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Genetic diversity and phylogenetic analysis of *Robinia pseudoacacia* L. populations using ISSR markers, ITS1 and trnL-F intergenic spacer sequences

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Abstract: *Robinia pseudoacacia* L. is a deciduous tree planted almost all around the world for a wide variety of uses such as ornamental in urban ecosystems and forest trees in afforestation. This study aims to evaluate the genetic diversity and phylogenetic relations of *R. pseudoacacia* using some selected populations in Istanbul and Kocaeli cities. For this aim, molecular marker-assisted and DNA sequence-based analyses were performed. According to the results, nine of 15 inter simple sequence repeats (ISSR) primers gave clear and distinguishable bands with a total of 100 loci. The percentage of polymorphic loci (*PPL*) was calculated as 100% for multi-populations and ranged from 46% to 76% for single populations. Nei's gene diversity value was calculated between 0.165 and 0.251. The lowest and highest *PPL* were found in populations of Barbaros Boulevard and Dilovası District, respectively. Population structure analysis showed seven different genetic structures for five populations. Internal transcribed spacer 1 region (ITS1) and trnL-F intergenic spacer region were used to examine the phylogenetic relationships of *R. pseudoacacia*, and both regions showed a high discriminative power at the family level. Based on the findings, *R. pseudoacacia*, as a forest tree residing in the urban ecosystem, may face the risk of population decline in the upcoming years due to its moderate/low genetic diversity and susceptibility to environmental pressures.

Keywords: DNA barcoding; forest tree; molecular marker; molecular phylogeny; urban population

Genetic diversity is crucial to the survival of the species under ever-changing environmental conditions (Govindaraj et al. 2015). Therefore, the information obtained from genetic diversity analyses has been very useful for breeding programs, germplasm diversification studies and conservation strategies (Filiz et al. 2015; Ebert, Engels 2020). In this sense, molecular markers are suitable tools for assessing genetic diversity in species. As one of the frequently used DNA markers, inter simple sequence repeats (ISSR) is widely used in plants (Filiz et al. 2018; Shaygan et al. 2021). ISSR is a polymerase chain reaction (PCR)-based method that targets areas between microsatellites and can produce highly polymorphic, informative and reproducible bands (Nadeem et al. 2018; Nasim et al. 2020). *Robinia*

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pseudoacacia L. is a perennial tree of the *Fabaceae* family, one of the most widely cultivated woody angiosperms in the world, and is sometimes considered an invasive species due to its ability to adapt to almost any ecosystem (Cierjacks et al. 2013; Moser et al. 2016; Wang et al. 2018). Although there are many ISSR-based molecular studies in the current literature, ISSR/SSR-based studies are not sufficient for *R. pseudoacacia*. Sun et al. (2009) conducted a genetic diversity study with 10 populations and 100 individuals using ISSR markers and reported that the genetic diversity of *R. pseudoacacia* was not correlated with geographical patterns. Guo et al. (2017; 2018; 2022a, b) developed and applied SSR markers for the assessment of genetic differentiation and diversity of *R. pseudoacacia*. Plant leaves were collected from seedlings covering 19 provenances from its natural distribution in China. At the end of these studies, the authors stated that (*i*) there was a high level of genetic diversity resulting from genetic variation within the populations, and (*ii*) geographic distribution among native *R. pseudoacacia* populations was not an important factor affecting genetic diversity. Another study reported a high level of genetic diversity among seven *R. pseudoacacia* populations from China and evaluated genetic diversity using SSR markers (Yang et al. 2020). The available literature for the molecular phylogeny of *R. pseudoacacia* based on internal transcribed spacer 1 region (ITS1) and trnL-F intergenic spacer region (trnL-F IGS) is limited, therefore this study attempts to fill in the gaps by analysing the phylogenetic relationships of *R. pseudoacacia* populations by sequencing ITS1 and trnL-F IGS. The main objectives of this study are (*i*) to analyse the genetic diversity of *R. pseudoacacia* populations in the provinces of Istanbul and Kocaeli, (*ii*) to understand the structure of the populations, and (*iii*) to reveal the phylogenetic relationships between *R. pseudoacacia* genotypes and its close relatives.

MATERIAL AND METHODS

Plant sample collection. Plant samples were collected from five different stations: Prince Islands (PRI/ADA), Bagdad Avenue (BAG), Barbaros Boulevard (BAR), Dilovası District (DIL), and Trans European Motorway (TEM). While the BAG, BAR, and TEM regions were selected as congested locations, PRI was specifically selected because it is a congestion-free location in Istanbul, and DIL is a specifically selected location for heavy industrial activities in Kocaeli/Turkiye (Figure 1).

Figure 1. Map of sampling stations showing the population of BAR (1), BAG (2), PRI (3), TEM (4), and DIL (5)

BAR – Barbaros Boulevard; BAG – Bağdat Avenue; PRI – Prince Islands; TEM – Trans European Motorway; DIL – Dilovası District
Source: The map was prepared with Google Earth software (Version 10.43.0.2, 2023)

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The total number of samplings was 58 genotypes/individuals. The GPS coordinates of the samples are given in the Electronic Supplementary Material (ESM), Tables S1–S5.

Genomic DNA isolation and amplification. Total genomic DNA was extracted from plant leaf samples using the cetyltrimethylammonium bromide (CTAB) method with some modifications (Doyle 1991). Modifications were applied as follows; using 0.2 to 0.5 g of fresh leaf sample without the addition of 2-mercaptoethanol, incubated for 60 min at 60 °C and centrifuged at 13 000 rpm. We used ultrapure water for the rehydration of the extracted DNA after air drying. The quality and quantity of DNA was analysed using NanoDrop (OPTIZEN NanoQ micro-volume photometer; Mecasys, South Korea). The integrity of the DNA was checked by ethidium bromide staining on a 1% *w*/*v* agarose gel in 1X-Tris-borate EDTA (TBE) buffer. PCR mixture for each ISSR, ITS, and trnL-F IGS analysis was 25 µL in total volume, including 2.5 µL 10 × PCR buffer, 1.25 µL primer (10 µM each), 2.5 µL 25 mM $MgCl_2$, 2 µL 2.5 mM each dNTP, 0.25 µL 5U TaqDNA Polymerase, 1 µL DNA template, and 15.5 µL ultrapure water. The sequences were amplified using a thermal cycler (Aeris Model 96; Esco, Singapore) programmed for 35–45 cycles of denaturation at 94 °C for 60 s, annealing at a specified temperature (refer to Table 1) for 60 s, and extension at 72 °C for 60 s, followed by a final extension at 72 °C for 7 min. Amplifications were carried out using two ITS (White et al. 1990), two trnL-F IGS (Taberlet et al. 1991) and 15 ISSR (Nagaoka, Ogihara 1997) primers (Table 1). PCR products were separated by 1.2% agarose gel electrophoresis with 1X TBE. DNA bands were visualised using a UV-transilluminator (Vilber Lourmat, France) and WiseUV WUV-L20 UV-transilluminator (Wisd-Witeg Laboratory Equipments, Germany).

ISSR analysis. Amplified ISSR bands were separated on agarose gel and visualised for scoring. Genetic parameters such as the percentage of polymorphic loci (*P*), the mean number of observed alleles (*Na*), effective alleles per locus (*Ne*), Nei's gene diversity (*h*), and Shannon's information index (*I*) were calculated using POPGENE (Version 1.32; Yeh, Boyle 1997). A dendrogram was generated using the unweighted pair group method (UPGMA) based on Jaccard's similarity coefficients by the

Table 1. Primer sequences and annealing temperatures used in the amplification of ITS, trnL-F IGS regions, and ISSR in *Robinia pseudoacacia*

Primer	Sequences	Annealing temperature (°C)
ITS1	TCCGTAGGTGAACCTGCGG	48
ITS2	GCTGCGTTCTTCATCGATGC	48
trnL-F forward	AAAATCGTGAAGGTTCAAGTC	48
trnL-F reverse	GATTTGAACTGGTGACACGAG	48
ISSR1/UBC807	AGAGAGAGAGAGAGAGT	48
ISSR2/UBC811	GAGAGAGAGAGAGAGAC	53
ISSR3/UBC817	CACACACACACACACAA	50
ISSR4/UBC818	CACACACACACACACAG	53
ISSR5/UBC820	GTGTGTGTGTGTGTGTC	53
ISSR6/UBC823	TCTCTCTCTCTCTCTCC	53
ISSR7/UBC827	ACACACACACACACACG	53
ISSR8/UBC825	ACACACACACACACACT	54
ISSR9/UBC848	CACACACACACACACARG	56
ISSR10/UBC849	GTGTGTGTGTGTGTGTYA	54
ISSR11/UBC855	ACACACACACACACACYT	54
ISSR12/UBC842	GAGAGAGAGAGAGAGAYG	56
ISSR13/UBC875	CTAGCTAGCTAGCTAG	59
ISSR14/UBC829	TGTGTGTGTGTGTGTGC	49
ISSR15/UBC844	CTCTCTCTCTCTCTCTRC	56

ITS – internal transcribed spacer; trnL-F IGS – trnL-F intergenic spacer region; ISSR – inter simple sequence repeats

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Multi-Variate Statistical Package MVSP (Version 3.2, 2013). Principal component analysis (PCA) showing the variance-covariance matrix from the marker data was performed using the MVSP package. Parameters such as polymorphic information content (*PIC*), marker index (*MI*), resolving power (*Rp*) and effective multiplex Ratio (*E*) were calculated to estimate the suitability of ISSR primers for genetic profiling (Ivanovych et al. 2017). STRUCTURE software (Version 2.3.4; Pritchard et al. 2000; Evanno et al. 2005) was used for inferring population structures. Analyses were conducted on an admixture model, with simulations of one to 10 inferred subpopulations (*K*) using a burn-in period of 10^4 replicates with 5×10^5 Markov Chain Monte Carlo (MCMC) simulations. Structure Harvester (Version 0.6.94, 2012) was used to identify meaningful values for the number of possible subpopulations (ΔK; Earl, VonHoldt 2012).

Sequencing and phylogenetic analysis. PCR products of ITS1 and trnL-F IGS regions were sequenced by Iontek Molecular Diagnostics (Türkiye). Sequencing was performed for the DNA of three random samples from each station. Sequenced DNAs were aligned by using ClustalW in BioEdit (Version 7.2.5, 1999) with default parameters (Hall 1999; Larkin et al. 2007). The phylogenies were constructed by MEGA X (Version 10, 2021) using the maximum likelihood (ML) method with 1 000 bootstrap replications (Tamura et al. 2013; Kumar et al. 2018). Phylogenetic trees were edited by using the online tool ITOL (Version 4; Letunic, Bork 2016).

RESULTS

ISSR band analysis. Among the 15 ISSR primers used, 9 of them produced visible and distinguishable bands, ranging from 200–1 800 bp in size (Table 2). A total of 100 ISSR bands were amplified, with an average of 11.1 bands per primer. When all 58 individuals were considered as a single population, the bands showed a 100% polymorphism rate. The number of loci per primer ranged from 8 (UBC820) to 13 (UBC818 and UBC823). In addition, polymorphic information content (*PIC*) was calculated as the highest (0.447) for primer UBC820 and the lowest (0.272) for primer UBC855. The resolving power (*Rp*) was calculated as the highest for primer UBC825 (12.97) and the lowest for primer UBC827 (5.66). The marker index (*MI*) was determined to be highest at 4.96 for primer UBC820 and lowest at 3.02 for primer UBC855. The effective multiplex ratio (*E*) had a standard value of 11.11 for all primers.

Furthermore, genetic variations within all populations (at multi-population level) and within every single population (station) were calculated separately using diploid ISSR data (Table 3). According to statistical data obtained from all individuals of studied populations; the percentage of polymorphic loci (*PPL*), the observed number of alleles (*Na*), the effective number of alleles (*Ne*), Nei's gene diversity (*h*), Shannon's information index (*I*), total gene diversity (*Ht*), gene diversity in the population (*Hs*), genetic differentiation coefficient (*Gst*),

Table 2. Notable ISSR primers (9 out of 15) and discriminatory values in *Robinia pseudoacacia* genotypes

No.	ISSR primer	Amplicon size (bp)	Total number of bands	Polymorphic bands	Polymorphism ratio (%)	Polymorphic information content (*PIC*)	Resolving power (*Rp*)	Marker index (*MI*)	Effective multiplex ratio (*E*)
1	UBC811	200–1 100	11	11	100	0.393	10.520	4.360	11.11
2	UBC818	200–1 100	13	13	100	0.371	7.210	4.130	11.11
3	UBC820	300–1 200	8	8	100	0.447	8.100	4.960	11.11
4	UBC823	300–1 200	13	13	100	0.322	10.030	3.570	11.11
5	UBC827	200–1 100	10	10	100	0.316	5.660	3.520	11.11
6	UBC825	250–1 300	12	12	100	0.347	12.970	3.850	11.11
7	UBC849	200–1 200	12	12	100	0.301	11.930	3.340	11.11
8	UBC855	300–1 200	10	10	100	0.272	8.900	3.020	11.11
9	UBC842	300–1 000	11	11	100	0.340	9.380	3.770	11.11
Average			11.11	11.11	100	0.345	9.410	3.837	11.11

ISSR – inter simple sequence repeats

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Table 3. Multi-population and single-population descriptive statistics

Populations	*PPL* (%)	*Na*	*Ne*	*h*	*I*	*Ht*	*Hs*	*Gst*	*Nm*
Multi-population (*N* = 58)	100	2.0000	1.5325	0.3169	0.4811	0.3169	0.2220	0.2993	1.1703
Single-population									
DIL (*N* = 10)	76	1.7600	1.4192	0.2511	0.3809	–	–	–	–
PRI (*N* = 11)	73	1.7300	1.4186	0.2454	0.3693	–	–	–	–
TEM (*N* = 13)	59	1.5900	1.4122	0.2317	0.3388	–	–	–	–
BAG (*N* = 12)	58	1.5800	1.3796	0.2166	0.3200	–	–	–	–
BAR (*N* = 12)	46	1.4600	1.2873	0.1654	0.2454	–	–	–	–

DIL – Dilovası District; PRI – Prince Islands; TEM – Trans European Motorway; BAG – Bağdat Avenue; BAR – Barbaros Boulevard; *N* – number of genotypes; *PPL* – percentage of polymorphic loci; *Na* – observed number of alleles; *Ne* – effective number of alleles; *h* – Nei's gene diversity; *I* – Shannon's information index; *Ht* – total gene diversity; *Hs* – within population gene diversity; *Gst* – genetic differentiation coefficient; *Nm* – gene flow

and gene flow (*Nm*) were calculated as 100%, 2.0, 1.5325, 0.3169, 0.4811, 0.3169, 0.2220, 0.2993, and 1.1703, respectively.

The findings showed that the DIL population had the highest genetic diversity among the sampling stations, whereas the BAR population had the lowest genetic diversity. In a similar study, Sun et al. (2009) calculated the ISSR based *PPL*, *h*, and *I* values as 93.41%, 0.4337, and 0.6145, respectively. They also concluded that intrapopulation genetic variation is relatively higher compared to interpopulation. The *PPL*, *h*, and *I* values of the mentioned study were higher than the results of this study.

Genetic distance is defined as the genomic diversity between species or populations (Neale, Wheeler 2019). Here, a dendrogram based on Nei's (1987) genetic distance was constructed using the UPGMA method to reveal genetic distances between populations. It was revealed that PRI and BAR genotypes were the closest groups genetically with a distance value of 1.7830, while TEM was the most distant group with a distance value of 10.9899 (Figure 2). Supportively, unbiased genetic distance measures of the lowest and highest Nei's were also distributed between 0.0357 (between PRI and BAR) and 0.2659 (between TEM and DIL). Furthermore, genetic identity ranged from 0.7665 (between TEM and DIL) to 0.9650 (between PRI and BAR).

In the PCA analysis, the distribution pattern of the *R. pseudoacacia* genotypes in Figure 3 showed

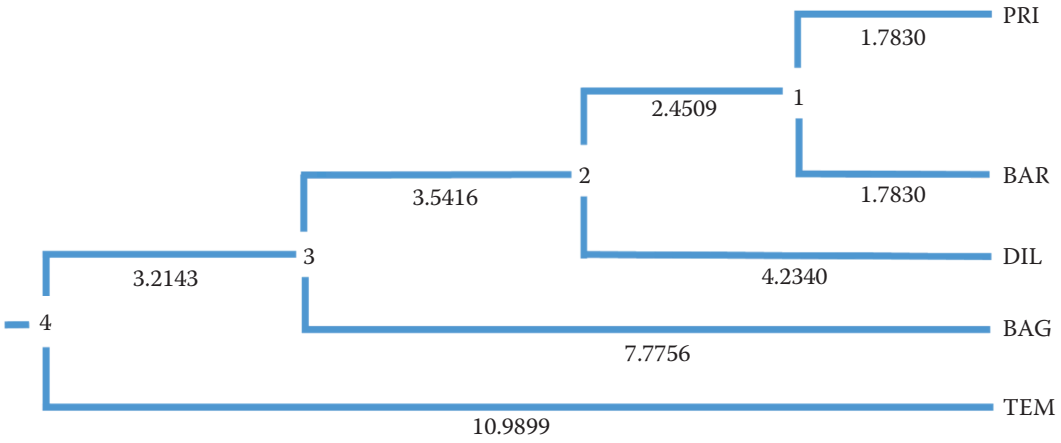

Figure 2. Dendrogram showing genetic distances between sampling stations, constructed based on Nei's (1978) genetic distance using the UPGMA method

The values on branches indicate genetic distances in each population; UPGMA – unweighted pair group method; PRI – Prince Islands; BAG – Bağdat Avenue; BAR – Barbaros Boulevard; DIL – Dilovası District; TEM – Trans European Motorway

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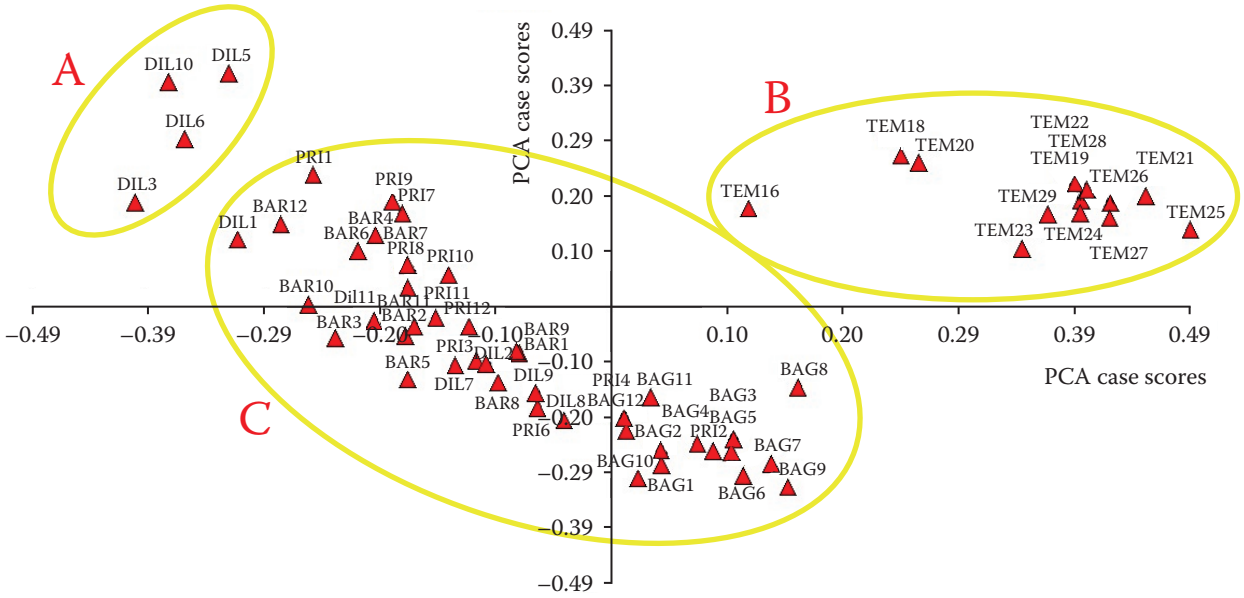

Figure 3. Principal component analysis (PCA) of *Robinia pseudoacacia* genotypes from sampling stations

Red triangles show individuals/genotypes; BAG – Bağdat Avenue; BAR – Barbaros Boulevard; DIL – Dilovası District; PRI – Prince Islands; TEM – Trans European Motorway

Source: The graph was generated by using MVSP software (Version 3.2, 2013)

three main groups, A, B, and C. Group A included only four genotypes/individuals from the DIL population. However, all genotypes in the TEM population clustered in group B, while group C contained the remaining genotypes from other populations. The distribution of populations in the PCA plot, except TEM, demonstrated that especially the BAG and BAR populations were settled at the two far ends of cluster C, and the DIL and ADA populations with higher levels of genetic diversity were also distributed among these populations in the same cluster. In addition, different DIL members in cluster A may be a result of the impact of high environmental pollution in the region (Bingol et al. 2013, 2018).

ISSR data are also used to infer the population structure of *R. pseudoacacia* (Figure 4). The possible number of subpopulations (ΔK) was calculated as seven. The genotypes of the PRI population mainly showed the structure of three subpopulations, while

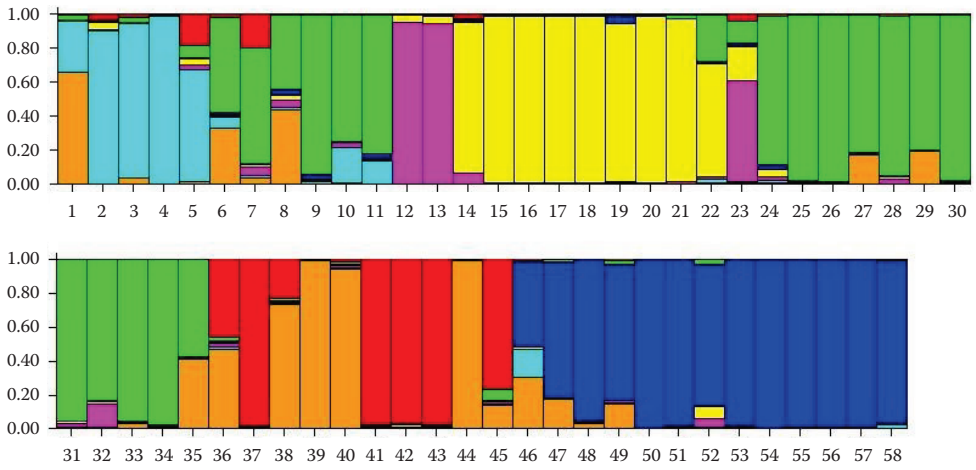

Figure 4. Genetic structure among *Robinia pseudoacacia* populations obtained using STRUCTURE (Version 2.3.4); Individuals: 1–11 PRI; 12–23 BAG; 24–35 BAR; 36–45 DIL; 46–58 TEM

PRI – Prince Islands; BAG – Bağdat Avenue; BAR – Barbaros Boulevard; DIL – Dilovası District; TEM – Trans European Motorway

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the genotypes from the BAG and DIL populations displayed two subpopulation structures. BAR and TEM populations showed mainly single subpopulation structures. The highest divergence was detected in the PRI population, while the lowest divergence was detected in the BAR and TEM populations.

Inferring phylogenetic relations of *R. pseudoacacia* by analysing nrDNA ITS1 and cpDNA trnL-F IGS regions. ITS regions are widely used as DNA barcodes in plants and fungi due to their higher discrimination potential (Shneyer, Rodionov 2019). Herein, ITS1 regions of 15 *R. pseudoacacia* genotypes, each with three genotypes from five populations, were sequenced. The length of all ITS1 sequences was found to be 239 bp, and the guanine-plus-cytosine (GC) content ranged from 52.72% to 56.56% (Table 4).

ISSR analyses were conducted to evaluate the genetic diversity of *R. pseudoacacia* populations. Maximum likelihood-based (ML) phylogenetic trees were built to investigate the phylogenetic relationships of *R. pseudoacacia* using the ITS1. The first tree (Figure 5) consisted of 15 ITS1 sequences of *R. pseudoacacia* plants from the studied populations, while the second tree also incorporated 61 ITS1 sequences from other plants, in addition to the 15 ITS1 sequences (Figure 6).

The phylogenetic analysis based on individuals (Figure 5) has demonstrated that the ITS1 region exhibits a limited degree of discrimination. The ITS1 region's phylogenetic analysis is not in agreement with the ISSR-based analysis results. In the combined tree (Figure 6), genotypes of *R. pseudoacacia* were classified with other *R. pseudoacacia* genotypes and members of the *Fabaceae* family retrieved from NCBI GenBank.

Phylogenetic relationships of *R. pseudoacacia* were also inferred using the ML method based on the trnL-F IGS region. Chloroplast trnL-F IGSs have been gaining popularity recently in molecular phylogeny and taxonomy studies as these noncoding regions are better at distinguishing lower ranks (Hocaoglu-Ozyigit et al. 2022). In this context, trnL-F IGS regions of 15 *R. pseudoacacia* genotypes of three genotypes each from five different stations were also sequenced. The length of the sequenced regions was determined as 453 bp with GC content between 29.14–29.80%. A phylogenetic tree based on 15 genotypes of *R. pseudoacacia* was constructed using the ML method (Figure 7). A second combined phylogenetic tree was then constructed to reveal the phylogenetic distribution of 15 trnL-F IGS in addition to 51 trnL-F IGS from other plants (Figure 8). All obtained ITS and

Table 4. NCBI accession numbers of ITS and trnL-F IGS in *Robinia pseudoacacia* genotypes

No.	Genotype name	ITS1			trnL-F IGS		
		size	GC content	NCBI AN	size	GC content	NCBI AN
1	PRI1 (ADA1)			KY311818			KY274204
2	PRI10 (ADA10)			KY311819			KY290234
3	PRI14 (ADA4)			KY311820			KY290233
4	BAG3			KY311821			KY290235
5	BAG4			KY311822			KY290236
6	BAG8			KY311823			KY290237
7	BAR10			KY311824			KY290238
8	BAR11	239 bp	52.72–56.56%	KY311825	453 bp	29.14–29.80%	KY290239
9	BAR12			KY311826			KY290240
10	DIL7			KY311827			KY290241
11	DIL8			KY311828			KY290242
12	DIL9			KY311829			KY290243
13	TEM19			KY311830			KY290244
14	TEM25			KY311831			KY290245
15	TEM26			KY311832			KY290246

PRI/ADA – Prince Islands; BAG – Bağdat Avenue; BAR – Barbaros Boulevard; DIL – Dilovası District; TEM – Trans European Motorway; ITS – internal transcribed spacer; trnL-F IGS – trnL-F intergenic spacer region; GC – guanine-plus-cytosine; NCBI AN – NCBI accession number

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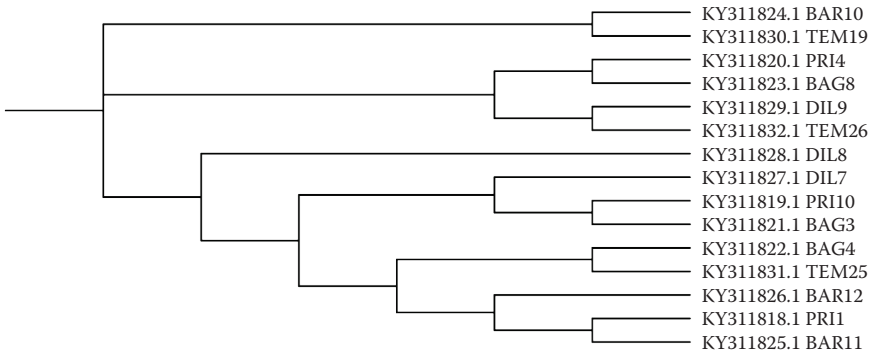

Figure 5. ITS1 sequence-based phylogenetic relationships of *Robinia pseudoacacia* genotypes

ITS1 – internal transcribed spacer 1 region; BAR – Barbaros Boulevard; TEM – Trans European Motorway; PRI – Prince Islands; BAG – Bağdat Avenue; DIL – Dilovası District

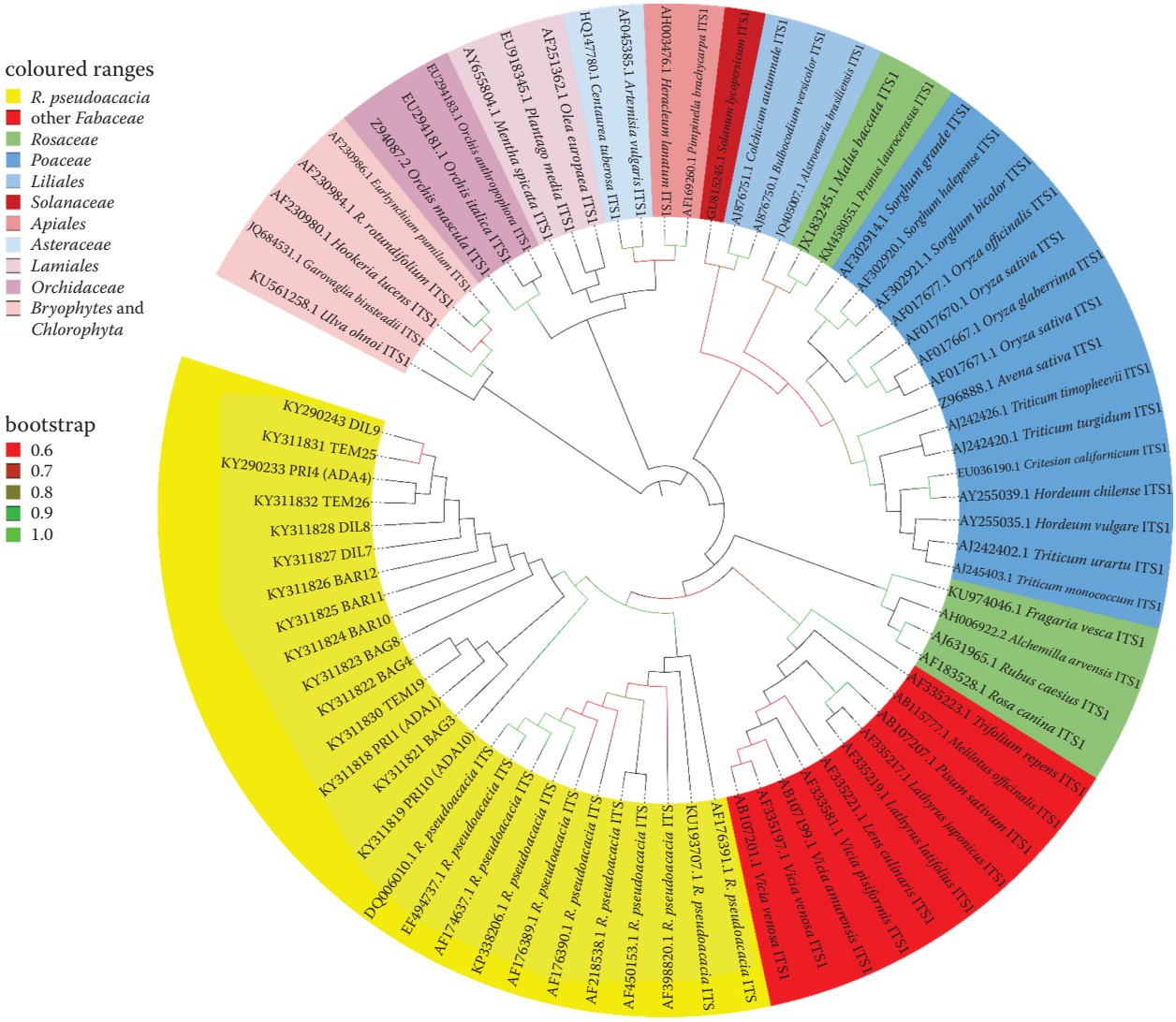

Figure 6. Phylogenetic distribution of ITS1 sequences in *Robinia pseudoacacia* and other plants

ITS1 – internal transcribed spacer 1 region; BAG – Bağdat Avenue; BAR – Barbaros Boulevard; DIL – Dilovası District; PRI/ADA – Prince Islands; TEM – Trans European Motorway

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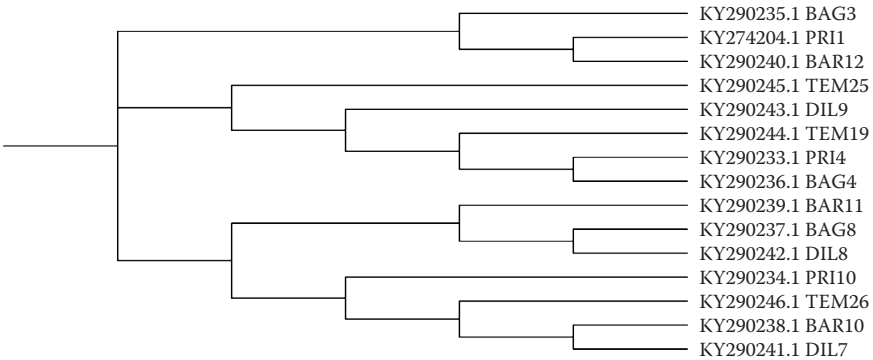

Figure 7. Phylogenetic distribution of trnL-F IGS sequences of *Robinia pseudoacacia* genotypes

trnL-F IGS – trnL-F intergenic spacer region; BAG – Bağdat Avenue; PRI – Prince Islands; BAR – Barbaros Boulevard; TEM – Trans European Motorway; DIL – Dilovası District

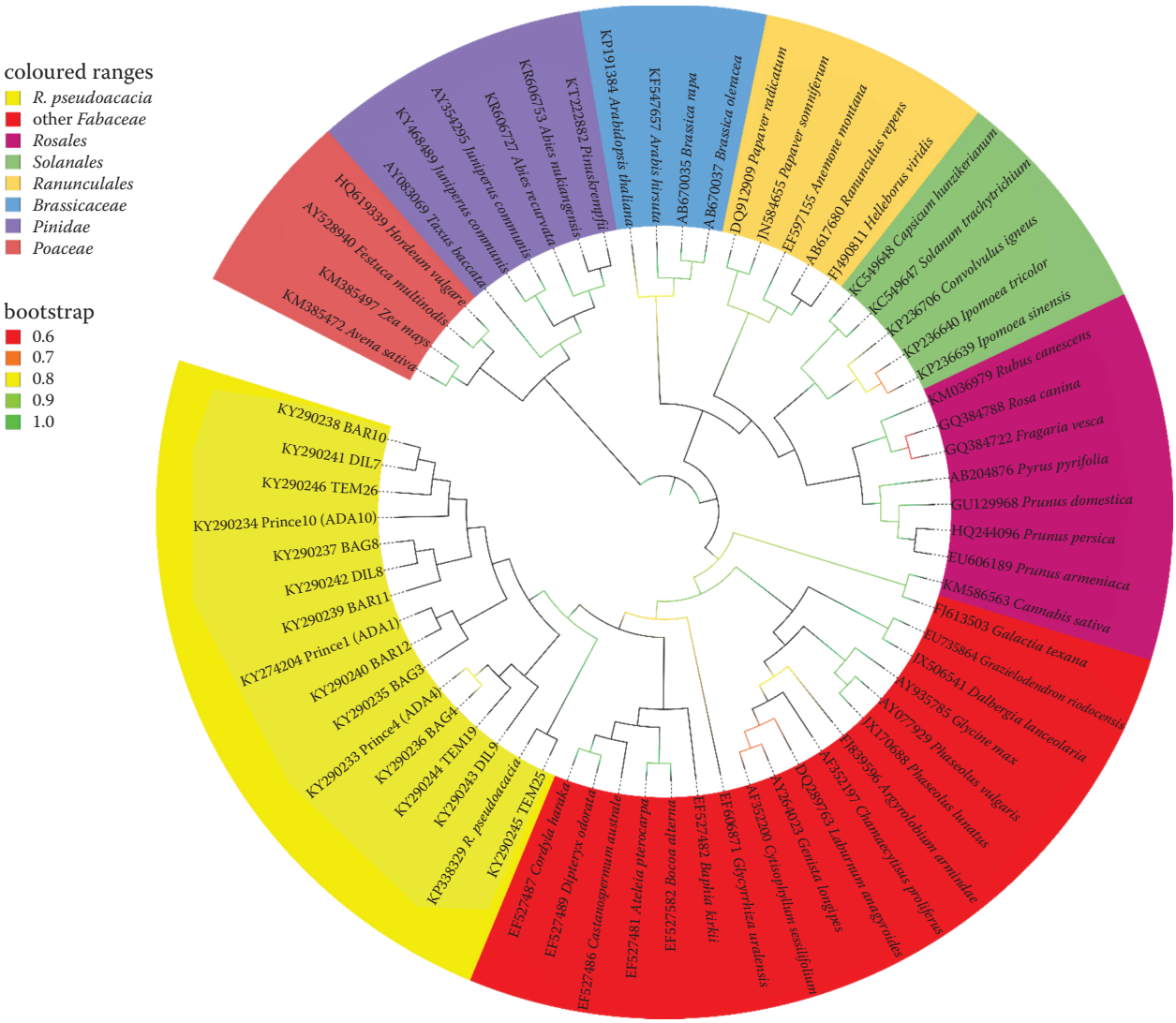

Figure 8. Phylogenetic distribution of trnL-F IGS sequences in *Robinia pseudoacacia* and other plants

trnL-F IGS – trnL-F intergenic spacer region; BAG – Bağdat Avenue; BAR – Barbaros Boulevard; DIL – Dilovası District; PRI/ADA – Prince Islands; TEM – Trans European Motorway

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trnL-F IGS sequences were uploaded to NCBI GenBank and the accession numbers of the sequences are given in Table 4.

The phylogenetic tree, based on trnL-F IGS sequences, exhibits low discriminatory power, consistent with findings from the ITS1-based analysis. Moreover, it does not correlate with the genetic diversity analyses based on ISSR markers. The combined tree, based on the trnL-F IGS sequence (Figure 8), suggests that the region shows significant discriminatory power at the family level.

DISCUSSION

Genetic diversity is an important component of inter/intraspecific adaptation and biodiversity, making it crucial for sustainable populations and ecosystems (Guo et al. 2018; Hocaoglu-Ozyigit et al. 2022). Therefore, gains from genetic diversity studies are promoting many topics such as plant breeding, population genetics, conservation, taxonomy, germplasm enrichment and forensic science, *etc*. (Hassan et al. 2012; Govindaraj et al. 2015; Li et al. 2015). In particular, determining the genetic diversity of tree species gains even more importance due to their long-life span, which requires adaptation to different environmental conditions (Filiz et al. 2015; Guo et al. 2018), so that the characteristics that support rich genetic variation could be successfully exploited for various breeding programs. Accordingly, this study aims to reveal the genetic diversity and phylogenetic relationships of *R. pseudoacacia* genotypes populating different urban habitats. Due to its strong adaptability and fast growth rate, attainments on the subject will be at many levels for further applications such as breeding seedlings tolerant to urban ecosystems, plant selection for landscaping and conservation planning. In urban environments, plants are subject to significant environmental pressures. The cumulative effects of the stress factors and reduced genetic diversity threaten plant populations. Monitoring genetic diversity is, therefore, crucial for conserving urban trees.

Overall, genetic diversity at the multi-populations level was significantly higher compared to single populations. When all members are considered as one population, the increase in the number of individuals leads to a corresponding increase in diversity, as observed in the *PPL* values. In addition, the test populations here are not from natural vegetation but were planted for landscaping purposes. Therefore, it is highly possible that these individuals were produced asexually by vegetative methods in nurseries.

There are some similar genetic studies on *R. pseudoacacia* populations. Sun et al. (2009) performed genetic diversity analysis in 10 different *R. pseudoacacia* populations by applying 10 ISSR primers to a total of 100 genotypes. In the study, the *h*, *I* value, and polymorphism rate were calculated to be 0.4337, 0.6145, and 93.41%, respectively. Compared with the results of this study, genetic diversity values were higher. The authors also stated that the genetic variation between populations of *R. pseudoacacia* plants was lower than the genetic variation within the population, and concluded that genetic variation was not affected by geographical patterns. Similarly, in this study, it can be said that genetic diversity is not directly affected by geographical conditions. In another study (Guo et al. 2018), the genetic diversity of native *R. pseudoacacia* samples was investigated using 'expressed sequence tag-derived simple sequence repeats' (EST-SSRs) and 'genomic DNA-derived simple sequence repeats' (G-SSRs) markers, and the Nei genetic diversity (*h*) value was calculated as 0.535 and 0.835, respectively. Mean *PIC* values were calculated as 0.503 for EST-SSR and 0.817 for G-SSR markers. Yang et al. (2020) investigated genetic diversity among seven native populations using SSR markers; the *PPL* value was calculated as 100%, the mean *PIC* value was calculated as 0.5862, and the *I* value was 0.779. *PIC* and *I* values calculated in these studies were higher than in this study. Guo et al. (2022a) examined the genetic diversity and population structure of six *R. pseudoacacia* varieties using 36 SSR markers. The authors analysed 687 strains from four improved varieties from two secondary provenances. Shannon's index was calculated at 1.302 and identified four main population structures. The authors stated that (*i*) there was a wide genetic variation between samples, (*ii*) there was a higher level of intra-population genetic variation than the interpopulation level, (*iii*) intra-population genetic variation may not be related to geographic locations. In another study by Guo et al. (2022b), 1 054 samples were analysed to extract the *R. pseudoacacia* germplasm core collection using markers of phenotype, physiology and genotyping. 13 phenotypic, 3 physiological traits, and 48 genotyping markers were used for

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evaluations. In accordance with the previous study, high genetic diversity was observed in the sample raw set, and 332 individuals were subsequently selected for the germplasm core collection.

In earlier studies mentioned above, the individuals used in the sampling were selected from natural populations from a large geographical area, whereas in this study, individuals from the urban ecosystem, bred for landscaping purposes, were specifically sampled. The genetic diversity value of plants in urban areas, whose plantation is mostly carried out by municipalities using *in vitro* and/or nursery-derived saplings, was lower than plants naturally propagated by seed dispersal in natural populations. The aforementioned case studies reported high genetic diversity at the intra-population level, but low genetic diversity at the inter-population level. On the contrary, in this study, low values of intra-population genetic diversity, and high values of inter-population genetic diversity indicate the main difference between natural populations and urban plants propagated by vegetative propagation. *R. pseudoacacia* plants are originally forest trees (Sitzia et al. 2016). Genetic diversity is generally observed more in long-lived forest trees (Ingvarsson, Dahlberg 2019). However, the use of vegetatively propagated *R. pseudoacacia* trees for landscaping has limited the plant's genetic diversity of the populations in urban ecosystems.

For inferring phylogenetic relations, in addition to ITS1 sequences from nrDNA, trnL-F IGS sequences from cpDNA were used to examine the phylogenetic relationships of the *R. pseudoacacia* plant. The *R. pseudoacacia* genotypes examined in this study were grouped with the *R. pseudoacacia* genotypes obtained from NCBI GenBank with high bootstrap values (ITS1 97%; trnL-F IGS 94%). In the ITS1 region sequence-based ML tree (Figure 5), the 15 sequenced genotypes were not sorted by population. This situation suggests that the region is conserved and demonstrates limited discriminatory ability at the species level. In the combined tree based on ITS1 sequences (Figure 6), both the *R. pseudoacacia* genotypes sequenced in this study and the *R. pseudoacacia* genotypes obtained from the database are divided into two separate subgroups. While the sequences were collected, all ITS region sequences of *R. pseudoacacia* individuals were added to the tree, since there was no ITS1 sequence belonging to the *R. pseudoacacia* plant in the NCBI database. Because of this situation, *R. pseudoacacia* genotypes may have formed two subgroups on the ITS1-based phylogenetic tree. One of the subgroups includes the *R. pseudoacacia* genotypes sequenced in this study, and the other includes the *R. pseudoacacia* genotypes obtained from the database. Although the length of the sequences differed in the two groups, a bootstrap value of 97% indicates that the region is highly protected. The trnL-F IGS sequence obtained from the 15 genotypes of *R. pseudoacacia* sourced from the researched populations (Figure 7) exhibited a limited capacity for species-level discrimination, consistent with the ITS1 sequence. However, in the combined tree based on the trnL-F IGS (Figure 8), the region showed high discriminatory power at the family level. In both combined trees, the closest group to *R. pseudoacacia* plants were, as expected, members of the *Fabaceae* family. *Fabaceae* groups were formed with a bootstrap value of approximately 70% in the ITS1-based tree and 94% in the trnL-F IGS-based tree. The bootstrap values calculated when constructing the ITS1-based tree were generally lower than the bootstrap values of the trnL-F IGS-based tree. Based on this information, it can be suggested that the trnL-F IGS sequence, which is longer than ITS1, has greater discriminatory power.

CONCLUSION

In this study, genetic diversity and phylogenetic relationships of *R. pseudoacacia* genotypes collected from urban habitats were investigated using ISSR markers, ITS1 and trnL-F IGS region sequences. According to the analysis based on ISSR data, *R. pseudoacacia* genotypes showed moderate/low genetic diversity in both intra/interpopulation. UBC820, UBC811, and UBC818 ISSR primers with related high *PIC* values can be recommended as ISSR primers suitable for use in genetic diversity studies of *R. pseudoacacia* and closely related species. According to ISSR analysis results, it is evaluated that genetic similarities and differentiations in perennial forest and urban plant populations such as *R. pseudoacacia* depend on the parents from which the trees are obtained and/or specifically the pollution they are exposed to in some regions. ITS1 and trnL-F IGS sequences were used as DNA barcodes to evaluate the phylogenetic relationships of *R. pseudoacacia* in the *Fabaceae* family. Phylogeny analysis based on ITS1 and

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trnL-F IGS sequences agreed with previous results. The sequences of both regions exhibited effective discriminatory power at the family level with high degrees of discrimination. The findings suggest that *R. pseudoacacia*, an urban forest tree, may be at risk of population decline in the future due to its low genetic diversity and vulnerability to environmental pressures. In order to ensure the survival of tree populations in urban ecosystems, it is crucial to monitor their populations, preserve existing individuals and introduce new members to increase genetic diversity. The findings obtained in this study not only contribute to the studies conducted in understanding the genetic diversity of *R. pseudoacacia* genotypes but also can help the authorities in developing protection programs for *R. pseudoacacia* and other tree species in urban areas.

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