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Microsatellite markers as a useful tool for species identification and assessment of genetic diversity of the *Tilia* species in the Czech Republic

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Abstract: Diagnostic morphological traits distinguishing *Tilia cordata* and *T. platyphyllos* are not always apparent. Precise species identification is crucial for conservation management and for establishing genetic resources of forest reproductive material. In this study, we employed fifteen microsatellite (SSR) markers to identify diagnostic loci for reliable species discrimination between *T. cordata* and *T. platyphyllos* species and to evaluate the genetic diversity. A total of 250 trees were sampled from eight natural autochthonous populations of *T. cordata* and *T. platyphyllos* in the Czech Republic, and additional ten individuals of *T. tomentosa* were included for comparison. Markers Tc8 and Tc918 were identified as diagnostic, exhibiting species-specific alleles for *T. cordata* and *T. platyphyllos*. Contrary to the previously published results, we obtained PCR amplicons at locus Tc918 with an allele size of 226 bp in *T. cordata*. An analysis of the fifteen SSR loci revealed a high level of genetic variability in both the *T. cordata* and *T. platyphyllos* populations. No clonally identical genotypes were detected across the eight study sites. The genetic differentiation (Fst) was higher between *T. platyphyllos* and *T. tomentosa* (0.316) than between *T. platyphyllos* and *T. cordata* (0.205). Bayesian clustering further revealed distinct genetic structures for *T. cordata*, *T. platyphyllos*, and *T. tomentosa*.

Keywords: genetic variability; protection and reproduction of genetic resources; species discrimination; SSR markers; *Tilia cordata*; *Tilia platyphyllos*

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The lime genus (*Tilia* L.) comprises broad-leaved trees predominantly occurring in the temperate regions of the Northern Hemisphere (Buriánek & Novotný 2018). Owing to their pronounced morphological uniformity and often ambiguous species boundaries, members of *Tilia* genus (Malvaceae s.l., Tilioideae) have long presented a challenge for taxonomists. This complexity is reflected in the more than 500 names historically assigned to a group currently recognised as comprising only about 30 species (Ala et al. 2024). According to the most recent taxonomic revision presented in Pigott's (2012) monograph, the genus *Tilia* comprises 23 species and 14 subspecies worldwide. As noted by Buriánek and Novotný (2018), the genus is particularly problematic from a taxonomic perspective due to pronounced intraspecific polymorphism and the frequent occurrence of natural hybrids.

In Europe, *Tilia* is represented primarily by three native species: *Tilia cordata* Mill., *Tilia platyphyllos* Scop. (as well as their relatively rare natural hybrid *Tilia* × *europaea* L., commonly referred to as the common lime), and *Tilia tomentosa* Moench. (Radoglou et al. 2009). In the Czech Republic, only the first two above-mentioned species and their hybrid occur as native species (Úradníček et al. 2010). The distribution of *Tilia cordata* is mainly in floodplain forests and in scree and ravine forests of lower altitudinal vegetation zones, and it rarely reaches mountainous localities, while *Tilia platyphyllos* is widespread mainly in the hills, where it is a diagnostic species of scree and ravine forests. It often reaches the mountains, while, in floodplain forests, it is probably non-native and is planted here only occasionally (Buriánek & Novotný 2018). *Tilia cordata* is a tree species with little variability among populations (Svejgaard Jensen 2003), whereas *Tilia platyphyllos* is an exceptionally variable species. Its variability is manifested by the existence of several subspecies, which are not morphologically sharply defined, making their distinction problematic. In the Czech Republic, three subspecies of *Tilia platyphyllos* are recognised. As their ecological requirements are largely similar, the subspecific differentiation is of limited practical relevance in forestry practice. The most widespread taxon is *T. platyphyllos* subsp. *cordifolia*, whereas subsp. *platyphyllos* occurs only sporadically to rarely. The rarest subspecies, *T. platyphyllos* subsp. *rubra*, reported exclusively from southern Moravia, may even be confused with *Tilia cordata* due to the overlapping morphological traits. *Tilia tomentosa* Moench is cultivated in the Czech Republic both as an avenue tree and as an ornamental species, and it is frequently planted in urban alleys (Buriánek & Novotný 2018).

There are a few morphological differences between the two native species *T. cordata* and *T. platyphyllos* (Table 1).

Although the morphology can well distinguish between *T. cordata* and *T. platyphyllos*, diagnostic traits are not always apparent, particularly under suboptimal or shaded conditions (Logan et al. 2015) or in the period of vegetation rest when leaves are not available (Buriánek & Novotný 2018). In such cases, molecular tools, particularly microsatellite markers, provide an effective means of identifying

Table 1. Differences in the morphological traits of *Tilia* species native in the Czech Republic (according to Úradníček et al. 2010; Buriánek & Novotný 2018)

Morphological traits	*Tilia cordata*	*Tilia platyphyllos*
Tufts of hair in the leaf vein axils	rusty brown	white
Leaves underside	hairless, greyish	with upright, scattered hairs, green
Leave blades	straight veins of 3rd order indistinct	overhanging edges veins of 3rd order distinct
Annual shoots	hairless	hairy
Buds	the lower scale exceeds half the size of the bud	the lower scale reaches a maximum of half the size of the bud
Inflorescences	upright 4–11 flowers blooming later by 2 weeks	hanging 3–5 flowers blooming sooner by 2 weeks
Fruits	fragile smaller 5–6.5 mm surface smooth or indistinct ribs	hard bigger 6–10 mm distinct ribs

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species and their hybrids (Phuekvilai & Wolff 2013; Tamošaitis et al. 2021; Villani at al. 2021; Cvrčková et al. 2025). Microsatellites are highly polymorphic, enabling both the detection of substantial genetic diversity and the quantification of the population genetic structure (Logan et al. 2015). Phuekvilai and Wolff (2013) developed fifteen microsatellite (SSR) markers for *T. platyphyllos*, twelve of which also successfully amplified in other *Tilia* species. The authors reported that the one locus (Tc918), which amplified in *T. platyphyllos*, but not in *T. cordata*, may have potential utility for species delimitation. Building on this, Logan et al. (2015) applied thirteen highly variable simple sequence repeat (SSR) markers to reliably differentiate *T. cordata* and *T. platyphyllos*, as well as to distinguish both from their hybrid. This hybrid, known as *Tilia* × *europaea*, arises from the occasional natural hybridisation between *T. cordata* and *T. platyphyllos*, particularly in areas of sympatric occurrence. Although relatively rare in natural forest stands, *T.* × *europaea* is widely planted in urban environments and managed landscapes across Europe (Svejgaard Jensen 2003).

This study investigated *T. cordata* and *T. platyphyllos* from eight natural populations and *T. tomentosa* from an urban plantation in the Czech Republic, with the objective of identifying diagnostic SSR markers for the reliable differentiation of *Tilia* species and assessing the genetic diversity of the studied populations.

MATERIAL AND METHODS

Origin of populations and their sampling. A total of 250 trees were sampled from eight natural local autochthonous populations of *Tilia cordata* and *Tilia platyphyllos* from the Czech Republic from April to June in 2023 and 2025 (Table 2). In addition, ten samples of non-native species *Tilia tomentosa* (Prague, locality Na Františku) for comparison were analysed for the possibility of distinguishing the species. In the selected populations, the species identification of the monitored individuals was verified based on the morphological traits, and only accurately identified individuals of the studied species were included in the SSR marker analysis to determine the loci with species-specific allele sizes. Coordinates have been recorded for every sampled tree to allow repeated species identification.

Approximately three newly sprouted shoots were taken from each tree, placed in a cooling box and transported to the laboratory for sampling. If the sampling was carried out before the shoots sprouted, the collected shoots were placed in glasses of water in the laboratory and left to sprout. Samples of young leaves were taken for DNA isolation.

DNA extraction and microsatellite genotyping. Genomic DNA was extracted from 100 mg of fresh collected material (young leaves) or 20 mg lyophilised material using a DNeasy® Plant Mini Kit (Qiagen,

Table 2. Basic information about the sampling plots and sampling populations

Locality	Species	Coordinates	Geology	Biogeography	Designation of populations	Number of sampling trees
Hradec nad Moravicí	*Tilia platyphyllos*	49.8746873N; 17.8590634E	carbonaceous offal	Hercynicum (near to Polonicum boundary)	LPV_1	31
Pálava	*T. platyphyllos*	48.8775876N; 16.6654173E	limestone	Pannonicum	LPV_2	33
Budíškovice	*T. platyphyllos*	49.081561N; 15.531232E	gneiss	Hercynicum	LPV_3	32
Tlustec	*T. platyphyllos*	50.723843N; 14.741772E	basalt	Hercynicum	LPV_4	30
Hostětín	*T. cordata*	49.0473571N; 17.864547E	flysch	Carpaticum	LP_1	21
Soutok	*T. cordata*	48.645486N; 16.936534E	quaternary sediments	Pannonicum	LP_2	30
Božejov	*T. cordata*	49.343850N; 15.165261E	migmatite	Hercynicum	LP_3	33
Stračov	*T. cordata*	50.325922N; 15.658401E	cretaceous calcareous marls	Hercynicum	LP_4	40

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Hilden, Germany) following the manufacturer's instructions. The quality and concentration of the extracted DNA were determined using a MaestroNano Pro spectrophotometer (MaestroGen, Las Vegas, NV, USA).

Fifteen nuclear polymorphic microsatellites described by Phuekvilai and Wolff (2013) were selected for the DNA analysis in order to study the genetic diversity of the lime individuals and for the possibility of the related species discrimination. Amplifications of microsatellite loci based on the polymerase chain reactions (PCRs) were carried out with specific fluorescent dye-labelled primers (FAM, VIC, PET and NED dyes). The fifteen nuclear SSR markers were assembled for PCR into three multiplexes from the viewpoint of the targeted allele sizes (Table 3).

The reaction mixture for every DNA sample in a volume of 15 µL contained for each multiplex: 1 µL of template DNA (≈5–68 ng/µL), 1.5 µL of 10 × PCR buffer (Mg-free), 2 mM $MgCl_2$, 0.37 U of Platinum® *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), 0.133 mM of dNTP mixture (Takara Bio

Table 3. Microsatellite markers

Locus/repeat motif	Primer sequence (5'–3')	Allelic size range (bp)	Fluorescent dye/ multiplex sets
Tc4/$T_6(GT)_{12}$	F: ATTTTAGAATGCCAACCTGCTAAG R: TATTGAAGTCCATTTCCAATTGTC	211–244	6-FAM/1
Tc5/$(AG)_{12}$	F: TTTTCATACATTTAGAGACTTTTAGCA R: TGCATGATTTGTATGTTTAGGG	136–176	6-FAM/2
Tc6/$(AG)_{12}$	F: CCATATCTTCTGCCAGTTTTCC R: GGACTAATTTCTTCCTTTTATTAGGC	120–166	6-FAM/1
Tc7/$(GA)_{13}$	F: TTTACTTTTGCCAGTTGTGAGG R: CACCTAGAATGCCTCCTATTCG	213–261	NED/2
Tc8/$(GA)_{13}$	F: CGAAGAAACTGTCAAAACAACG R: AGCTGGGTTTTAGAGGATAGGG	142–176	PET/1
Tc11/$(AG)_{13}$	F: AGCTATGAAAGAACTATCAAGAGAAAG R: CCCCAAGACATTGCAGTAGAAC	129–155	VIC/3
Tc31/$(GA)_{12}$	F: TTTGCAAAGACTACTCCAAGAATC R: AAATCGATGGTCAAGAACTAAATC	192–228	PET/2
Tc915/$(CT)_{16}$	F: ACATCGATTGTATTTCCCTTTAAC R: GTTGTATTTTGCCCTTAACATTG	135–195	VIC/2
Tc918*/$(AC)_9(TC)_2$	F: AACGGCTAATTACTCCTAGTTTCG R: TGTTCAGCTCACTACTACCTTTCAC	226–234	VIC/3
Tc920/$(GA)_2(GT)_{15}(AG)_4$	F: AAATGTCTTCAGAGTGACTAGATGG R: TGCCTCATTATTCTCCTAATTCTC	215–239	NED/1
Tc927/$(AG)_{10}$	F: AGTCCTCCTGTCAAATGCTG R: ATCACACTCGTTTATGACATCTTG	140–190	6-FAM/3
Tc937/$(AG)_{13}$	F: AGCCAACCAACTTTTACAATACAG R: AGATAAAAGCACATAAATCGATGG	146–182	VIC/1
Tc943/$(CA)_{10}$	F: ATTTCATCTTTCTCTAAAGCCTTG R: GGGAAAGCCTGTGTTAGTTTC	140–154	PET/3
Tc951/$(CT)_{12}$	F: TGTTATGACCTCACTTATAACCAAGT R: GGGTGAGCTGACAATATAGAAGAG	141–177	NED/3
Tc963/$(CT)_{11}$	F: CTAACCCCATTCTCTTTAATTCTG R: GCTTTCATTTCAGTTTTCCTCTAG	208–268	VIC/1

*For the purpose of species discrimination, PCR amplification of the Tc918 locus was conducted separately with the same conditions as all the loci

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Inc., Otsu, Shiga, Japan), and combinations of the forward and reverse primers. The primer concentrations of loci Tc7, Tc8, Tc11, Tc918, Tc927, Tc937, Tc943, Tc951 were 0.1 µM, of loci Tc4, Tc5, Tc6, Tc31, Tc915, Tc920 were 0.2 µM, and of locus Tc963 was 0.3 µM. Thermocycling was performed according to a protocol of Phuekvilai and Wolff (2013). The same PCR conditions were applied to all the loci as follows: initial denaturation for 5 min at 95 °C, followed by 15 cycles for 15 s at 95 °C, for 15 s at 54 °C, for 15 s at 72 °C, and 20 cycles for 20 s at 89 °C, for 20 s at 52 °C, for 20 s at 72 °C, and a final extension for 30 min at 72 °C. Amplification was performed in a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA). One (1) µL of the PCR product was added to 11 µL HiDi™ formamide (Applied Biosystems) and 0.4 µL size standard GeneScan™ 600LIZ® (Applied Biosystems) before the fragmentation analysis, and capillary electrophoresis was run on an ABI 3500 genetic analyser (Applied Biosystems, Foster City, CA, USA). Alleles were sized using the GeneMapper® 4.1 software (Applied Biosystems, Foster City, CA, USA), allele binning was performed manually after plotting the fragment size distribution for each locus (Guichoux et al. 2011). Micro-Checker software version 2.2.3 (Van Oosterhout et al. 2004) was used to identify the genotyping errors in the microsatellite data as evidence of a scoring error due to stuttering, as evidence for large allele dropout, and to estimate the null allele frequencies according to the three observed *Tilia* species. The processed data were subjected to a statistical analysis using the CERVUS software package (Kalinowski et al. 2007) and GenAlEx 6.503 program (Peakall & Smouse 2006, 2012). The genetic characteristics of the SSR markers, including the polymorphic information content (PIC) of the SSR markers and the species distinction of the monitored lime trees, were evaluated. The evaluation of the genetic differentiation among the studied lime species were determined as *Tilia cordata* and *Tilia platyphyllos* and the samples of *Tilia tomentosa* was performed on the basis the pairwise F_{ST} values that were visualised by the principal coordinate analysis (PCoA). For distinguishing the lime species, the Bayesian clustering method implemented in the STRUCTURE 2.3.4 software (Pritchard et al. 2000; Falush et al. 2003, 2007; Hubisz et al. 2009) was used to derive the structures based on the multi-locus data of the monitored SSR markers. The admixture model with correlating allele frequencies without Lockprior specification were used. The burn-in period was set to 50 000, followed by 500 000 Markov Chain Monte Carlo (MCMC) repetitions. Simulations were run for values of K from 1 to 10, each repeated ten times. The optimal number of clusters (*K*) was determined using the web-based StructureSelector tool (Li & Liu 2018).

RESULTS

Analyses of the SSR markers. The genetic characteristics of the SSR markers (Tc4, Tc5, Tc6, Tc7, Tc8, Tc11, Tc31, Tc915, Tc918, Tc920, Tc927, Tc937, Tc943, Tc951 and Tc963) used for study of diversity were obtained from the analyses of 240 *Tilia* individuals with reliably determined species identity based on morphological characteristics and show a high degree of their variability. The number of different alleles (*k*) ranged from 4 (Tc918) to 30 (Tc963). The mean number of effective alleles over the populations per locus (*Ne*) ranged from 1.08 (Tc918) to 7.56 (Tc963). Shannon's information index calculated for the allelic and genetic diversity ranged from 0.12 at locus Tc918 to 2.01 at locus Tc963. The values of the observed heterozygosity ranged from 0.0 (Tc918) to 0.86 (Tc5), the expected heterozygosity ranged from 0.06 (Tc918) to 0.78 (Tc963). The values of the polymorphic information content (PIC) for the markers ranged from 0.43 (Tc918) to 0.92 (Tc963), with a mean value of 0.76 (Table 4). The polymorphic information content (PIC) was used to estimate the quality of the SSR marker by measuring the number of alleles and allele frequency for each marker. PIC is classified as satisfactory (PIC > 0.5), medium (0.25 ≤ PIC ≤ 0.5), or low (PIC < 0.25) (Botstein et al. 1980). According to this criterion, all the markers analysed in this study except marker Tc918 were satisfactory (Table 4).

Evidence of null alleles was detected by the Micro-Checker software in *Tilia cordata* at loci Tc4, Tc5, Tc11, Tc31, Tc927, Tc943, Tc951 and Tc963, and in *Tilia platyphyllos* at loci Tc5, Tc7, Tc31, Tc918, Tc920, Tc927, Tc937, Tc943, Tc951 and Tc963. No evidence of null alleles was found in *Tilia tomentosa*. The adjusted allele frequencies at loci with evidence of null alleles can be used to study population differences.

Evaluation of the genetic diversity of lime trees and the detection of species by selected SSR markers. The genetic investigation of 250 trees from eight natural local autochthonous populations of *Tilia cordata* and *Tilia platyphyllos* from the Czech Re-

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Table 4. Genetic characteristics of 15 nuclear SSR loci across 240 lime individuals

Locus	*k*	*Ne*	*I*	H_o	H_e	PIC
Tc4	11	3.45	1.37	0.73	0.67	0.75
Tc5	24	6.82	1.87	0.86	0.75	0.91
Tc6	19	4.93	1.51	0.56	0.62	0.86
Tc7	21	4.29	1.23	0.5	0.5	0.86
Tc8	14	3.24	0.75	0.29	0.29	0.65
Tc11	12	2.61	1.09	0.66	0.57	0.78
Tc31	15	3.27	1.2	0.67	0.57	0.75
Tc915	21	5.19	1.56	0.56	0.58	0.91
Tc918	4	1.08	0.12	0.00	0.06	0.43
Tc920	12	3.28	1.11	0.47	0.51	0.76
Tc927	22	4.15	1.32	0.56	0.59	0.79
Tc937	15	3.33	1.26	0.71	0.59	0.77
Tc943	9	1.65	0.68	0.18	0.36	0.65
Tc951	14	1.79	0.74	0.18	0.35	0.64
Tc963	30	7.56	2.01	0.75	0.78	0.92

k – number of different alleles; *Ne* – mean number of effective alleles; *I* – Shannon's information index; H_o – mean observed heterozygosity; H_e – mean expected heterozygosity; PIC – polymorphic information content

public was conducted using analyses of fifteen nuclear SSR markers as described by Phuekvilai and Wolff (2013). Following the initial SSR marker analyses, individuals with suspected species misidentification were phenotypically verified at the sampling sites. Twenty individuals from the studied populations were excluded from the overall analysis due to belonging to a different autochthonous species. A total of 230 morphologically confirmed individuals were included in the subsequent evaluations: 122 of *Tilia cordata* and 108 of *Tilia platyphyllos*. In addition, 10 *Tilia tomentosa* individuals (designation LPS) were analysed using SSR markers. The data obtained from the analysed loci are provided in the Supplementary Materials. No clonally identical individuals were detected across the eight studied sites of the two autochthonous lime species. In *T. tomentosa*, two genotypes were identified, with nine of the ten individuals being clonally identical.

The species determination was based on selected loci with species-specific allele sizes. In all the individuals of *T. cordata* and *T. tomentosa*, allele 226 was present in a homozygous form at locus Tc918. In *T. platyphyllos*, three homozygous allele variants (230, 232 and 234 bp) were detected. Among the 108 analysed individuals, one carried allele 234, ten carried allele 232, and 97 carried allele 230. Unlike the pilot study of Phuekvilai and Wolff (2013), the PCR analyses of locus Tc918 were conducted separately to obtain unambiguous amplicons.

The allele size 142 was consistently present in a homozygous form at locus Tc8 in *T. cordata* and *T. tomentosa*. In *T. platyphyllos*, 14 alleles ranging from 142–176 bp were detected. The 142 bp allele occurred in only one individual (labelled as LPV_1_26) in a heterozygous form, while allele 160 bp was most frequent (21.3%) across the investigated individuals of *T. platyphyllos*. As the sample LPV_1_26 also had allele sizes at the Tc7 (215 bp) and Tc927 (140 bp) loci, which were only observed in the *T. cordata* samples, we hypothesise that it could be a *Tilia* × *europaea* hybrid. In *T. tomentosa*, homozygosity was observed at loci Tc8, Tc7, Tc915, Tc918, Tc920, and Tc951. The allele sizes of loci Tc8 and Tc918 were identical to those in *T. cordata*, while the sizes of loci Tc937, Tc31, Tc915, and Tc951 corresponded to the ranges observed in *T. platyphyllos*. A summary of the allele size ranges in the studied species is provided in Table 5.

Genetic differences among the lime species and investigated populations. The evaluation of the genetic differentiation among the lime species was carried out based on the result of the pairwise comparisons of the F_{ST} values obtained from the analysis of fifteen selected microsatellites. The highest value of genetic differentiation was observed between

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Table 5. Number of different alleles and allele size ranges of the SSR markers obtained from analyses of the lime species

Locus	Range of allele sizes in number of base pairs (bp) and number of different alleles are given in parentheses		
	Tilia cordata 122 individuals	*Tilia platyphyllos* 108 individuals	*Tilia tomentosa* 10 individuals as comparative samples
Tc4	211–238 (10)	214–244 (9)	220, 226 (2)
Tc6	120–142 (11)	122–166 (18)	132, 142 (2)
Tc8	142 (1)	142–176 (14)	142 (1)
Tc920	219–235 (7)	215–239 (10)	227 (1)
Tc937	146–166 (6)	148–182 (12)	162–174 (3)
Tc963	235–285 (21)	227–279 (22)	257–267 (3)
Tc5	136–176 (15)	132–176 (23)	138–170 (3)
Tc7	213–249 (10)	215–261 (20)	226 (1)
Tc31	192–212 (5)	194–228 (12)	208–220 (3)
Tc915	149–179 (16)	141–189 (20)	145 (1)
Tc11	129–151 (4)	131–155 (11)	141, 147 (2)
Tc918	226 (1)	230–234 (3)	226 (1)
Tc927	140, 142, 150 (3)	140–190 (22)	148, 152 (2)
Tc943	140–152 (6)	142–154 (6)	146–150 (3)
Tc951	153–159 (4)	141–177 (13)	149 (1)

T. platyphyllos and *T. tomentosa* (0.316), the genetic differentiation between *T. cordata* and *T. tomentosa* was 0.305. The lowest value of genetic differentiation was observed between *T. platyphyllos* and *T. cordata* (0.205).

According to the pairwise comparisons of the investigated populations of two autochthonous lime species (Table 6), low levels of genetic differentiation were observed among *Tilia platyphyllos* populations (LPV_1–LPV_4), and low and medium levels of genetic differentiation were observed among *Tilia cordata* populations (LP_1–LP_4). The highest F_{ST} value was noted between the LP_1 and LP_4 (F_{ST} = 0.065), while the lowest value was found between the LPV_1 and LPV_3 (F_{ST} = 0.02). The genetic distance among the investigated populations of *T. cordata* and *T. platyphyllos* samples and *T. tomentosa* samples are graphically represented by the principal coordinate analysis (PCoA) in Figure 1.

The genetic structures of the observed lime trees *T. cordata, T. platyphyllos* and *T. tomentosa* were obtained by Bayesian clustering. The proportions of individual genetic clusters were quantified as percentages to compare the genetic structures of the different lime species. The optimal number of clusters delta K = 2 was determined using the StructureSelec-

Table 6. Pairwise F_{ST} values among investigated populations of *Tilia cordata* and *T. platyphyllos*

Populations	LPV_4	LPV_2	LPV_1	LPV_3	LP_4	LP_3	LP_2	LP_1
LPV_4	0.000							
LPV_2	0.035	0.000						
LPV_1	0.036	0.030	0.000					
LPV_3	0.029	0.021	0.020	0.000				
LP_4	0.231	0.196	0.201	0.201	0.000			
LP_3	0.230	0.197	0.194	0.199	0.029	0.000		
LP_2	0.285	0.249	0.246	0.252	0.057	0.031	0.000	
LP_1	0.290	0.255	0.258	0.261	0.065	0.039	0.023	0.000

LPV – *T. platyphyllos*; LP – *T. cordata*

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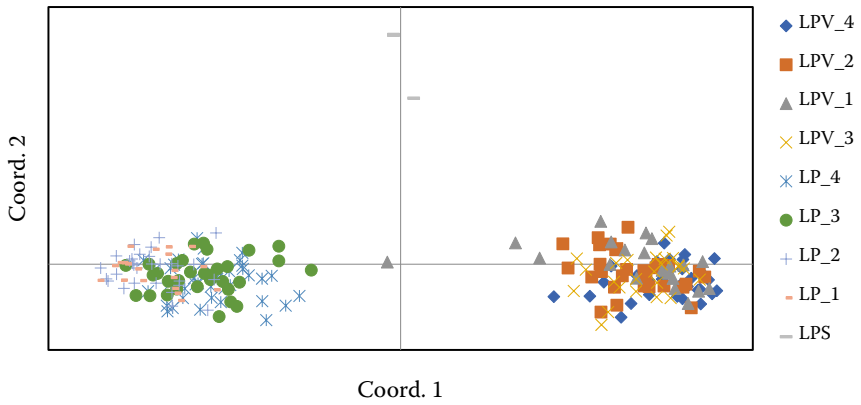

Figure 1. The genetic distance among the investigated populations of the *Tilia cordata* and *T. platyphyllos* samples and *T. tomentosa* samples illustrated by the principal coordinates analysis (PCoA)
LPV – *T. platyphyllos*; LP – *T. cordata*, LPS – *T. tomentosa*

tor program. The number of clusters $K = 5$ provided the more detailed resolution of the genetic profiles, allowing for clearer differentiation among the studied lime species (Figure 2). For $K = 5$, the mean proportions were: the violet cluster 96.6% in *T. tomentosa*, 0.3% in *T. platyphyllos*, 0.2 % in to *T. cordata*, the blue cluster 98.7% in *T. platyphyllos*, 1% in *T. cordata*, 0.4% in *T. tomentosa*, the green cluster 34.7% in *T. cordata*, 0.2% in *T. platyphyllos*, 0.1% in *T. tomentosa*, the orange cluster 34.7% in *T. cordata*, 0.5% in *T. platyphyllos*, 0.3% in *T. tomentosa* and the purple cluster 29.3% in *T. cordata*, 0.4% in *T. platyphyllos*, 2.5% in *T. tomentosa*.

DISCUSSION

Research on *Tilia cordata* and *Tilia platyphyllos* is increasingly important due to their ecological roles, genetic complexity, and relevance in the face of climate change and urbanisation. Similar morphological traits within the genus *Tilia* can lead to ambiguity in species designation (Pigott 2012). The morphological identification of *Tilia* taxa is often problematic due to phenotypic plasticity, overlapping diagnostic traits, and frequent hybridisation (Logan et al. 2015, 2019). Molecular analyses represent an indispensable methodological approach for investigating populations of *Tilia cordata* and *Tilia platyphyllos*, as they enable the precise detection of clonal structures and facilitate reliable species discrimination (Erichsen 2019; Danusevičius et al. 2021; Rungis & Krivmane 2021; Wolff et al. 2021). The present study demonstrates that a set of fifteen nuclear microsatellite markers (Tc4, Tc5, Tc6, Tc7, Tc8, Tc11, Tc31, Tc915, Tc918, Tc920, Tc927, Tc937, Tc943, Tc951, and Tc963) provide a powerful tool for species discrimination and for assessing the genetic diversity in selected lime populations originating from different habitat conditions in the Czech Republic. Most of the loci used exhibited high polymorphism, with PIC values above 0.5, confirming their informativeness for genetic studies (Botstein et al. 1980). In total, 243 alleles were obtained from 240 lime samples, with an average of 16.2 alleles per locus. Comparable high diversity values have previously been reported: Logan et al. (2019) detected 19.7 and 13.7 alleles per locus in *T. platyphyllos* (229 trees, 12 markers) and *T. cordata* (376 trees), respectively; Erichsen et al. (2019) reported 15 alleles per locus from nine markers in 774 individuals (*T. cordata*, *T. platyphyllos*, *Tilia* × *europaea*); and Rungis and Krivmane (2021) found 10.57 alleles per locus using 14 markers in 244

Figure 2. Structure analysis results with $K = 5$ for *Tilia cordata, T. platyphyllos* and *T. tomentosa*

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T. cordata individuals. The highest genetic diversity in our study was observed for marker Tc963, consistent with the findings of Rungis and Krivmane (2021) for Latvian *T. cordata* and Erichsen et al. (2019) for Danish *T. cordata* populations. The least polymorphic locus was Tc918, with only one allele of size 226 detected in our sample of *T. cordata* and *T. tomentosa*, which was used as a diagnostic marker. Although locus Tc918 is not polymorphic, it proved to be particularly important for species identification, as it enabled a clear separation between *T. cordata* and *T. platyphyllos*. Studies on the use of SSR markers for genetic analyses in lime trees are usually based on the initial work of Phuekvilai and Wolff (2013), who reported the amplification of the Tc918 locus only in *Tilia platyphyllos* individuals and noted the absence of amplification products in *T. cordata* and *T. tomentosa*. Consequently, most authors do not use this locus in their studies of *T. cordata*. In our study, however, we found that when PCR amplifications of this marker were performed separately, this locus appeared in a homozygous form with an allele size of 226 bp in *T. cordata* and *T. tomentosa*, and in a homozygous form with an allele size of 230 bp in most *T. platyphyllos* trees. This observation contrasts with previous reports (e.g., Erichsen et al. 2019; Rungis & Krivmane 2021), which failed to amplify Tc918 in *T. cordata*. Our results highlight the usefulness of this marker in the Central European gene pool of the *Tilia* genus. In our study, we found that another marker, Tc8, was highly diagnostic. *T. cordata* and *T. tomentosa* were fixed for a single allele of size 142 bp, while *T. platyphyllos* exhibited a wide allelic spectrum (146–176 bp), with the exception of one sample (*T. platyphyllos* LPV_1_26), a putative hybrid, which also carried the 142 bp allele. Logan et al. (2015, 2019) and Danusevičius et al. (2021) likewise identified locus Tc8 as useful for differentiating between *T. cordata* and *T. platyphyllos*, reporting the monomorphic character of the allele size in *T. cordata*. These results emphasise the significance of locus-specific markers for confirming morphological species identification in natural populations. For our lime samples, the mean observed heterozygosity was 0.53 and the mean expected heterozygosity was 0.57. Wolff et al. (2021) reported similar expected heterozygosity values for *T. cordata* and a higher value of 0.74 for *T. platyphyllos* growing in the Bavarian Forest National Park. In Latvian *T. cordata* populations, the values were comparable, with H_o = 0.56 and H_e = 0.60 (Rungis & Krivmane 2021). In nine Danish populations, the observed and expected heterozygosity values were identical (0.62) (Erichsen et al. 2019). Similar values of H_o and H_e, around 0.5, were also reported for *T. cordata* populations from different countries (Denmark, Finland, Russia, the UK) (Logan et al. 2019).

In the past, monocultural commercial forests were preferred and naturally occurring lime trees were significantly reduced. Today, greater emphasis is placed on restoring species-rich and genetically diverse forest stands. The use of the presented SSR markers will support the conservation of high-quality, genetically variable resources with precisely determined species *T. cordata* and *T. platyphyllos*. These sources of reproductive material should be used in artificial forest restoration to improve the forest composition.

CONCLUSION

The objective of this paper was to develop a reliable method to reveal and analyse the genetic background of a less economically valuable broadleaf temperate forest tree genus, the common lime (*Tilia* sp.), in the context of the Czech Republic. Locus-specific microsatellite markers (SSRs) still promise a reliable and relatively cost-effective method to solve the problem of clear species delineation and genetic diversity assessment for taxonomically challenging plant genera with suspected hybridisation between species. A selection of 15 previously explored SSR markers was tested on a sample size of 250 individual trees collected in the Czech Republic. This collection was made in three species (*Tilia cordata, Tilia platyphyllos, Tilia tomentosa*) that were determined based on morphology. The microsatellite analysis confirmed the reliability of the two loci (Tc8 and Tc918) for clearly distinguishing *T. cordata* and *T. platyphyllos*, the locus Tc918 newly showed unequivocal PCR amplification in *T. cordata*. One possible hybrid (*Tilia* × *europaea*) was also identified in the dataset. These results also emphasise the importance of thorough methodology optimisation, as even the less polymorphic loci can prove to be useful for species delineation in some contexts. Genetic profiling by SSR markers can help in the future reliable selection of variable reproductive material for minor broadleaf species, such as the common lime, that also respects the local ecological conditions of the Czech Republic.

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Data availability. Source data are available upon request from the corresponding author.

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