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Genetic diversity loss and homogenization in urban trees: the case of *Tilia* × *europaea* in Belgium and the Netherlands

An Vanden Broeck[1] · Karen Cox[1] · Iwona Melosik[2] · Bert Maes[3] · Koen Smets[4]

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Abstract

Urban trees form a vital component of sustainable cities but the use of a restricted range of species and genotypes may pose a risk to global biodiversity. Despite several studies investigating tree species diversity, intraspecific genetic diversity of urban trees remains largely unexplored. Here, we characterized the genetic diversity of *Tilia* × *europaea*, one of the most widely planted urban tree species in Northwest Europe. We compared the genotypic diversity of historical plantings of *Tilia* spp. from the 17th century with the genotypic diversity of currently available planting stock in Belgium and the Netherlands. In total, 129 trees were sampled and genotyped with 14 microsatellite loci and 150 polymorphic Amplified Fragment Length Polymorphism markers. In Northwest Europe, homogenization of urban *T.* × *europaea* plantings already started at the 17th century. Genetic diversity within contemporary commercial planting stocks was extremely narrow and consisted mainly of two clones, sold under the name 'Pallida' and 'Zwarte linde'. The genetic diversity found within the historical plantings was about four times higher than in the current commercial planting stocks. We recommend that tree nurseries should enlarge the genetic diversity of *T.* × *europaea* commercial planting stocks. The old clones have shown long-term disease resistance and could provide tree breeders with the valuable new genetic material. The range of available *Tilia* species and genotypes needs to be explored in future urban tree planning to optimize desired ecosystem services.

Keywords AFLP · Clones · Historical gardens · Single sequence repeats, *Tilia* · Tree breeding

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✉ An Vanden Broeck
an.vandenbroeck@inbo.be

Extended author information available on the last page of the article

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Introduction

The importance of biodiversity in the provision of ecosystem services in urban forests, parks, and gardens is widely recognized. Though several studies focus on species diversity of urban trees to examine their long-term impacts on urban ecosystem sustainability (e.g. Morgenroth et al. 2016; Richards 1993; Sjoman et al. 2012), little work has been done to understand the effects of intraspecific genetic diversity (Zhang et al. 2014). Indigenous species usually have substantial genetic variation within the species, which may also be important for their function in urban ecosystems (Richards 1993), for example in limiting disease spread (King and Lively 2012). However, in the tree nursery industry a few cultivars that have superior qualities are selected and clonally propagated, yielding plants of known qualities, but with little intraspecific genetic diversity (Morton and Gruszka 2008; Polakowski et al. 2011). The use of a few, widely distributed, cultivars and clones may pose a risk to global biodiversity via biotic homogenization (King and Lively 2012; Richards 1993). Homogeneous plantings, including those based on a restricted number of genotypes, are at heightened risk of disease attack (e.g. Boyd et al. 2013). Across Europe and the U.S., insects, and pathogens, such as *Ophiostoma novo-ulmi* that is responsible for the Dutch elm disease and *Cryphonectria parasitica*, the causal agent of chestnut blight, have caused epidemic rates of tree deaths (e.g. Desprez-Loustau et al. 2007). These recent pest outbreaks and the environmental changes resulting for example from air and soil pollution and climate changes, highlight the need for diversity at the gene level to achieve a resilient urban tree stock as an important contributor to urban ecosystem stability and to the functioning of trophic interactions (Boyd et al. 2013; Cardinale et al. 2006).

For centuries, *Tilia* × *europaea* (syn. *Tilia* × *vulgaris*) has been widely used as an ornamental tree in avenues, urban forests, parks and gardens in Northwest Europe (Bengtsson 2005; Maes 1990; Maes and van Vuure 1989; Pigott 2012). For example in Helsinki, *T.* × *europaea* comprises over 44% of all the current street and park trees (Sjoman et al. 2012). *T.* × *europaea,* the common lime or common linden, is a hybrid of *T. platyphyllos* and *T. cordata*. It occurs at scattered localities in the wild in Europe where *T. cordata* and *T. platyphyllos* occur in sympatry, although natural hybrids seem to be rare suggesting reduced hybrid fertility compared to the parental species (Phuekvilai 2014). *T.* × *europaea* was exported from Europe and introduced to North America where it has been occasionally planted (Pigott 2012). The hybrid can be easily vegetatively propagated by layering, where a branch grows adventitious roots when it touches the ground and is later detached from the parent plant. The domestic production of the *T.* × *europaea* is collectively known as 'The Dutch lime' (Bengtsson 2005).

Pigott (1992, 2012) describes several clonal groups of *T.* × *europaea*, on the basis of their general morphology. In England, two clonal groups were distinguished in trees planted before 1750: *T.* × *europaea* 'Pallida' (syn. 'Koningslinde', 'Kaiserlinde') and *T.* × *europaea* 'Hatfield'. A third clonal group *T.* × *europaea* 'Zwarte linde' was widely planted in the Netherlands and exported to Germany and Sweden, but rarely planted in Britain (Pigott 2012). Bengtsson (2005) investigated the morphological variation of *T.* × *europaea* in Swedish gardens created in the 17th & 18th centuries and extended the Pigott's (1992) classification system to six clonal groups by adding the following groups 'Ulriksdal', 'Malmvik' and 'Crimea' which is also called *T.* × *euchlora* 'Euchlora'. *T.* × *euchlora* is a hybrid whose parentage is unclear, but it is considered a hybrid of either *T. cordata* and *T. dasystyla*, or of *T. cordata* and *T. platyphyllos* (Pigott 2012).

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Large numbers of *T.* × *europaea* trees were exported from the Low Countries (corresponding to the present-day Netherlands and Belgium) for planting tree avenues in England and Sweden during the 17th & 18th centuries (Bengtsson 2005; Pigott 1992). These historical plantings almost certainly originated by propagation from historic village trees (Pigott 2012). Similarly, according to old archives, large numbers of *T.* × *europaea* trees from the Low Countries were imported in Denmark during the 17th century. Hansen et al. (2014) analyzed historical plantings of *T.* × *europaea* in Denmark using DNA markers and found that the same clones have been produced for decades or even centuries (starting in the 17th and 18th centuries) by private nurseries in the Dutch Republic. Hansen et al. (2014) suggested that, in Northwest Europe, the *T.* × *europaea* trees imported 400 years ago consisted of a very limited number of clones and that some of these clones are nowadays still sold commercially. If this is the case, it would mean that the same genetic material has been used in different parts of Northwest Europe for centuries, resulting in *T.* × *europaea* plantings of limited genetic variation (Hansen et al. 2014). This might be problematic in the long-term response to biotic stresses, like diseases, and abiotic stresses, such as drought or salt tolerance (e.g. Cavers and Cottrell 2015). Although some information exists about the planting of this tree, information on varietal identification and genetic relationships, particularly for old *Tilia* trees, is lacking.

Genetic markers are powerful tools for identifying clones and clonal lineages or cultivars. They allow clustering of individuals into homogeneous groups based on their genotype. Single sequence repeat (SSR) polymorphisms (microsatellite loci) and amplified fragment length polymorphisms (AFLPs) are both frequently used for this purpose (e.g. Buiteveld et al. 2016; Cox et al. 2014; Cretazzo et al. 2010). Microsatellite markers are characterized by hyper variability. transferability across species and the capacity for data comparison across laboratories. On the other hand, AFLPs generate a very high number of polymorphisms in a single assay and are characterized by a reasonable coverage of the genome (Vos et al. 1995).

Here, we used both marker systems, SSRs and AFLPs to identify the number of clones used in historical and contemporary urban plantings of *T.* × *europaea* in Belgium and the Netherlands. The objectives of this study were to: (1) provide a better understanding of the genotypic diversity within historical and contemporary urban plantings of *T.* × *europaea* in Belgium and the Netherlands, including the lime avenue at the Tongerlo Abbey (Westerlo) of the 17th century that is one of the oldest plantings of *T.* × *europaea* in the Low Countries; (2) provide recommendations to park managers, urban planners and the tree nursery industry to improve practices for maintaining *T.* × *europaea* genetic diversity to protect the trees from existing and future diseases which will contribute maintaining urban ecosystem adaptability for the future.

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Materials and methods

Samples and sampling locations

We sampled the trees in the entrance avenue at the Tongerlo Abbey (Westerlo) and several other monumental *T.* × *europaea* trees of historical and/or cultural interest, further called 'heritage trees', in Belgium and the Netherlands. To assess the genetic diversity of the historical plantings of *T.* × *europaea,* we compared these trees with planting stock of *T.* × *europaea* currently available at eight commercial tree nurseries in Belgium and the

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Netherlands. In total, we studied 110 individual trees of *Tilia* species, mainly of *T.* × *europaea*. Furthermore, 19 samples of *T.* × *europaea, T. platyphyllos, T. cordata* and the cultivar 'Euchlora' collected at botanical gardens and arboreta, called 'reference samples', were included in this study, resulting in a total of 129 trees. An overview of the number of samples successfully analyzed, excluding replicates, is shown in Table 1 and a complete sample list is given in Table S1.

Characterization of the lime tree avenue at the Tongerlo Abbey

The lime tree avenue at the Tongerlo Abbey (Lat.: 51,105669N/Long.: 4.906287E, Westerlo, Belgium) is one of the oldest plantings of *T.* × *europaea* in the Low Countries. The avenue was planted in the winter of 1676–1677 (Smets 2015). The avenue leads up to the abbey entrance and is a part of protected landscape area. The knowledge of the genetic diversity of the original remaining trees is considered a critical component of an effective long-term park management strategy. The avenue consists of 32 lime trees including 20 original *T.* × *europaea* trees planted in 1676 or 1677. One original tree, included in this study (tree A5; Table S1), was felled by a storm in 2014 and soon afterward propagated by cuttings. In total, 21 samples of the original *T.* × *europaea* trees planted in 1676–1677 were included in this study. In 1910, the trees were beginning to show signs of deterioration due to old age (Chalon 1910). They were in a generally good condition, but their trunks

Table 1 Summary of the samples successfully analyzed with SSRs and AFLP markers

	Sample category	Number of trees sampled	Number of trees analysed with SSR & AFLPs
1	Lime avenue Tongerlo Abbey, original trees planted in 1676–1677		
	Tilia × *europaea*	21	20
2	Lime avenue Tongerlo Abbey, replacements of original trees		
	Tilia platyphyllos	5	5
	Tilia × *europaea*	6	6
3	Heritage trees		
	Tilia platyphyllos	18	18
	Tilia × *europaea*	35	34
	Tilia × *euchlora*	3	3
4	Commercial planting stock		
	Tilia × *europaea* 'Pallida'	14	13
	Tilia × *europaea* 'Zwarte linde'	4	3
	Tilia × *euchlora* 'Euchlora'	4	4
5	Reference samples		
	Tilia cordata	4	4
	Tilia platyphyllos	5	5
	Tilia × *europaea*	6	6
	Tilia × *euchlora*	4	4
	Total	129	125

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were already hollow. On postcards and pictures dating from this period (1911–1920), a few young trees are visible, indicating that some of the original trees had already been replaced at the beginning of the 19th century. To date, the remaining 20 original trees have hollow trunks and heavy branches. The current circumference of the largest tree is 5.61 m at breast height. The lime tree avenue of the Tongerlo Abbey is described on https://inventaris.onroerenderfgoed.be and www.monumentaltrees.com.

In July 2015, leaf samples were collected from the crown of all 32 trees currently creating the lime tree avenue at the Tongerlo Abbey. In addition, second leaf samples were also collected from eight trees (six original trees, one planted in the 19th century and one planted in the 20th century; samples indicated by '_2' in Table S2) from young shoots at the tree base in order to determine whether trees were grafted, planted as seedlings or whether they were vegetatively propagated.

Additional heritage trees

In order to increase the samples size of historical *T.* × *europaea* plantings, we additionally collected leaf samples from 56 ancient heritage lime trees from Belgium (42 samples) and the Netherlands (14 samples) during the summers of 2007 and 2015 (Table S1). A heritage tree is defined here as a tree of historical or cultural interest because of its age, size or condition. The majority of the samples collected in Belgium are described on https://inventaris.onroerenderfgoed.be. For the heritage trees from the Netherlands, leaf samples were collected from the original older trees or from younger trees propagated by cuttings taken from the original heritage trees and grown in the Dutch national lime tree arboretum (Nationaal Linde Arboretum, Corle –Winterswijk, the Netherlands, www.lindearboretum.nl), holding one of the largest collections of lime trees in Europe. The samples collected were taken from the oldest known *T.* × *europaea* trees in the Netherlands, for example from a tree occurring in Sambeek (Boxmeer) (planted in 1580–1623, trunk circumference measured at breast height: 790 cm), a tree near the farm in Warken (Zutphen) (1650, 756 cm), a tree from Tilburg (Heuvel) (17th century, 540 cm) and removed in 2011 (Bert Maes, personal communication), a tree occurring near the castle of Nemelaer in Haaren (1800, 500 cm), a tree located in the park Oud-Bussem in Huizen (1828, circumference: 390 cm), and a tree called 'Kozakkenlinde' in Diepenveen (17th century, 650 cm) (www.monumentaltrees.com).

Current commercial planting stock

To assess the extent to which cultivation of *T.* × *europaea* alters the level of genetic diversity in contemporary urban plantings, we sampled trees from the current commercial planting stock. In July 2015, 22 leaf samples were collected from the current commercial planting stock of *T.* × *europaea* and *T.* × *euchlora* 'Euchlora' from eight commercial tree nurseries of which five were located in Flanders and three in the Netherlands. In the case when *T.* × *europaea* samples were provided by different tree breeders and/or propagators, one sample per origin was collected in the nursery. For *T.* × *europaea,* two different cultivars or clones were available within the commercial planting stock, namely 'Pallida' and 'Zwarte linde'. We sampled 12 trees of *T.* × *europaea* 'Pallida' in seven tree nurseries and originating from ten tree breeders and/or tree propagators. The cultivar *T.* × *europaea* 'Zwarte linde', was only available in two Dutch commercial nurseries. Furthermore, four leaf samples of trees of *T.* × *europaea* that originated from Dutch tree breeders were

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obtained from the Dutch national lime tree arboretum (Corle—Winterswijk): two samples from 'Pallida' and two from 'Zwarte linde'. This resulted in a total of 14 samples of *T.* × *europaea* 'Pallida' and four samples of *T.* × *europaea* 'Zwarte linde'. In addition, four samples from the cultivar *T.* × *euchlora* 'Euchlora' were collected from four tree nurseries located in Belgium and the Netherlands (Table 1).

Reference samples for taxonomic identification

It is sometimes difficult to identify the tree species on the basis of morphological characters because the environment and pruning treatments may have a major effect on leaf morphology (Bengtsson 2005; Pigott 2012). We, therefore, included samples of *T.* × *europaea* and its parental species *T. platyphyllos* and *T. cordata*, as well as *T.* × *euchlora* from botanical gardens as reference samples in the genetic analysis. In total 19 reference samples were collected; six samples from the Botanical Garden Meise (Meise, Belgium), six samples from the Arboretum Bokrijk (Genk, Belgium), and seven samples from Adam Mickiewicz University Botanical Garden (AMUBG) (Poznań, Poland, see also Melosik et al. (2014).

DNA extraction

Fresh leaves were collected and stored on silica gel until DNA extraction, except for seven samples obtained from AMUBG. From the latter, dry-preserved herbarium material was used for DNA extraction. Total DNA was extracted with the QuickPickTM Plant DNA kit (Isogen Life Science, De Meern, the Netherlands). The integrity of the DNA was assessed on 1.5% agarose gels, and DNA quantification was performed with Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies) using a Synergy HT plate reader (BioTek).

Microsatellite analysis

We used 14 microsatellite loci or single sequence repeats (SSRs) to identify clones. The microsatellite loci were developed for the genus *Tilia* by Phuekvilai and Wolff (2013); *Tc4, Tc5, Tc6, Tc8, Tc11, Tc31, Tc915, Tc918, Tc920, Tc927, Tc937, Tc943, Tc951* and *Tc963* (Table S3). Forward primers were labeled with FAM, HEX, VIC, NED or PET (Applied Biosystems). PCR-conditions were performed as described in Phuekvilai and Wolff (2013) and PCR-products were run on an ABI 3500 analyzer with the GeneScan-600 LIZ size standard. Microsatellite fragments were detected with GeneMapper v3.7 software and verified manually. Eight samples were repeated two to five times (mean: 4.4) in different PCR-reactions and on different PCR-plates starting from the same DNA extract to calculate the genotyping error rate. Three negative controls (blanco samples) were also included.

AFLP assay

In addition, AFLP markers were used for clonal assignments. AFLPs were generated according to Vos et al. (1995), but with restriction and ligation conducted in one single step. Initially, 16 primer combinations (EcoRI/MseI) were tested on 16 samples. Two primer combinations were selected for the selective amplification on the basis of clarity and reproducibility of amplified bands, and the presence of polymorphism: *Eco*RI-AGC/*Mse*-CAT and *Eco*RI-ACT/*Mse*-CAC. Amplicons were fluorescently labelled with

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two dyes: NED and FAM. PCR products were run on an ABI 3500 analyser (Applied Biosystems) with the GeneScan-600 LIZ size standard (Applied Biosystems) and analysed using the RawGeno v 2.0.2, an R package for automating AFLP scoring implemented in R CRAN freeware R (Arrigo et al. 2009). We used GeneMapper v3.7 (Applied Biosystems) for the sizing of DNA fragments using the size standard and RawGeno for normalization of peak intensities, applying the mean intensity for each marker. A binary matrix (presence/absence of each marker in each sample) for a user-specified range of marker detection and peak-calling thresholds was then generated using the same program. In a pair of profiles two equally long fragments of a different genomic origin may have been scored, appearing as identical bands in the two profiles (Gort et al. 2006). This is called size homoplasy. Size homoplasy causes bias in the estimation of genetic similarity (Gort et al. 2006). However, avoidance of fragments in the small-size classes (< 150 bp) reduces the risk of size homoplasy (Vekemans et al. 2002). A negative correlation between fragment size and band-present frequency is an indication for potential size homoplasy (Vekemans et al. 2002). We assessed for each primer combination the correlation between AFLP band size and frequency among samples using RawGeno. Estimating the genotyping error rate is essential for distinguishing between experimental error and true genotypic differences among accessions. Fourteen samples, selected randomly, were replicated to assess reproducibility, starting from leaf tissue (four samples) as well as from DNA extract (ten samples). They allowed us to estimate the error rate according to Bonin et al. (2004). Normalisation and phenotype scoring were undertaken for each pair of primers separately. Results for the two primer pairs were then combined for subsequent analyses.

Data analysis

Clone assignment

Clone assignment was performed with the software GenoDive 2.0b23 (Meirmans and Van Tienderen 2004). GenoDive uses pairwise genetic distances to classify samples as members of a clonal group on the basis of a threshold value selected by the user. Samples coming from known clonemates or ramets (i.e. individuals produced asexually) do not always have genetically identical microsatellite multilocus genotypes or identical AFLP-fingerprints, due to genotyping errors and somatic mutations (Douhovnikoff and Dodd 2003). Particularly in long lived, vegetative propagated plants, it is expected that repeated cycles division of mitotic cells over long periods of time leads to the accumulation of somatic mutations (Ally et al. 2010). It is, therefore, necessary to establish a threshold level of genetic distance among individuals, below which they are considered to be the result of clonal reproduction and are thus classified as part of the same clone. The threshold indicates the maximum dissimilarity (genetic distance) that is allowed between two individuals to still be considered as clonemates. While a too low threshold value overestimates the clonal diversity, a too high one may lead to underestimation of clones. There is no single optimal procedure for defining clones using molecular markers (Ally et al. 2010). We, therefore, explored the sensitivity of clonal assignments to the threshold used, by using several threshold-values in GenoDive ranging from 0 to 10 and from 0 to 12, for SSRs and AFLPs, respectively. The threshold value consists of the number of mutations that are needed to transform the genotype of one individual into the genotype of the other, summed over all loci. For the SSR-data, we used the stepwise mutation model (Ohta and Kimura 1973); alleles that differ by only a few base pair repeats are considered to be of more recent common ancestry than those that are more markedly different in terms

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of allele size (Meirmans and Van Tienderen 2004). For the AFLP-data, we used the infinite allele model as mutation model and the Manhattan distance (the sum of absolute differences) as the genetic distance measure between all pairs of individuals, following the recommendations of Meirmans and Van Tienderen (2004). In a dataset containing replicates of the same clone, the frequency distribution of pairwise genetic distances is expected to be bimodal, with a peak at the lower end of the range representative of genetic dissimilarity among individuals within the same clonal group (due to somatic mutations and genotyping errors), and one peak at greater genetic distances indicating differentiation among genets (e.g. Arnaud-Haond et al. 2005; Douhovnikoff and Dodd 2003). We finally defined the appropriate threshold value for the identification of cultivars and the discussion of the results on the basis of this frequency distribution and, for AFLPs, on the basis of dissimilarities among AFLP-fingerprints of the 17 replicated samples (estimated error rate) while taking into account genetic divergence generated by somatic mutations (Fig. 1).

Identical genotypes can also be produced by sexual reproduction when the amount of genetic variation is low or when few genetic markers are used (Meirmans and Van Tienderen 2004). The power of the microsatellite data for identifying individual genotypes was quantified by calculating the probability of identity (P_{ID}) (Paetkau et al. 1995) using GenAlEx 6.5 (Paetkau et al. 1995; Peakall and Smouse 2012). The information content of the AFLP-data for identifying genotypic diversity was evaluated through the clonal population structure test implemented in GenoDive. The null hypothesis (H_0: the observed clonal diversity is due to sexual reproduction) was tested by comparing the observed genotypic diversity (G_0) with the distribution of the expected genotypic diversity (G_e) in a sexual, panmictic population at Hardy–Weinberg equilibrium obtained with Monte Carlo simulations following the general procedure of Hoffman (Hoffmann 1986). This was done by randomizing alleles over individuals using 999 permutations and the corrected Nei's measure of genetic diversity as a statistic (see Gómez and Carvalho 2000). The *p* value obtained is the probability of obtaining the observed or a smaller value of G_0 under sexual reproduction, assuming the random association of alleles at different loci (e.g. no linkage disequilibrium).

Cluster analysis

To identify cultivars and to investigate the genetic differentiation between them, we performed a cluster analysis using the unweighted pair group method analysis (UPGMA) (Sokal and Michener 1958). For the microsatellite data, UPGMA was performed with the program Populations 1.2.31 (Langella 1999, available via http://bioinformatics.org/~tryphon/populations/) and based on pairwise genetic distances between individuals calculated using Cavalli-Sforza and Edwards chord distance (Dc) (Cavalli-Sforza and Edwards 1967). UPGMA based on AFLP-data was performed with the program TREECON 1.3b (Van De Peer and De Wachter 1994) on a similarity matrix calculated from the simple matching coefficient. The reliability and the robustness of the dendrograms were tested by bootstrap analyses with 1000 replications, to assess the statistical support for the topology at a node.

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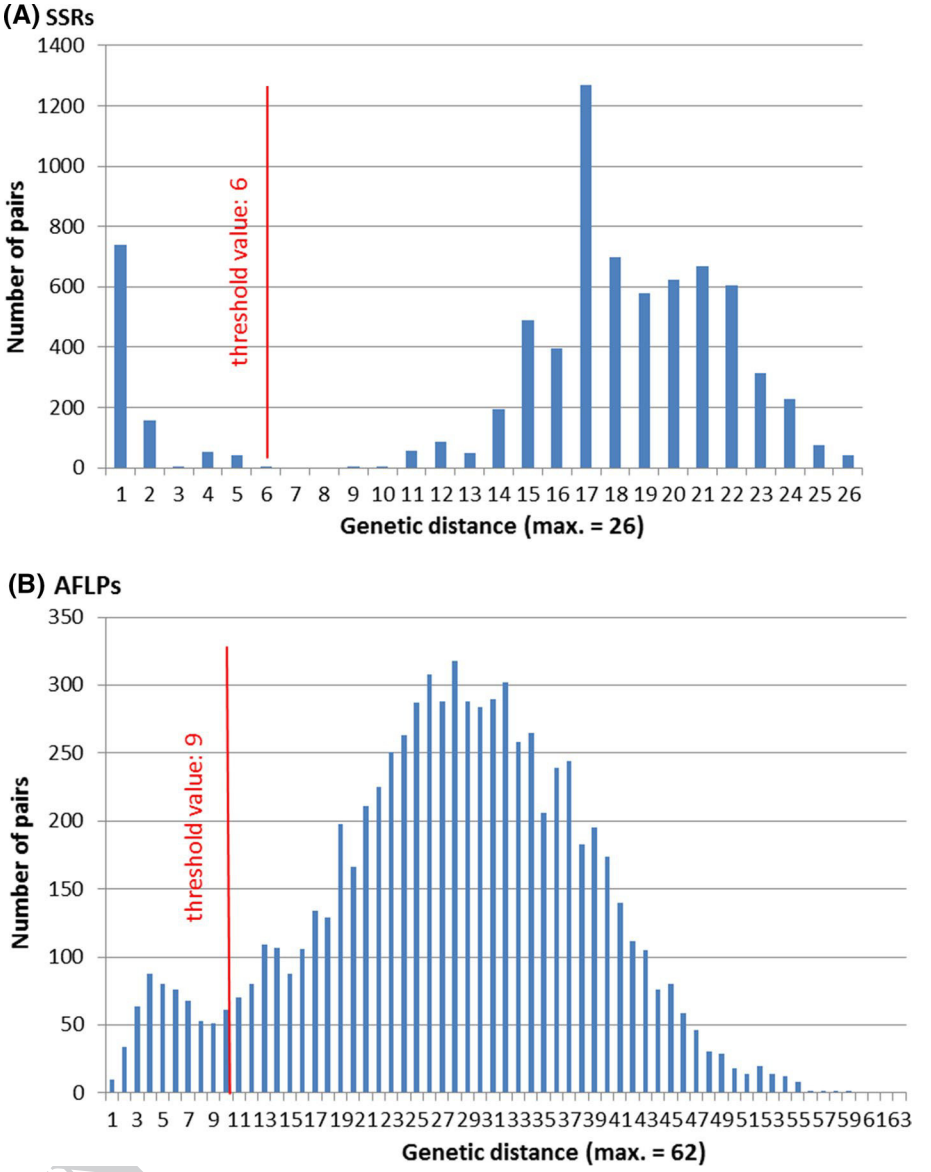

Fig. 1 Histogram of the frequency distribution of the genetic distances based on A. SSRs and B. AFLPs. Histogram of the frequency distribution of pairwise genetic distances (Cavalli-Sforza and Edwards chord distance and Manhatten distance for SSRs and AFLPs, respectively) calculated for 114 individuas of *Tilia*×*europaea*, *T. cordata,* and *T. platyphyllos* and based on 14 microsatellite loci and 150 polymorphic AFLP-markers, respectively. The user-defined threshold for clonal assignment is indicated by the vertical red line

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Results

Marker information and error rates

After removal of samples that produced poor quality DNA and/or banding patterns, microsatellite and complete AFLP markers provided genotypes for 125 of the 129 individual trees sampled. For 11 samples of *T.* × *euchlora*, the amplification of the majority of the microsatellite loci resulted in amplified non-target sequences. This group of samples represented a single genotype with identical microsatellite profiles and are likely to be triploid (see also Pigott 2012). The latter samples were therefore excluded from the genetic distance estimation and the cluster analysis.

All the 14 microsatellite loci were polymorphic, with 2–25 alleles per locus (Table S3). No differences were observed between the genotypes of the repeated samples thereby indicating the consistency of the approach. The locus *Tc918* did not amplify in the samples of *T. cordata*, as also reported by (Phuekvilai and Wolff 2013). The percentage of missing data, locus Tc918 excluded, was 0.0087%.

The two AFLP primer pairs yielded 158 AFLP loci scored, 73 and 85 for the first and second primer pair, respectively, of which 150 (95%) loci were polymorphic. The average polymorphism information content (PIC) (Roldán-Ruiz et al. 2000) of the AFLP markers was equal to 0.17 (S.E.: 0.013). The mean typing error calculated on the 14 replicates was 2.2% corresponding to a mean of 3.5 pairwise differences between loci for the replicated samples. The negative correlation between fragment size and the frequency of AFLP markers among samples (r^2) was not significant suggesting no evidence for size homoplasy (*Eco*RI-AGC (NED) + *Mse*-CAT; $r^2 = -0.155$, $p = 0.189$, *Eco*RI-ACT (FAM) + *Mse*-CAC: $r^2 = -0.146$, $p = 0.181$).

The combined probability of identity $P_{(ID)}$ summed over all microsatellite loci was 4.8×10^{-4} and the observed genotypic diversity based on AFLPs was significantly lower than expected under sexual reproduction ($p = 0.001$) in a hypothetical population. This suggests a sufficient information content of both microsatellite and AFLP -marker data for identifying individual clones.

Cultivar identification

The results for the clone assignments for a range of threshold values for both SSRs and AFLPs are given in the Table S2 and in Fig. S1. For the microsatellite data, we discuss the results of the clonal assignment on the basis of a threshold of six meaning that a maximum of six mutation steps, summed over all 14 loci, are allowed to consider individuals as clonemates. For the AFLPs, we chose a threshold higher than the mean typing error calculated between replicates (i.e. higher than 3.35 pairwise differences) to account for somatic mutations. Considering the frequency distribution of all pairwise genetic distances calculated based on the AFLP-data, there was a valley between the first and second peak at a threshold value of nine (Fig. 1). We further discuss the results of the assignment of clones based on the AFLP-data on the basis of a threshold-distance of nine pairwise band differences between two individuals.

The results of the cluster analysis based on the SSR-data and on the AFLP data, respectively, are represented in Fig. S1. A dendrogram representing the results of the

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Fig. 2 Dendrogram representing the results of the UPGMA cluster analysis based on 14 microsatellite loci for *Tilia*×*europaea*. Numbers above the branch points represent bootstrap values (1000 replications). The number before the sample name represents the clonal group for a defined threshold for clonal assignment of six. The threshold indicates the maximum dissimilarity (genetic distance) that is allowed between two individuals to still be considered as clonemates. UPGMA was performed with the program Populations 1.2.31 (Langella 1999, available via http://bioinformatics.org/~tryphon/populations/) and based on pairwise genetic distances between individuals calculated using Cavalli-Sforza and Edwards chord distance (Dc) (Cavalli-Sforza and Edwards 1967)

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cluster analysis and the clone assignment based on the microsatellite data and for the subset of *T.* × *europaea* samples (82 samples), is given in Fig. 2.

Discussion

Little attention is given so far to the genetic diversity within tree species in urban landscapes (Zhang et al. 2014). We compared the level of genotypic variation of *Tilia* × *europaea* in historical plantings in Belgium and the Netherlands with current commercial planting stock from eight commercial tree growers in Flanders (Northern Belgium) and the Netherlands, using AFLP-markers.

Here, we demonstrated that the microsatellite markers, as well as AFLPs, are effective tools for the identification of *T.* × *europaea* clones or cultivars. Although, the number of clonal groups identified was slightly different between both marker systems with 42 and 46 clones identified with microsatellites and AFLPs, respectively, the clustering of analyzed accessions gave fairly similar dendrograms. However, the assignment of clones was more sensitive to the chosen threshold value for the AFLPs compared to the clone assignment using the microsatellite data. We therefore further discuss the results of the clustering and clone assignment based on the microsatellite data for a chosen threshold of six mutations allowed between two individuals to still be considered as clonemates.

The original *T.* × *europaea* trees from the avenue at the Tongerlo Abbey planted in the 17th century were all genetically different from current commercial planting stock. Considering all 68 heritage *T.* × *europaea* trees successfully analyzed, including these from the avenue at the Tongerlo Abbey, we found in total 12 clones. In contrast, we found only three clones within the current *T.* × *europaea* commercial planting stock (*T.* × *euchlora* excluded). Of the latter, one clone (SSR clone no. 32, sold as 'Koningslinde') was represented by one sample only. The other two clones (nos. 8 and 11, Table S2) were represented by five and ten samples of commercial planting stock, respectively, and were identified as the cultivars 'Zwarte linde' and 'Pallida'. The number of clones found within the historical plantings is thus about four times higher compared to the number of clones found within the current commercial planting stock of *T.* × *europaea*. This indicates that the nursery industry has further reduced the number of commercially available clones, thereby reducing the genetic diversity. The cultivar *T.* × *europaea* 'Zwarte linde' was difficult to find in the selected tree nurseries and in one commercial nursery, trees identified in this study as 'Zwarte linde' were labeled as 'Pallida'. It seems that, nowadays, 'Zwarte linde' is rarely propagated and sold. In the past 'Zwarte linde', almost certainly of Dutch origin, was widely planted in Northwest-European parks from about 1690–1720 (Pigott 2012). Several authors described a clearly different phenology and morphology for 'Zwarte linde' and 'Pallida' (Bengtsson 2005; Pigott 2012), suggesting indeed a different genetic background. Remarkably, a significant part of the additional heritage trees sampled (51%, 18 out of the 35 trees) also belongs to the 'Pallida'-group. Among these are several trees planted in the 17th–18th centuries, like a tree located in the park 'Oud-Bussem' and a tree near an old farm in Anloo (Drenthe, the Netherlands). We identified four heritage trees planted in the 17th & 18th or begin 19th century as 'Zwarte linde'. This confirms the hypothesis of Hansen et al. (2014) that the same clones, specifically the 'Pallida'-group, have been produced since the 17th century by private tree nurseries and continue to be sold commercially today. The original trees of the *T.* × *europaea* avenue at Tongerlo Abbey planted about 340 years ago and represented by a single clone, originate from vegetative propagation, since the genotypes of

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leaves collected in the tree crown and at the tree base of a subsample of trees belonged to the same clone. This supports that layering was likely the main method of propagation for *T.* × *europaea* trees in the 17th & 18th centuries (Bengtsson 2005; Hansen et al. 2014; Pigott 2012), although also grafting was used for propagation Within the tree avenue, four different clones of *T.* × *europaea*, including the clone 'Zwarte linde' were used during replacements of six original trees performed in the 19th and 20th centuries. The replacements in the avenue planted with *T. platyphyllos* (five trees) dated from the 19th–21th centuries, with all five trees showing unique genotypes. Considering their unique genotype and the otherwise extensive use of clones, it is likely that these five trees originated from seeds or sprouts of trees which had arisen as seedlings. A combination of *Tilia* species like *T. platyphyllos* and *T. cordata* together with *T.* × *europaea* were also found within historical plantings established in the 19th century in Denmark (Hansen et al. 2014) and England (Pigott 1992). This study supports the hypothesis of Pigott (1992) that, in the 19th century, seedlings from *T. platyphyllos* and *T. cordata* were occasionally used. Replacements in uniform plantings with different *Tilia* species also suggest that species and cultivars from lime trees are relatively difficult to distinguish using morphological characters (Hansen et al. 2014).

Historical sources indicate that during the 17th and 18th centuries, plant material of lime trees for avenues, parks and gardens in Denmark (Hansen et al. 2014), Sweden, England and Wales (Bengtsson 2005; Pigott 1992) was purchased in the Netherlands where it was produced by layering of only a few clones and thus representing very limited genetic diversity. This resulted in an almost perfect uniformity of the trees within the plantings, of which several still exist and are 200–300 years old. The same limited number of *T.* × *europaea* clones from the Netherlands was also used in plantings in the 19th and early 20th century (Hansen et al. 2014; Pigott 1992). As a consequence, genetic diversity in Northwestern European historical *T.* × *europaea* plantings is assumed to be very limited (Hansen et al. 2014). The use and distribution of vegetatively propagated *T.* × *europaea* over short and larger distances in the early 17th century were also confirmed in this study. By comparing the genetic fingerprints of old *T.* × *europaea* trees located in Belgium and the Netherlands, we found that the original trees of the *T.* × *europaea* avenue from the Tongerlo Abbey belonged to the same clone as four other ancient *T.* × *europaea* trees from the Netherlands, located up to 144 km away from Tongerlo.

If the historic integrity and uniformity of an existing old common lime tree avenue are deemed essential, the genetically identical stock could be produced from old trees by layering or from suckers. Replacements with new trees or new tree avenues could also be created with a different genetic stock. If the objective remains to continue planting *Tilia* species, seedlings of local provenances from *T. cordata* or *T. platyphyllos* should be considered. The advantage of more genetic variation within avenues planted with seedlings is the maintenance of stand adaptability, i.e. the ability to face an unexpected catastrophic perturbation due to biotic or abiotic causes. Furthermore, urban street trees of native species could improve the landscape connectivity of the species' natural populations thereby contributing to the evolutionary process of adaptation.

Conclusion

During the last centuries, commercial tree nurseries have further reduced the number of different genotypes of *T.* × *europaea*, resulting in extremely narrow genetic diversity within the current *T.* × *europaea* planting stock. Tree nurseries play an important role in the supply

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and quality of plant material. We recommend that the tree nursery industry reuse the old clones to enlarge the genetic diversity in commercial nursery stock of *T.* × *europaea*. The remaining ancient *T.* × *europaea* trees have shown long-term disease resistance and could provide nurseries with the valuable new genetic material. The range of available *Tilia* species and genotypes needs to be explored in future urban tree planning to optimize desired ecosystem services. There is a need to raise awareness among urban planners and policymakers on the importance of genetic diversity within tree species planted in the urban environment.

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Data Archiving Statement The microsatellite data and the AFLP data are available from the Dryad Digital Repository: XXX (to be completed after acceptance). A full list of accession numbers is also included as supplemental material (Table S1).

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Affiliations

An Vanden Broeck[1] · Karen Cox[1] · Iwona Melosik[2] · Bert Maes[3] · Koen Smets[4]

Iwona Melosik
melosik1@amu.edu.pl

Bert Maes
maes.dool@planet.nl

Koen Smets
koen.smets@vlaanderen.be

[1] Research Institute for Nature and Forest (INBO), An Vanden Broeck; Gaverstraat 4, 9500 Geraardsbergen, Belgium

[2] Department of Genetics, Adam Mickiewicz University in Poznań, Umultowska Str. 89, 61-614 Poznan, Poland

[3] Ecologisch Adviesbureau Maes, Achter Clarenburg 2, 3511 JJ Utrecht, Netherlands

[4] Flanders Heritage Agency, Havenlaan 88 bus 5, 1000 Brussels, Belgium

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