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Assessment of genetic variations of silver lime (*Tilia tomentosa* Moench.) by RAPD markers in urban and forest ecosystems

Ertugrul Filiz[a]*, Seda Birbilener[b], Ibrahim Ilker Ozyigit[c], Semsettin Kulac[d] and Fatma Cigdem Sakinoglu Oruc[a]

[a]*Department of Crop and Animal Production, Cilimli Vocational School, Duzce University, 81750 Cilimli, Duzce, Turkey;* [b]*Institute of Pure and Applied Sciences, Biology Department, Marmara University, 34722 Goztepe, Istanbul, Turkey;* [c]*Department of Biology, Faculty of Science and Arts, Marmara University, 34722 Goztepe, Istanbul, Turkey;* [d]*Department of Forest Engineering, Faculty of Forestry, Duzce University, 81620 Duzce, Turkey*

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In the present study, the genetic diversity analysis of *Tilia tomentosa* plants was performed by using random amplified polymorphic DNA (RAPD) primers. Twenty eight plant samples, collected from urban (25 members) and forest (3 members) ecosystems, were used in this study. A total of 53 bands were obtained from eight RAPD primers, of which 48 (90.6%) were polymorphic. The percentage of polymorphic loci (*P*) was found to be 94.29%, the observed number of alleles (Na) was 1.94, the effective number of alleles (Ne) was 1.60, Nei's gene diversity (*h*) was 0.34 and Shannon's information index (*I*) was 0.50. Unweighted pair group method with arithmetic mean (UPGMA) cluster analysis revealed two major groups. Members of the urban and forest ecosystems showed high genetic similarity (28%–92%) and they did not separate from each other in UPGMA tree. Furthermore, urban and forest genotypes clustered together in principal component analysis.

Keywords: genetic diversity; principal component analysis (PCA); UPGMA; urban ecosystem

Introduction

Lime tree (*Tilia* spp.), which is a member of Malvaceae family, is a long-lived tree that can live up to 500 years. They can grow in loamy sites, but can also be found on sandy and infertile soils.[1,2] *Tilia* species are found in the forests of the northern hemisphere in Europe, Asia, Central and Eastern North America.[3] Naturally, four *Tilia* species are found in Europe: Caucasian lime –*Tilia dasystyla* (Stev.), silver lime –*Tilia tomentosa* (Moench.), small-leaved lime –*Tilia cordata* (Mill.) and large-leaved lime –*Tilia platyphyllos* (Scop.).[4] *T. tomentosa* and *T. dasystyla* are distributed in south-eastern Europe and around the Black Sea regions.[2]

Deciduous trees are widely used as ornamentals in municipal parks and avenues of large cities.[5] Climatic conditions and human impact have affected the distribution of *Tilia* species in Europe in the past 2000 years. Farming and low seed fertility are the primary reasons for the decline of *Tilia* species in Europe.[2] Up to now, *Tilia* species have been used in various genetic studies, which include *random amplified polymorphic DNA* (RAPD) markers,[6,7] *simple* sequence repeats markers,[8] chloroplast DNA markers,[9] isozyme markers [10,11] and *internal* transcribed spacer sequences.[12] The assessment of the genetic diversity is a key factor for establishing a long-term conservation in their natural habitat.[13] RAPD technique is one of the efficient methods for determining genetic similarities and inter- and intra-species relationship degrees.[6,14] In this study, selected *T. tomentosa* genotypes (individuals) from different regions of the urban ecosystem in Duzce city, Turkey, were evaluated by using RAPD markers to understand the genetic variation level of *T. tomentosa* living in urban areas.

Materials and methods

Study area and plant materials

Duzce is located on the west of the Black Sea region (N 40° 50′ 30″, E 31° 09′ 30″) on the north-western part of Turkey and it covers 3.641 km^2 land area (Figure 1). The area is affected by the Black Sea climatic conditions with hot summers and cold and humid winters, thus mostly humid forest vegetation covers the area.[15] The average annual temperature is 13 °C, the average annual rainfall is 825 mm and the average relative humidity is 75%.[16]

The leaf samples were collected from 28 different individuals of *T. tomentosa* in Duzce (Figure 1). Twenty five of the collected samples were from the city centre (indicated as A1, A2, A3, A4, M2, M4, M6, M7, M8, M9, M10, M11, M12, M13, K1, K2, K3, K4, K5, F, Ç2, Ç3, Ç4, Ç5 and O) and three of them were from the forest (indicated as Y2, Y3 and Y5) as out-group samples. The

*Corresponding author. Email: ertugrulfiliz@duzce.edu.tr

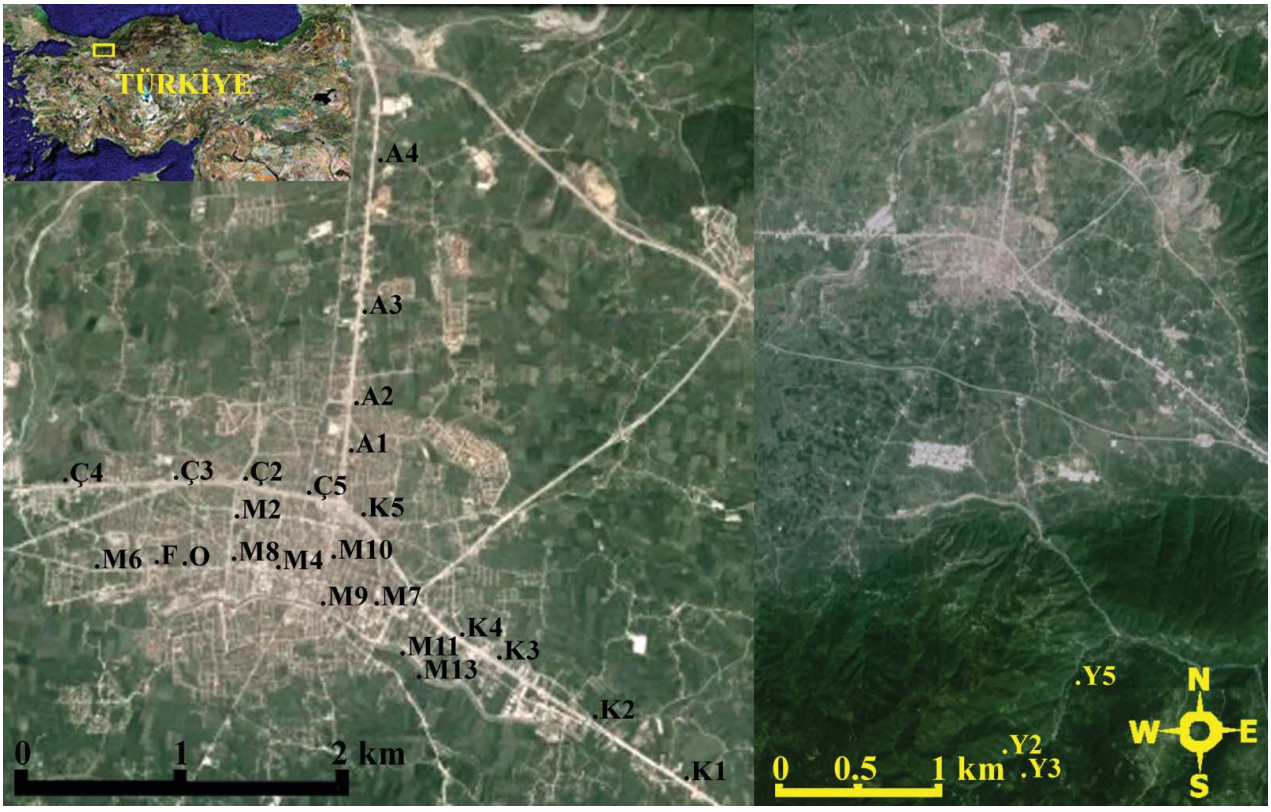

Figure 1. The geographic location of *T. tomentosa* samples.

collected leaves were stored at −80 °C until the DNA extraction process.

DNA isolation and RAPD reactions

Total genomic DNA was isolated from the frozen leaf material by using GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA). The DNA concentration of each sample was determined by using BioSpec-nano (Shimadzu, Japan) and the elutions were diluted with sterile distilled water to a final concentration of 50 ng μL^{-1}. As a preliminary study, 25 RAPD primers (Operon Technologies Inc.) were tested and eight of them were selected for polymerase chain reaction (PCR) (Table 1).

RAPD reactions were performed in a final volume of 25 μL, including 2.5 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} dNTPs, 0.5 μmol L^{-1} primers, 1 U *Taq* DNA polymerase (Thermo Sci, USA), 2.5 μL 10 × *Taq* DNA polymerase buffer (pH 8.3) and 50 ng template DNA. The amplification was performed in a programmed thermo cycler (Thermo Scientific, USA) with initial denaturation at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 36 °C for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. The amplification products were separated by electrophoresis in a 1% agarose gel with 1 × Tris-borate-Ethylenediaminetetraacetic acid (EDTA) (TBE) −Life Technologies buffer and digitally photographed under UV light. The used marker was a 100-bp DNA ladder (Thermo Sci, USA).

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 Jaccard similarity index.[17] The data were analysed using Popgene version 1.31 [18] and MVSP 3.2 (multi-variate statistical package). The percentage of polymorphic loci (*P*), mean number of observed (Na) alleles and effective (Ne) alleles per locus, [19] Nei's gene diversity (*h*) [20] and Shannon's information index (*I*) [21] were calculated as genetic parameters. A principal component analysis (PCA) test, which is providing a graphical representation of the RAPD relationship between individuals, was demonstrated with the variance-covariance matrix calculated from marker data using MVSP 3.2 program. A dendrogram was generated based on Jaccard's similarity coefficients [17] using the unweight pair group method with calculating the arithmetic average (UPGMA) by MVSP 3.2.

Results and discussion

A total of 25 RAPD primers were tested and eight of them produced suitable and reproducible banding patterns. We

Table 1. List of the used primers for the RAPD-PCR reactions.

No.	Primer code	Sequence (5′−3′)	Annealing temperature (°C)	Total number of bands	Number of polymorphic bands	Polymorphic bands (%)
1	R5	CTGGGGCTGA	35	6	6	100
2	R8	GGTGGTGATG	35	5	5	100
3	R9	AAAGTGCGGC	35	6	6	100
4	R12	AATGCGGGAG	35	8	7	87.5
5	R16	TGCGCCCTTC	35	7	6	85.7
6	R17	TGCTCTGCCC	35	6	5	83.3
7	R18	GGTGACGCAG	35	8	7	87.5
8	R23	TCGGCGATAG	35	7	6	85.7
	Total	–	–	53	48	–

Note: The polymorphism percentages were obtained by analysing 28 genotypes of *T. tomentosa*.

obtained 53 fragments that could be scored (Table 1). Out of the 53 fragments, 48 were polymorphic with an average of 6 polymorphic fragments per primer. The highest number of polymorphic bands (7) was obtained with primer R12 and R18, whereas the lowest number of bands (5) was obtained with primers R8 and R17. An example for RAPD banding patterns is given for primer R17 in Figure 2. The percentage of *P* was found to be 94.29%. Also, the observed number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity (*h*) and Shannon's information index (*I*) were found to be 1.94, 1.60, 0.34 and 0.50, respectively.

Based on UPGMA analysis, two major groups were observed: O and M12 were clustered together in the first major group, whereas the other 26 genotypes were grouped together in major group two (Figure 3). The forest genotypes (Y2, Y3 and Y5, indicated in blue colour) were clustered together with the urban genotypes in the second major group. Notably, the highest similarity value was found to be between forest genotype Y5 and urban genotype Ç3 with 92.3%, followed by Y2−Ç3 with 84% and Y3−K4 with 75%. In addition, the lowest similarity value was found to be between forest genotype Y3 and urban genotype M11 with 28%, followed by Y2−M11 with 32% and Y5−M11 with 37%. In the urban genotypes, the highest similarity was found to be 87.5% between A3−K4, followed by M8−M10 with 87% and M7−M10 with 86.7%, whereas the lowest value was 20% between M12−M13. The dendrogram was not in accordance with the geographic distributions of *Tilia* genotypes.

PCA was performed to visualize the genetic relationship between *Tilia* genotypes (Figure 4). Wide range distribution profile with three subplots with forest and urban genotypes was obtained. Two subplots (2 and 3) comprised urban genotypes, whereas the other one (1) comprised both urban and forest genotypes. The members of subplots 2 and 3 were separated from the other genotypes.

Biological diversity may be indicated at three levels, such as genetic diversity, species diversity and ecosystem diversity.[22,23] Urban biodiversity is altered by habitat fragmentation and loss, introduction of new species, urban climate change, soil type, hydrology and biogeochemical cycles.[24,25] Urban trees are fundamental structural components in the urban environment and are important factors for determining the ecological function of the urban forests.[26−28] Genetic variations in plant populations are considered to be shaped by location and time.[29] Long lifespan, phenotypic plasticity, gene flux among populations (pollen and seeds) support the resistance of trees.[30] Up to date, the genetic studies realized with *Tilia* species have been limited.[6,7] Thus, in this study,

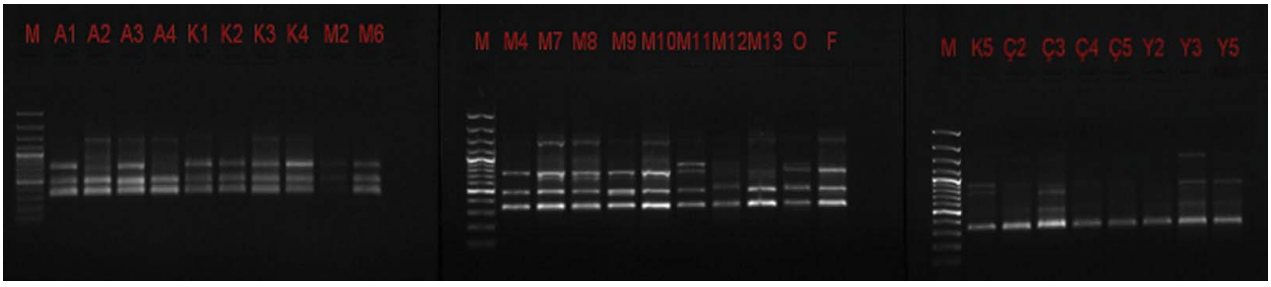

Figure 2. RAPD patterns of 28 *Tilia* genotypes with primer R17.
Note: 100 bp (base pair) gene ruler (M).

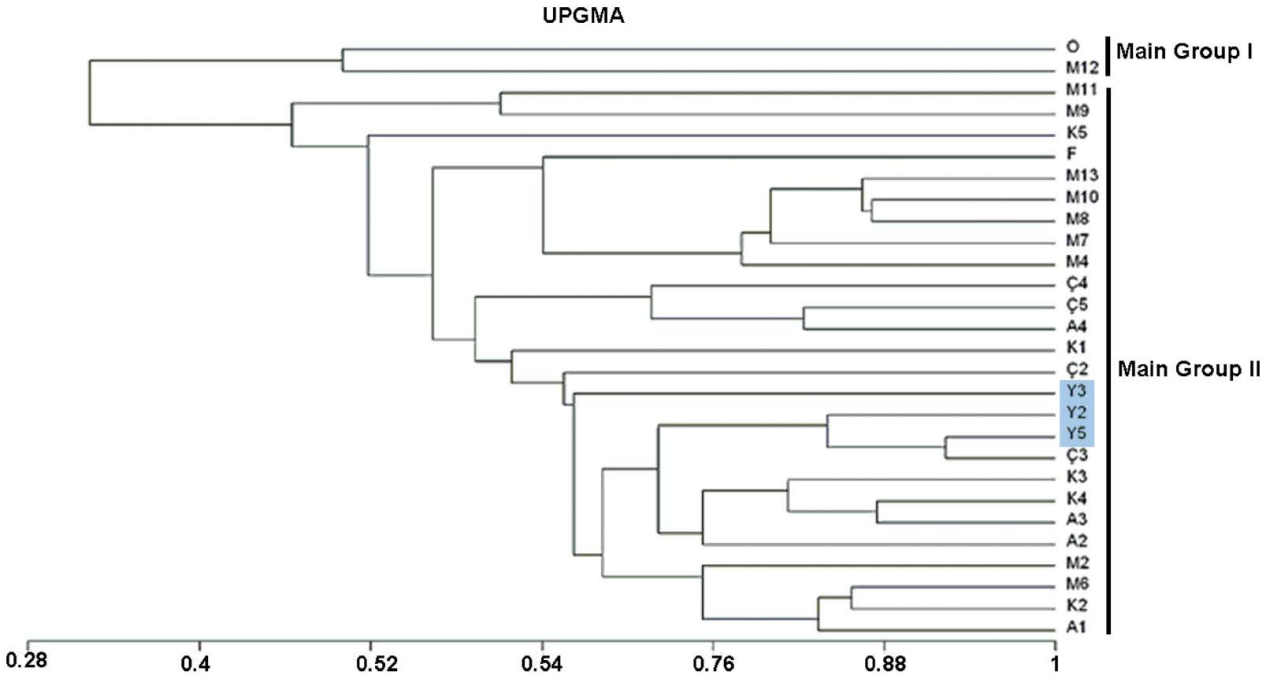

Figure 3. Dendrogram of the 28 *T. tomentosa* genotypes by using RAPDs data with UPGMA cluster analysis.
Note: The *x*-axis shows similarity values. Blue colour indicates the forest genotypes, whereas the others resembled genotypes from the urban ecosystem.

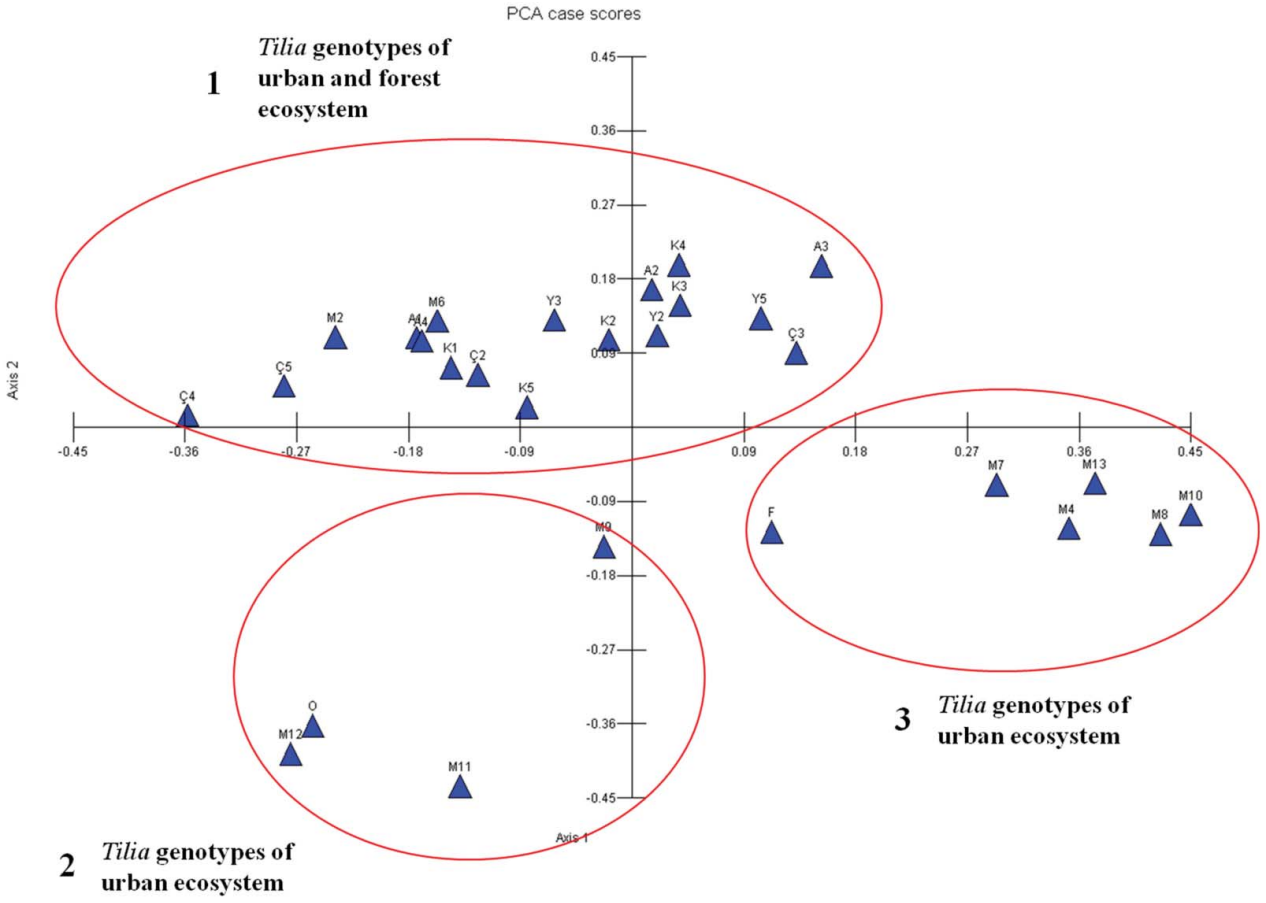

Figure 4. Principal component analysis plot of the RAPDs data among 28 genotypes of *T. tomentosa*.

the genetic variations of *T. tomentosa* genotypes were investigated by using RAPD markers in urban ecosystem.

In this study, polymorphic bands and the average population diversity were found to be 94.29% and 0.50, respectively in *T. tomentosa* by using eight different RAPD primers. In a similar study, conducted with *T. rubra*, populations from Hyrcanian forests of north of Iran, polymorphic bands and average population diversity were found to be 41.4% and 0.22, respectively, by using seven RAPD primers.[7] Our values were two times higher than those of *T. rubra*. Ecogeographic factors and breeding systems can affect the genome structure of plants.[22] Also, life history and ecological characteristics are important factors for shaping the genetic structures in woody plant species.[31] These factors may affect the genetic variations of *T. tomentosa* in urban ecosystems. In another study, *Tilia* taxa, such as *T. cordata*, *T. platyphyllos*, *T. dasystyla*, *T. euchlora*, *T. europaea*, *T. tomentosa*, *T. americana*, included 36 genotypes, 403 polymorphic bands, obtained by using 17 RAPD primers and the mean number of bands per primer was about 23.7.[6] These findings were higher than our results and it can be suggested that wide gene pool of different *Tilia* taxa may cause high polymorphism levels. There are some other studies, conducted with different woody plant species, which performed genetic diversity analysis by using RAPD markers, including *Robinia pseudoacacia*,[32] *Ginkgo biloba*,[33] *Pinus halepensis*,[34] *Pinus longaeva*,[35] *Pinus oocarpa* [36] and *Populus euphratica*.[37] In our study, the amount of RAPD variations observed in *T. tomentosa* ($h = 0.34$) was lower than that in *G. biloba* ($h = 0.44$) and *P. oocarpa* ($h = 0.358$), whereas it was higher than that in *P. halepensis* ($h = 0.32$ and 0.30), *P. longaeva* ($h = 0.134$) and *P. euphratica* ($h = 0.124$).[32–37]

The UPGMA dendrogram revealed that urban and forest genotypes did not separate from each other (Figure 3) and Y5 (forest genotype) and Ç3 (urban genotype) showed the highest similarity rate −92.3%. The PCA confirmed the UPGMA tree data. The rates of asexual reproduction could result in the reduction of genotypic diversity and population differentiation, but, on the other hand, may increase heterozygosity. Furthermore, clonal propagation is also critical for the effects on heterozygosity and genetic structure.[38–40] Vegetative reproduction is more common than generative reproduction in *Tilia* taxa. Nearly 77%–80% of the young trees in the southwest region of Russia and almost 100% in the north-east region of Europe were detected as a result of vegetative reproduction.[41,42]

Conclusions

The high similarity between urban and forest genotypes in UPGMA tree could be a result from the high rates of asexual reproduction in the life history of *T. tomentosa* and showed that similar genetic structures appear in genotypes of different ecosystems. This situation could also explain the origin of *T. tomentosa* individuals. The ones, collected from urban areas, could be generated by vegetative reproduction by using *Tilia* seedlings originated from forests. However, as a result of the fast urbanization and enlargement of the city through the forests, the city partly covered the forest and some individuals from the forest became placed in the urban ecosystems. Based on low similarity values among the urban genotypes, it can be suggested that sexual reproduction in *Tilia* genotypes may cause low genetic similarities among genotypes.

Disclosure statement

No potential conflict of interest was reported by the authors.

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