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A contribution to the systematics of the genus *Tilia* with respect to some hybrids by RAPD analysis

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Abstract: The putative hybrid character of two *Tilia* varieties selected as avenue trees ('Szent Istvan' and 'K3') should be clarified for further breeding activities. Their ancestor species should be identified by a genetic comparison with reference material (*Tilia cordata, T. platyphyllos, T. dasystyla, T.* × *euchlora, T.* × *europaea* and others). RAPD marker data were evaluated by UPGMA cluster and neighbour-joining analysis. The genetic comparison of the two selected clones with the collection of reference material offers insights into some systematic relationships within the genus *Tilia*. It was supplemented by a critical discussion of methods of RAPD data evaluation for taxonomic purposes.

Additional key words: *Tilia* L.; linden; taxonomy; hybrids; DNA; UPGMA cluster analysis; neighbour-joining; resampling

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Introduction

Trees and shrubs of the genus *Tilia* L. (Malvaceae) were found in the forests of the northern hemisphere in Europe, Asia and Eastern North America and Central America. They are growing in temperate, subtropical and tropical climates and occur from moist to dry regions(Muir 1984).

Representatives of these deciduous trees became increasingly important in municipal parks of large cities and play a central role as avenue trees (Sukopp and Wurzel 2000). Growing damages of lime-trees were observed in European cities because of the ubiquitous stress factors as salt and drought [Helsinki: Terho and Hallaksela (2005), Warsaw: Chmielewski et al. (1998), Budapest: Sinkó (unpublished)]. Former plantings were undertaken more for ornamental reasons. Since the 1960s, *T. cordata* Miller and *T. platyphyllos* Scop. were increasingly replaced by *T. tomentosa* Moench and *T.* × *euchlora* K. Koch from southeast Europe and the Caucasian region because of their better adequacy to urban conditions in Central Europe. Actually the breeding efforts focus on better-adapted plant material with high drought and salt resistance and more tolerance to soil compaction for urban locations (Karnosky et al. 1982).

In Budapest, several trees from an avenue with positive traits in stress resistance were selected (Jámborné et al. 2001). They are about 30 years old and originate from seedling offsprings after free pollination. They are putative hybrids but their parents could not be reconstructed. Presumed ancestors seem

to originate from the group of European and Caucasian lime-trees. One selected variety ('Szent István') develops morphological characteristics similar to *T. dasystyla* Steven or *T.* × *euchlora*. The other variety ('K3') is more similar to *T. platyphyllos*. However, their morphological characteristics of twigs, leaves, flowers and fruits did not allow a doubtless taxonomic classification to fulfil the standards for traded ornamental plants (Sinkó 2005). The morphological findings of the selected clones of interest should now be completed by genetic data to get more information on their taxonomic status for further breeding. This suggestion bases on the hypothesis that phylogenetic relationships could be reflected by suitable genetic markers and compared to adequate reference material. Several methods of data evaluation should be critically reviewed to reconstruct the evolutionary relationships of the investigated group of organisms.

Only some genetic studies in the genus *Tilia* (*T. cordata* and *T. platyphyllos*) were carried out by isozyme markers (Maurer and Tabel 1995; Fromm and Hattemer 2003). Isozyme markers in the context of population genetic studies of outcrossing species are known to possess a high level of within population variation. An additional difficulty for codominant nuclear markers is the polyploidisation in the phylogeny of these lindens (Fromm and Hattemer 2003). However, sufficient population based material from the Caucasian species group could not be provided and thus isozyme markers did not come into question. Nuclear microsatellite markers for *Tilia* species are not available so far. Even if they existed, they would not be suitable for our study for the same reason like isozyme markers.

High variation in chloroplast DNA haplotypes was detected for European *T. cordata* populations (Fineschi et al. 2003). However, chloroplasts were mostly maternal inherited in Angiosperms (Harris and Ingram 1991; Rajora and Dancik 1992) and therefore did not reveal taxonomic relationships in case of possible hybridisation between closely related species. Secondary, it is known that several related species share their chloroplast genotypes within the genus [i.e. *Tilia* (Brunner et al. 2001), *Quercus* L. (Dumolin-Lapegue et al. 1997), *Betula* L. (Palme et al. 2004), *Hedera* L. (Grivet and Petit 2002)]. Thus, chloroplast markers are not appropriate for our subject.

Markers widely distributed within the genome would be the method of choice for visualisation of phylogenetic relationships and to get information on the origin of putative hybrids, even if the number of reference accessions is low: species-independent RAPD (Random Amplified Polymorphic DNA) and AFLP markers (Amplified Fragment Length Polymorphism). Due to the fact that AFLPs need an extensive laboratory input, the risk of somewhat lower reproducibility was accepted. For the application of RAPD markers no molecular information is needed for short, arbitrary primers. As a fingerprinting method these markers are well established for clonal and cultivar identification of numerous plant species. Representative examples amongst others are given for poplar, melon and *Citrus* (Rajora and Rahman 2003; Tanaka et al. 2007; Rao et al. 2007). By application of a sufficient number of primers and evaluable bands, RAPDs reveal good estimations of genetic similarities and degree of relationship.

Methods

Plant material

The putative hybrid clone 'Szent István' (No. 1 in Table 1) and the clone 'K3' (No. 2), which is very close to *T. platyphyllos* regarding morphological traits, are selected clones for urban locations with a problematic taxonomic status. A collection of plant material from the genus *Tilia* was compiled to cover the putative relatives, which were assumed among the European and Caucasian group of *Tilia* species based on morphological observations (*T. dasystyla*, *T. dasystyla* Steven *subsp. caucasica* (V. Engl.) Pigott and *T.* × *euchlora*). *T.* × *euchlora* is a putative hybrid of *T. cordata* and *T. dasystyla*.

A number of individuals of available species were included to estimate within and among population variation at the species level as far as possible (*T. cordata* and *T. platyphyllos*). The specimens No. 26 and No. 27 are labelled as *T. dasystyla* in the Botanical Garden Berlin-Dahlem, but they were identified as *T. cordata* by morphological traits (Sinkó unpublished). Two other species with only 3 individuals were included into the experiment: *T. tomentosa* and *T. amurensis* Rupr.

Furthermore some selected clones were involved: *T. cordata* 'Wega', *T. tomentosa* 'Szeleste', *T.* × *europaea* L. 'Pallida', and one unknown *Tilia* specimen from the Späth Arboretum in Berlin. *T.* × *europaea* is possibly a hybrid of *T. cordata* and *T. platyphyllos*.

In addition, species far distant from the putative relatives of the interesting clones were integrated as outgroups to test the reliability of the method (America: *T. americana* L., Asia: *T. henryana* Szyszyll.).

RAPD analysis

Twigs were harvested in February and March 2004. They were cultivated in greenhouse up to the development of first young leaves. Leaves were frozen in liquid nitrogen and homogenised. DNA was isolated with the help of DNeasy Plant Mini Kit (QIAGEN) according to manufactures instructions. The samples of 20–50 mg leaf tissue yielded in respectively 100–250 ng DNA estimated by photometric assay.

A standard protocol was used to carry out the RAPD analysis. A total of 36 arbitrary primers were used for the amplification (Table 2). Each PCR (Polymerase Chain Reaction) was performed in 25 μl reaction volume containing 100–250 pg template DNA, dNTP mix (200 μM each nucleotide), 0.4 μM primer, reaction buffer (including $MgCl_2$ to a final concentration of 2.5 mM) and 1 Unit Taq polymerase (Amersham Pharmacia Biotech, NJ, USA).

The amplification reactions were carried out in a thermocycler (Biometra GmbH, Göttingen, Germany) with the following program: Initial denaturation 5 minutes at 95°C; 30 cycles with 30 sec 95°C denaturation, 30 sec 35°C annealing, 1 min 72°C elongation; final extension 7 min 72°C; hold 4°C.

PCR products were separated in 1.8% agarose gel by electrophoresis and stained with ethidium bromide. The gels were photographed using the Kodak Edas 290 System.

A replication of DNA extraction was carried out with tissues from buds and cambium in one case and replications of DNA amplification were performed with 17 relevant primers and a subset of samples to confirm reproducibility.

Table 1. Material collection of genus *Tilia* including selected clones

No.	Species and/or cultivar	Abbreviation	Location in Germany	Age (years)	Place of origin
1	*T.* 'Szent István'	T.'SzIs'	Berlin, Humboldt University	3	Hungary, Budapest
2	*T.* 'K3'	T.pla'K3'	Berlin, Humboldt University	3	Hungary, Budapest
3	*T. cordata* 'Wega'	T.c'Wega'	Berlin, Humboldt University	8	Commercial nursery clone
4	*T. tomentosa* 'Szeleste'	T.t'Szel'	Berlin, Humboldt University	8	Hungary, Szeleste
5	*T.* ×*europaea* 'Pallida'	T.xvu'Pal'	Berlin, Humboldt University	15	Commercial nursery clone
6	*T.* ×*euchlora*	T.xeuc1	Berlin, Botanical Garden Dahlem	29	Unknown
7	*T.* ×*euchlora*	T.xeuc2	Berlin, Arboretum Späth	~ 70	Unknown
8	*T.* ×*euchlora*	T.xeuc3	Berlin, Humboldt University	15	Commercial nursery clone
9	*T. dasystyla*	T.das3	Berlin, Arboretum Späth	103	Russia, Novospasskoye
10	*T. dasystyla caucasica*	T.cauc	Tharandt, Forest botanical garden	~ 25	Iran, Aschchabad
11	*T. platyphyllos*	T.plat1	Göttingen, Forest botanical garden	~ 130	Germany, Höxter
12	*T. platyphyllos*	T.plat2	Göttingen, Forest botanical garden	~ 100	Germany, Höxter
13	*T. platyphyllos*	T.plat3	Göttingen, Forest botanical garden	~ 100	Germany, Höxter
14	*T. platyphyllos*	T.plat4	Mixed forest	80–100	Germany, Pritzhagen
15	*T. platyphyllos*	T.plat5	Mixed forest	80–100	Germany, Pritzhagen
16	*T. platyphyllos*	T.plat6	Mixed forest	80–100	Germany, Pritzhagen
17	*T. platyphyllos*	T.plat7	Mixed forest	80–100	Germany, Pritzhagen
18	*T. platyphyllos*	T.plat8	Mixed forest	80–100	Germany, Pritzhagen
19	*T. cordata*	T.cor1	Berlin, Botanical Garden Dahlem	1	France
20	*T. cordata*	T.cor2	Berlin, Botanical Garden Dahlem	22	Russia, Zhiguli
21	*T. cordata*	T.cor3	Mixed forest	80–100	Germany, Pritzhagen
22	*T. cordata*	T.cor4	Mixed forest	80–100	Germany, Pritzhagen
23	*T. cordata*	T.cor5	Mixed forest	80–100	Germany, Pritzhagen
24	*T. cordata*	T.cor6	Mixed forest	80–100	Germany, Pritzhagen
25	*T. cordata*	T.cor7	Mixed forest	80–100	Germany, Pritzhagen
26	*T. cordata*	T.das1	Berlin, Botanical Garden Dahlem	~ 25	Caucasus
27	*T. cordata*	T.das2	Berlin, Botanical Garden Dahlem	~ 25	Caucasus
28	*T. amurensis var. amurensis*	T.amu1	Tharandt, Forest botanical garden	~ 30	Kasachstan, Karaganda
29	*T. amurensis var. amurensis*	T.amu2	Tharandt, Forest botanical garden	~ 30	Russia, Moscow Pan-Kudryashov Botanical Garden
30	*T. amurensis*	T.amu3	Tharandt, Forest botanical garden	~ 30	Georgia, Laghodeki (Caucasus)
31	*T. tomentosa*	T.tom1	Berlin, Botanical Garden Dahlem	18	Hungary, Villány-Siklós
32	*T. tomentosa*	T.tom2	Berlin, Botanical Garden Dahlem	18	Hungary, Villány-Siklós
33	*T. tomentosa*	T.tom3	Berlin, Botanical Garden Dahlem	18	Hungary, Villány-Siklós
34	*T. americana*	T.amer	Berlin, Botanical Garden Dahlem	~ 50	USA
35	*T. henryana var. subglabra*	T.henr	Berlin, Botanical Garden Dahlem	18	China
36	*T. sp.*	T. ???	Berlin, Arboretum Späth	~ 50	Unknown

Table 2. Random primers used for RAPD analysis of *Tilia* specimens

Primer designation	Sequence (5'–3')	Primer designation	Sequence (5'–3')
L 01	GGC ATG ACC T	L 19	GAG TGG TGA C
L 02	TGG GCG TCA A	L 20	TGG TGG ACC A
L 03	CCA GCA GCT T	OPA 03	AGT CAG CCA C
L 04	GAC TGC ACA C	OPA 09	GGG TAA CGC C
L 05	ACG CAG GCA C	OPA 13	CAG CAC CCA C
L 06	GAG GGA AGA G	OPB 10	CTG CTG GGA C
L 07	AGG CGG GAA C	OPD 11	AGC GCC ATT G
L 08	AGC AGG TGG A	OPE 01	CCC AAG GTC C
L 09	TGC GAG AGT C	OPE 02	GGT GCG GGA A
L 10	TGG GAG ATG G	OPE 03	CCA GAT GCA C
L 11	ACG ATG AGC C	OPE 05	TCA GGG AGG T
L 12	GGG CGG TAC T	OPE 11	GAG TCT CAG G
L 13	ACC GCC TGC T	OPE 16	GGT GAC TGT G
L 14	TGT ACA GGC T	OPE 20	AAC GGT GAC C
L 15	AAG AGA GGG G	OPG 14	GGA TGA GAC C
L 16	AGG TTG CAG G	OPG 15	ACT GGG ACT C
L 17	AGC CTG AGC C	OPK 20	GTG TCG CGA G
L 18	ACC ACC CAC C	OPY 07	AGA GCC GTC A

Data evaluation

Clear and well resolved fragments were scored manually to construct a zero and one matrix of all well resolved bands, where "one" represented the presence of a certain band and "zero" their absence. The pairwise DICE coefficient was calculated (similarity = 2a/(2a + b + c)), whereas "a" is the number of matches and "b" and "c" are the numbers of mismatches comparing two specimens. The corresponding distance matrix (distance = 1 – similarity) was used to execute a hierarchical cluster analyses (UPGMA, unweighted pair group method using arithmetic averages) and as an alternative the neighbour-joining (NJ) analysis for construction of dendrograms. The UPGMA clustering method produces branch lengths proportional to genetic distances, whereas the neighbour-joining method minimises the total length of the tree.

Several resampling methods for the RAPD fragments as well as for the *Tilia* taxa were carried out to test the robustness of the trees (program package FreeTree by Pavlicek et al. (1999), N=1000 replicated datasets for all tests). The software TreeView (Page 1996) was used for graphical displays of NJ trees. A real scaled dendrogram of UPGMA cluster analysis was built with the SAS statistic package (SAS Institute Inc. 2003) using proc distance, proc iml, proc cluster and proc tree.

Table 3. Minimum pairwise DICE distances of *Tilia* clones of interest to reference material (Abbreviations see Table 1)

'Szent István'		'K3'	
T.xeuc1	0.477	T.plat1	0.432
T.xeuc2	0.481	T.plat2	0.472
T.xeuc3	0.506	T.'SztIs'	0.522
T.pla'K3	0.522	T.plat3	0.533
T.das2	0.566	T.plat5	0.566

Results

Out of the 36 random primers tested, the amplifications with 17 primers resulted in suitable and reproducible banding patterns. The remaining 19 primers did not produce PCR products or reliable resolved bands. The data evaluation for banding patterns of 17 primers yielded in 403 polymorphic bands that were scored in a zero and one matrix. The fragment lengths ranged between 230 bp and 2200 bp, and the mean number of bands per successful primer equals to 23.7. An example for RAPD banding patterns is given for primer L 12 in Figure 1.

The DICE similarity coefficients were used to calculate the corresponding distance matrix. As expected, maximum distances occur between the two outgroup specimens *T. americana* and *T. henryana* to all other *Tilia* objects (distances 0.75 … 0.88). The minimum distances (0.01 … 0.09) were observed between the three *T.* × *euchlora* specimens. All other distances range between these extremes.

The clones of special interest 'Szent István' and 'K3' should be regarded for their single pairwise distances to the next relatives among the *Tilia* collection.

The five minimum distances to other specimens extracted from the total pairwise distance matrix (data not shown) are given in Table 3. The clone 'Szent István' is closest associated to *T.* × *euchlora* and the clone 'K3' is closest associated to *T. platyphyllos*. This first result should be viewed in the context of the whole available reference material.

The UPGMA dendrogram (Fig. 2) displays all 36 objects arranged in a hierarchy. The clustering is in a good accordance with the known taxonomy, because the individuals of a certain species are clustered together. Different degrees of relationship covered by the collected material could be assigned to different ranges at the distance scale.

The clustering at the species level was observed to be in the distance range of approx. 0.40 to 0.55. This was evaluated for the species *T. platyphyllos* (T.plat1 … T.plat8, maximum distance 0.40), for *T. cordata* (T.cor1 … T.cor7, T.das1, T.das2, T.c'Wega', maximum distance 0.51) and for *T. amurensis* (T.amu1 … T.amu3, maximum distance 0.55). The within population variation in the mixed forest Pritzhagen is lower: 0.18 for *T. cordata* and 0.22 for *T. platyphyllos*. The maximum distance of 0.18 between the four *T. tomentosa* specimens (T.tom1 … T.tom3, T.t'Szel') is in the same magnitude. They originate from a narrow geographic region in Hungary. The selected clone 'K3' meets the *T. platyphyllos* cluster at a distance of 0.54. This is just in range of the assumed within-species variation.

Another cluster in the upper part of the dendrogram was formed by the hybrid individuals of *T.* ×

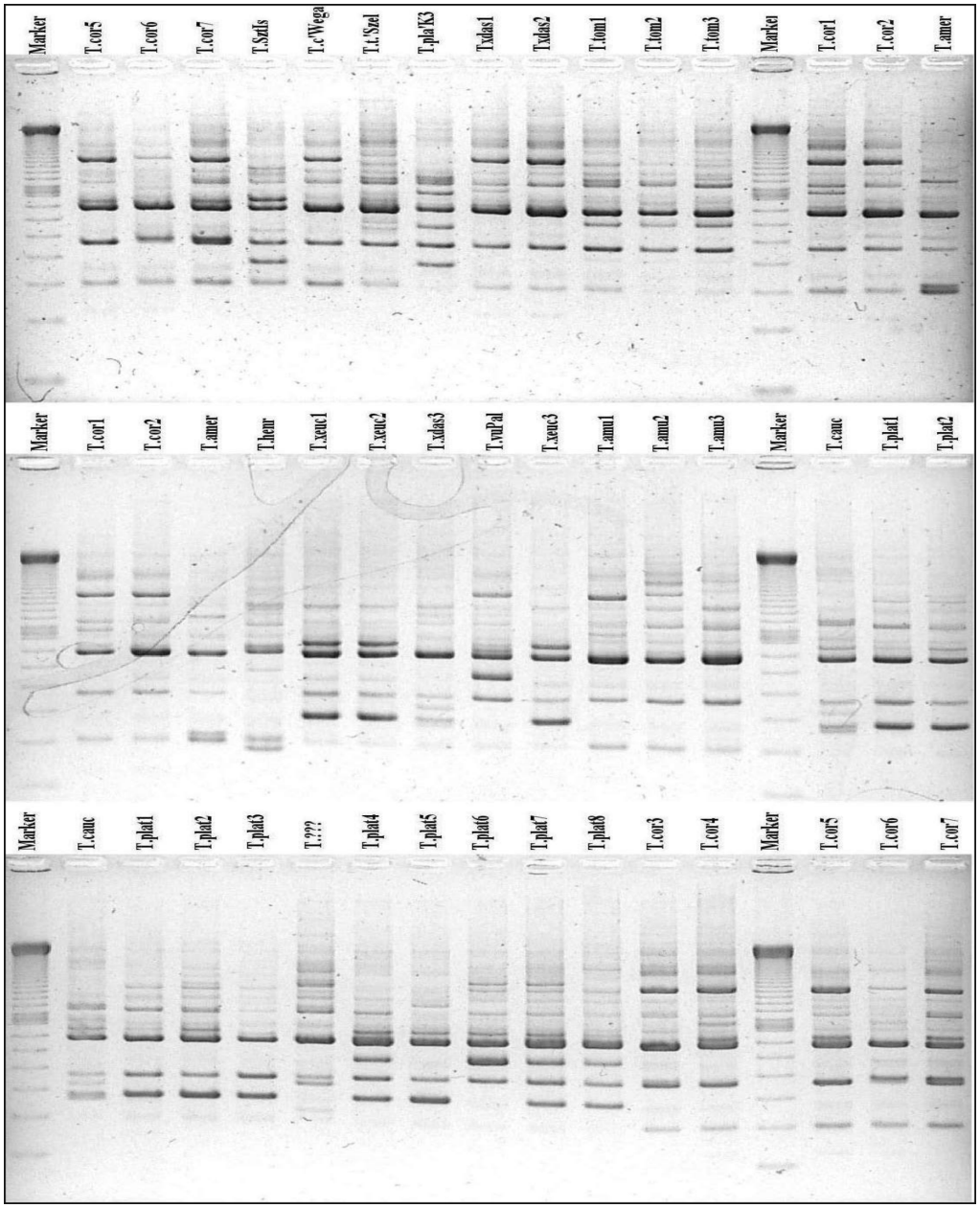

Fig. 1. RAPD pattern of 36 *Tilia* specimens after amplification with primer L 12 Abbreviations see Table 1)

euchlora, the clone 'Szent István', *T. dasystyla* and the hybrid *T.* × *europaea* 'Pallida' (maximum distance 0.55).

Two clusters at the above-species level were ascertained: One for *T. cordata* and *T. amurensis* (distance 0.63) and the other for a *T. platyphyllos – T. dasystyla* complex (distance 0.65). This *T. platyphyllos – T. dasystyla* complex consists of *T. platyphyllos*, *T. dasystyla*, *T. dasystyla caucasica*, the clone 'Szent István' and all hybrids containing *T. platyphyllos* or *T. dasystyla*.

The species *T. tomentosa*, *T. americana* and *T. henryana* as well as the unknown specimen from the Späth Arboretum are singular in the dendrogram without a grouping.

The robustness of the above described UPGMA tree topology was evaluated with several resampling methods offered in the package FreeTree and by comparing with the NJ dendrogram. Firstly the rearrangement of taxa (modifying the input order) was checked. No influence on the structure was observed, all nodes were confirmed with a percentage 100% in the UPGMA as well in the NJ tree.

The comparison of UPGMA and NJ dendrogram confirms the stability of all clusters at the species level (Figs 2, 3): *T. cordata* with 10 specimens (small differences in arrangement of *T. cordata* No. 5, 6 and 7 within population Pritzhagen), *T. amurensis* with 3 specimens, *T. platyphyllos* with 9 specimen (small differences in arrangement of *T. platyphyllos* No. 7 and 8 within population Pritzhagen) and *T. tomentosa* with 4 specimens. The hybrid group consisting of *T.* × *euchlora*, *T. dasystyla*, *T.* 'Szent István' and *T.* × *europaea* 'Pallida' is present in both dendrograms, but with changing positions of *T.* 'Szent István' and *T.* × *europaea* 'Pallida'. The most obvious difference between both dendrograms was found above the species level. The hybrid group meets the *T. cordata* cluster in the NJ tree just before *T. amurensis* comes together with *T. cordata*, whereas the hybrid group is combined with the *T. platyphyllos* cluster in the UPGMA dendrogram.

To assess the stability of clustering a resampling of *Tilia* specimen was carried out for the UPGMA as well as the NJ tree. The method of jackknifing over individuals determines whether their skipping can change the topology of others branches. The number of skipped taxa was successive increased, starting from one skipped taxon, up to no further change of jackknifing percentages was observed. This was the case for n=6 skipped taxa, even though the authors of the software recommended only 1 to 3 depending from the total number. The fusion between the *T. platyphyllos* group and the hybrid group is confirmed in the UPGMA dendrogram with 100% (Fig. 2). In contrast, the association of the hybrid group to the *T. cordata* cluster in the NJ tree is very weak with only 12% (Fig. 3).

Additionally, two methods of resampling the RAPD fragments were applied. The common used bootstrapping (sampling with replacement) produces repeated data sets that contain some of the RAPD fragments more than one times whereas other RAPD fragments are not present. The jackknifing of RAPD fragments randomly drops a part of the fragments and retains the other part, commonly 50% (202 out of 403 RAPD fragments). For each dendrogram the results of both resampling methods are very similar (data not shown). However, remarkable differences were found between the UPGMA and NJ dendrograms. The fusion of the *T. platyphyllos* cluster and the hybrid group is highly supported with 68 resp. 70% (bootstrapping, jackknifing) in the UPGMA dendrogram. The NJ tree supports the association of the hybrid group to the *T. cordata* cluster with only 6%. Thus, above species level the results of the NJ tree construction method are not reliable within the genus *Tilia*.

Discussion

The RAPD technique could be applied to any objects without prior information on DNA sequences. This relatively simple method with short arbitrary primers could involve sometimes lower reproducibility of banding patterns compared to other PCR based techniques with longer and more specific primers. The major disadvantage, however, is the common dominant nature of the data obtained (Nybom 2004). This disadvantage is rather important in population genetic studies than in a taxonomic investigation.

Several possibilities are available for calculation of distance matrices from binary datasets. Comparisons of several similarity coefficients were reported e.g. by Duarte et al. (1999) and by da Silva Meyer et al. (2004). They concordantly suggest the use of coefficients that do not include negative co-occurrences, because the negative co-occurrences do not necessarily mean that the regions of the DNA are identical. The reported distance levels of clustering within populations (0.18 … 0.22), at the species level (0.40 … 0.55) or between related species (0.63 … 0.65) are a good consensus for the populations and species in this study, but they are not to generalise. They depend on the similarity coefficient, on the collected reference material and on the markers used. They cannot be carried forward to other subjects.

Starting from the DICE similarity matrix two methods of tree construction were tested: the cluster analysis method UPGMA and the neighbour-joining method (Saitou and Nei 1987). Their respective algorithms are responsible for their different advantages and disadvantages. The UPGMA method is directly using the genetic distances for clustering and to scale the branch lengths. For this reason it is only suitable for datasets consisting of objects with relatively constant rates of evolution. The NJ method works without such a precondition, therefore it is adequate to

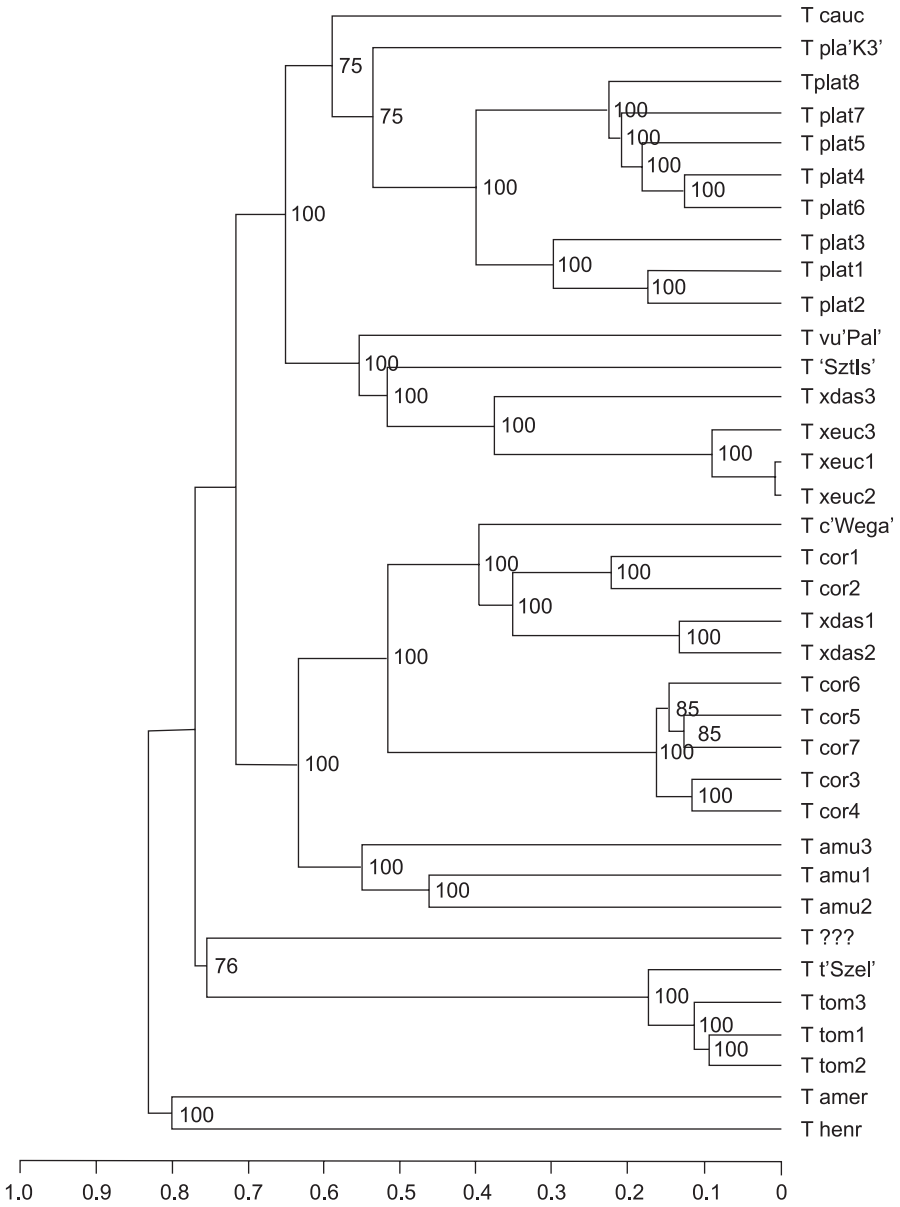

Fig. 2. Dendrogram of UPGMA cluster analysis based on DICE similarities of 36 *Tilia* specimens (Abbreviation see Table 1), numbers indicating jackknife values over the *Tilia* objects (percentage of n=1000 replicated datasets)

phylogenetic studies covering higher taxonomic levels (Mohammadi and Prasanna 2003). The NJ algorithm minimises the total length of the tree. That implies possible changes of tree topology, if OTUs (Operational Taxonomic Units) were added or dropped. An example of such a change is given by Nei (1996) for vertebrates.

Regarding the *Tilia* material in this study, concordant sub-trees at the population and species level were found with both tree construction methods. Yet, the high sensitivity of the NJ method against the skipping of taxa (OTU based jackknifing) leads to substantial doubts. In contrast, UPGMA regarding the real genetic distances is much more stable against the skipping of taxa. The aspect of stability of tree topology against the random omitting of taxa seems to be very important due to the fact that the collected reference material might be incomplete. The collected reference material was selected with respect to the target of the study; nevertheless, it was depended on the availability in botanical gardens. Since there is no reason to assume largely different rates of evolution within the genus *Tilia*, the UPGMA tree is the more reliable tree in this case and should be considered as the basis for conclusions.

The UPGMA dendrogram as a whole is in a good agreement with the taxonomy of the *Tilia* genus. Regarding the clustering above species level, *T. platyphyllos* and *T. dasystyla* are belonging to the subsection Trabeeculares and *T. cordata* and *T. amurensis* are grouped together in the subsection Reticulares (Muir 1984). These both subsections form the section

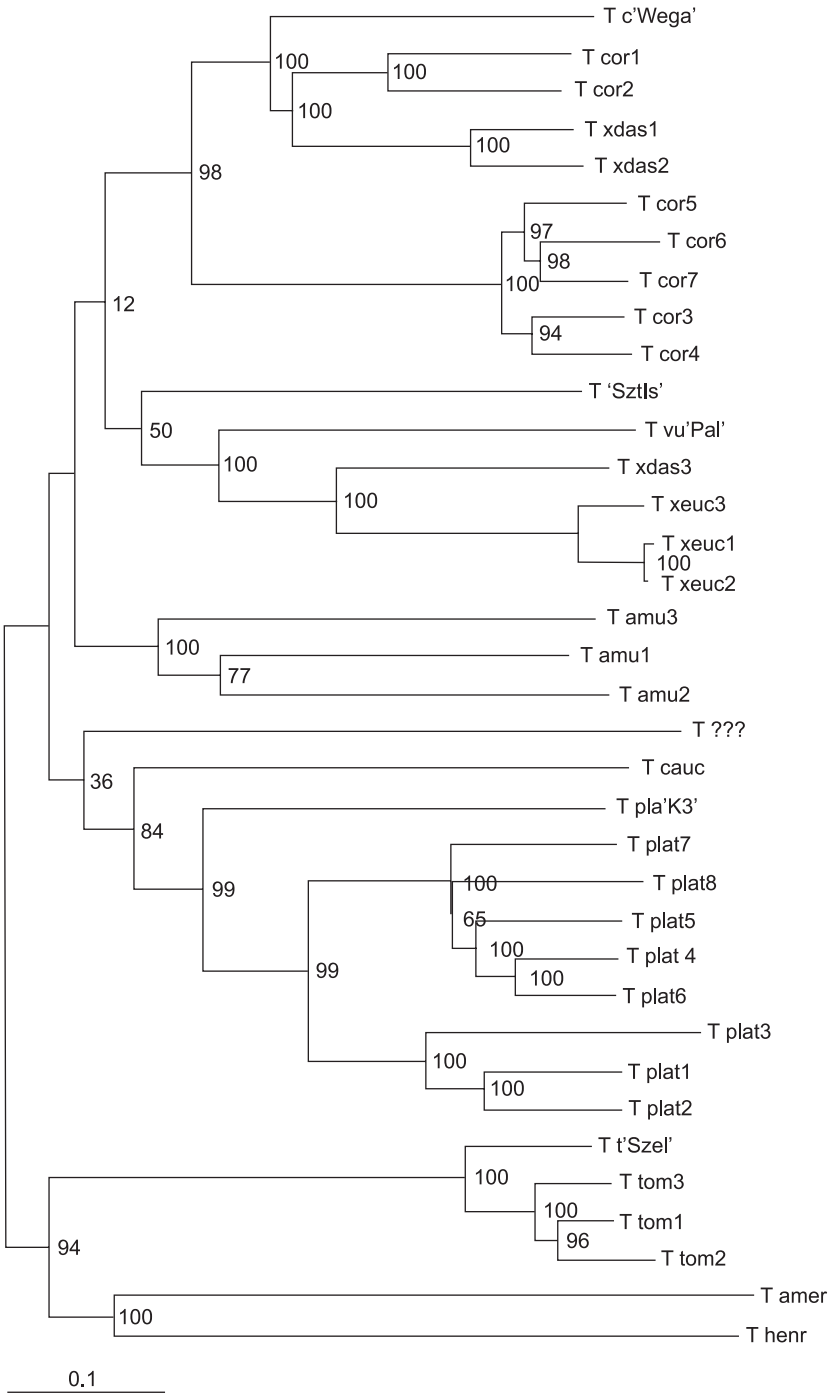

Fig. 3. Dendrogram of NJ analysis based on DICE similarities of 36 *Tilia* specimens (Abbreviation see Table 1), numbers indicating jackknife values over the *Tilia* objects (percentage of n=1000 replicated datasets)

Anastraea, whereas *T. tomentosa*, *T. henryana* and *T. americana* are members of the section Astrophilyra (Muir 1984).

The most problematic part of the dendrogram is the hybrid group consisting of *T.* ×*euchlora*, *T. dasystyla*, *T.* 'Szent István' and *T.* × *europaea* 'Pallida'.

The clone *T.* × *europaea* 'Pallida' has been regarded as a hybrid of *T. platyphyllos* and *T. cordata*. Its seedlings are very variable. Partially they develop morphological traits as *T. platyphyllos*, but never as *T. cordata*. Pigott & Sell (1995) concluded from their observations that *T. platyphyllos* is one parent, but there is no evidence for *T. cordata* as the other parent.

T. × *euchlora*, commonly accepted as a possibly hybrid between *T. cordata* and *T. dasystyla*, exists only in culture. Another source (Wikipedia, http://en.wikipedia.org/wiki/Tilia, unfortunately no reference given) listed *T.* × *euchlora* as the hybrid of *T. dasystyla* and *T. platyphyllos*. *T.* × *euchlora* seems to be one commercial clone with numerous ramets in Europe. Its seeds do not germinate (Muir 1984). One putative ancestor *T. cordata* (or *T. platyphyllos*) is a diploid spe-

cies with 2n=82 chromosomes (Uhrikova and Schwarzova 1980; Pigott 2002), whereas the other ancestor *T. dasystyla* is a tetraploid species with 4n=164 chromosomes (Pigott and Francis 1999). The hybrid *T.* × *euchlora* is probably triploid and its sterility could be explained from the ploidy level.

Ramets of clones should be genetically identical *per se*. However, the small genetic distances based on RAPD markers between the 3 specimens in this study could not be regarded as definitive arguments for or against a single clone in this special case.

It is alleged that RAPD patterns are not always complete reproducible from laboratory operation causes (Nybom 2004). This might be one cause for our results. But additionally two other facts should be regarded. One reason comes from the real existing ecosystem "tree". Numerous microorganisms like bacteria and fungi are located within plant tissues, the so-called endophytes. These endophyte spectra could differ in dependence from age, substrate and other environmental factors as it was detected for a single poplar clone (Ulrich et al. 2007). Thus, short arbitrary primers could amplify DNA from the tree, but as well from endophytes. The other fact is, that there is no observation up to now how variable RAPD patterns of a definitive clone could become after at least 140 years of vegetative propagation, when somatic mutations proceeded within this period.

Perhaps both hybrids, *T.* × *euchlora* and *T.* × *europaea* 'Pallida', do not descend from *T. cordata*. This perspective is in accordance with the cluster analysis in our study. The taxon name *T.* × *euchlora* is occupied from the specimen firstly described by K. Koch 1866 and its vegetative offspring. It is assumed that the clone 'Szent István' is a sister of *T.* × *euchlora*. The putative parents could be T. *dasystyla* and *T. platyphyllos*.

The other clone of interest 'K3' is closest associated to *T. platyphyllos* as it was shown by the single pairwise distances to the next relatives among the *Tilia* collection. The selected clone 'K3' meets the *T. platyphyllos* cluster in the UPGMA dendrogram at a distance of 0.54. This is just in range of the assumed within-species variation. Nevertheless, a small contribution of *T. dasystyla* within the pedigree should be not excluded, because of the genetic similarity to *T.* 'Szent István' (see table 3) and some morphological traits.

Further breeding effort for *Tilia* avenue trees should focus on crossings between *T. dasystyla* as one parent and *T. cordata* and *T. platyphyllos* as the other parent. Offspring material from both hybrid families should be tested for stress resistance traits and for their adequacy to urban environments. Furthermore, the ability for vegetative propagation should include into the selection criterions.

Parents and offspring material from these both hybrid families could be a good addition to the reference material for further taxonomic investigation at the RAPD marker level and a better characterisation of the present selections 'Szent István' and 'K3'. Moreover, a perspective for more specific DNA markers than RAPDs for species identification could be the development of SCAR markers (Sequence Characterized Amplified Region) based on single specific RAPD bands (Scheef et al. 2003). This would be a step to more reliable results with lower number of markers, but the suitability of SCAR markers to identify hybrids is not self-evident and should be tested in every special case.

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