphic bands and average population diversity were found to be 41.4% and 0.22, respectively, by using seven RAPD primers [COLAGAR & al. 2013]. In another study, *T. cordata*, *T. platyphyllos*, *T. dasystyla*, *T. euchlora*, *T. europaea*, *T. tomentosa*, *T. americana*, 36 genotypes were genotyped using 17 RAPD primers that yielded 403 polymorphic bands [LIESEBACH & SINKO, 2008]. Results were comparable with our findings, although higher polymorphism levels are generated by a wide gene pool of different *Tilia* taxa. Also, FILIZ & al. (2015) found 53 polymorphic fragments and average population diversity of 94.29% in *T. tomentosa* by using eight different RAPD primers. Our results were similar to *T. tomentosa* previous reports suggesting that eco-geographic characteristics and life history are important factors for genome structure in woody plant species.

The cluster analysis using the UPGMA dendrogram revealed a clear differentiation among genotypes based on geographical origin (Figure 1). The principal component analysis confirmed the UPGMA tree data and clustered P7 separately. As expected, P1 to P6 clustered together in the PCA. Clonal propagation results in the reduction of genotypic diversity and population differentiation, but, on the other hand, may increase heterozygosity [BALLOUX & al. 2003]. Moreover, asexual multiplication is critical for studying the effects of heterozygosity and genetic structure [HALKETT & al. 2005]. Most *Tilia* taxa are produced

through vegetative reproduction, summing approx. 77%-80% of the young trees in the southwest region of Russia and almost 100% in the north-east region of Europe [FILIZ & al. 2015].

Conclusions

The high rates of asexual reproduction of *T. tomentosa* could be clearly seen in the UPGMA tree and PCA results. Similar genetic structures could also explain the origin of *T. tomentosa* individuals as some are influenced by eco-geographical factors. A further breeding effort for *Tilia* trees should focus on crossings between parental lines with high-stress tolerance and adequacy to urban environments. Furthermore, the ability for vegetative multiplication should be included in the selection criterions for breeding purposes. Further studies are necessary for a good classification of reference material and further taxonomic investigation. New developments in the area of the molecular marker should be included used to obtain reliable results and sustainable outcomes, according to breeding objectives.

Notes on contributors

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