Plant Ecology and Evolution 149 (2): 165–176, 2016
http://dx.doi.org/10.5091/plecevo.2016.977

PLANT ECOLOGY EVOLUTION

REGULAR PAPER

Human impact on the genetic diversity of Dutch field elm (*Ulmus minor*) populations in the Netherlands: implications for conservation

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Background and aims – Field elms (*Ulmus minor* Mill.) can easily reproduce vegetatively by root suckers or sprouting. They also have a long history of propagation and planting in the Netherlands. Both natural vegetative reproduction and cultivation may significantly influence the genetic structure of *U. minor* populations and insight in these phenomena is of utmost importance for appropriate conservation management of this species. In this study we examined the presence and extent of clonality and patterns of genetic variability within and among field elm populations in the Netherlands.
Methods – We used microsatellites (SSRs) to describe the clonal diversity and structure and to calculate genetic diversity parameters in the Dutch *U. minor* populations. Additionally, we compared Dutch populations with two *U. minor* reference collections from Belgium and France.
Key results – We found high levels of clonality in the Dutch field elm populations. Out of the 159 Dutch trees analysed for clonal structure only 66 multilocus genotypes were identified. Clonal richness (R = 0.06–0.96) and diversity (D = 0.44–1.0) varied considerably among locations. Six genotypes were shared between locations indicating human-mediated translocations. We revealed a low to moderate genetic diversity in the populations (H_e = 0.483–0.628 and A_r = 2.4–2.9). At four locations some individuals were found that differed in assignment probabilities based on the STRUCTURE clustering analysis including parental species, suggesting that these might be hybrids or at least not pure *U. minor* specimens. This also indicates that morphological identification is difficult. When omitting these individuals genetic structure analyses still indicated the presence of two genetic clusters.
Conclusions – However artificial establishment has played a major role in the distribution of the species and its current genetic diversity in the Netherlands. These findings help facilitate Dutch gene conservation management programs for *U. minor*, in particular, the identification of high priority clones for *ex situ* conservation and efforts to restore remnant populations and hedgerows.

Key words – *Ulmus minor*, clonality, genetic variation, field elm, microsatellites, plantings.

INTRODUCTION

Field elm (*Ulmus minor* Mill. *sensu latissimo*), also referred to as the *U. minor* complex (Richens 1983), is a deciduous forest tree species distributed mainly in Southern Europe. Its distribution stretches from the Atlantic Ocean to the Caspian Sea (Jalas & Suominen 1988). The species is one of the three elm species that are native to Western Europe, the other being Wych elm (*U. glabra* Huds.) and European white elm (*U. laevis* Pall.). The natural range of field elm overlaps that of *U. glabra* in the North, where it easily hybridizes with this species to form the hybrid elm *Ulmus* × *hollandica* Mill. (Richens 1983).

Field elm can be found in the floodplain forests along the main European rivers, where it grows in association with oak and ash. Here it is able to tolerate floods as well as drought. It can also be found on the adjacent dry agricultural areas and on dry calcareous slopes. Flowers are hermaphroditic and wind-pollinated. Seeds are dispersed by wind, but in the riparian habitats also by floating, which enables colonization of new sites downstream (Heybroek et al. 2009). Like other elms, field elm is moderately to highly self-sterile (Heybroek 1993).

A number of features distinguishes *U. minor* from the other two European native species. Firstly, field elm is taxo-

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nomically complex. Within the *U. minor* complex (Richens 1983) a large variability exists and there is a lack of consensus on how all these forms should be classified in separate entities. Different viewpoints existed for describing the species, varying from one large species, namely *U. minor* Mill. *sensu latissimo* (Richens 1968), five different species including some varieties and hybrids (Melville 1975, 1978) to a description of forty microspecies according to Armstrong (1992). The most commonly adopted classification in recent years is the one of Richens, although it is not used in Flora Europaea, which maintains *U. minor* and *U. procera* as separate taxa (Tutin et al. 1964). More recently, based on molecular studies some of the distinctive forms within the field elm complex that were earlier elevated to species turned out to be single clones that have been propagated by cuttings or root suckers, such as *U. plotii* in England (Coleman et al. 2000), the Cornish elm in Cornwall (Hollingsworth et al. 2000) and the English elm (*U. procera* 'Atinia') (Gil et al. 2004).

Moreover, *U. minor* has a long history of human exploitation. According to Richens (1983) field elms have been widely propagated and transported throughout Europe by humans since prehistoric times. The species was used in winery, for feeding cattle, as a source of timber, for ornamental and many other uses.

The third important feature of *U. minor* is its ability to propagate vegetatively by root suckers and resprouting from the stump (Richens 1983). The suckering can result in clusters of trees that are identical in genotype. After stem cutting or injuries, sprouting is frequent near the trunk. Field elms can also sprout abundantly as a response to trunk death due to Dutch elm disease (DED) infection (López-Almansa 2004). The regeneration after elm disease infection enables the species to maintain itself in the landscape.

Due to its taxonomic complexity and the long history of plantings it is difficult to determine the exact natural range of the species and several hypotheses regarding its natural distribution exist. According to Richens (1983) the species is probably not native in North Europe and introduced into Britain in prehistoric times. He suggested that the species is composed of a series of clonal populations whose distribution is explicable in terms of human migration and trading contacts (Richens 1980). Indeed several field elms such as Cornisch Elm, Plot's Elm and English Elm have turned out to be single clones. On the other hand it has been suggested that introduction seems unlikely as many taxa within *U. minor* have apparently natural distributions (Armstrong & Sell 1996). On the mainland, field elm occurs in the large floodplain forests along the major rivers and even among minor streams, which are obviously natural forests. For example, before DED, *U. minor* was a dominant species in riparian forests in most parts of lowland Germany (Mackentum 2000).

In the Netherlands, the native status of field elm is also disputed. In contrast to the other two native elm species (*U. glabra* and *U. laevis*) it is common in the Netherlands but rare for wild populations. Although it was extensively planted for economic and ornamental reasons alongside roads, trails and in the landscape and used for coppice, we hypothesize that *U minor* is native to the Netherlands as populations occur in an assortment of habitats typical for this species. Typical habitats in the Netherlands are the alluvial forests on rich sandy soils along the major rivers and brook valleys, inner dunes and on loess covered terraces. The major part of this vegetation is coppice forest (Heybroek 1957). Also a typical semi-natural habitat for *U. minor* in the Netherlands are old hedgerows, which were probably created using plants of local provenance. Centuries ago, since about the 16th century, these hedges were used to separate pasture for livestock and arable land plots.

Its taxonomic complexity, human exploitation and clonality due to excellent regeneration ability have all possibly impacted the genetic diversity in this species and make *U. minor* a difficult species for conservation (Gil et al. 2004, Fuentes-Utrilla et al. 2014). Knowledge of the genetic structure of populations and the extent of clonality is needed in order to have a better insight in the human influence on this species in the Netherlands and is essential for setting up a conservation program.

The aims of this study were: (1) to investigate patterns of genetic variability within and among *U. minor* populations in the Netherlands; (2) to examine the occurrence of clones within populations; and (3) to investigate if there is evidence for anthropogenic influence on these field elm populations. Additionally, we compared the variation in the Dutch populations with two *U. minor* reference collections from Belgium and France in order to investigate if the Dutch material is genetically distinct from material located outside the Netherlands. Finally, conservation implications were drawn from these investigations.

MATERIAL AND METHODS

Study sites and sampling

In total 164 *U. minor* trees were sampled in six locations across the Netherlands. (see table 1 & fig. 1). They were selected based on inventories of autochthonous trees and shrubs conducted by Maes (N.C.M. Maes, Ecologisch Adviesbureau Maes, the Netherlands, pers. comm.) and reported in Rövekamp & Maes (2002), taking in account historical data (e.g. landscape element present on map of 1850) and growth site characteristics. These six locations represent the *U. minor* populations in The Netherlands at natural habitats such as alluvial forests, inner dunes and mixed forests on loess covered terraces or in typical hedgerow habitats. Some of these populations were used by humans for coppicing in the past. The populations varied in size from approximately 21 to over 180 trees, which was estimated by roughly counting the trees within the populations. As the trees have a more or less scattered or clumped distribution within the locations, the different locations represent different scales and sampling schemes. At each location a subset of 17 to 41 trees was sampled, depending on the population size. Trees were randomly sampled along the whole area of the entire population, but with a minimal distance of 10 m between trees to reduce the risk of sampling multi-stem individuals, except in the small Drenthe population where some sampled trees were separated by only 4 m. Here almost the entire population was sampled, including trees in close proximity. The

Table 1 – Characteristics of the *Ulmus minor* localities sampled in the Netherlands.
N = Number of individuals sampled, N_c = estimate of the total number of individuals present on the locality.

Sampling locality	Location (Lat/Long)	Type of landscape element/habitat	N	N_c
Drenthe	53º03′N/6º41′E	forest edge, brook valley	17	21
Zalkerbosch	52º32′N/6º01′E	mature stand, coppice, floodplains	30	150
Cortenoever	52º06′N/6º12′E	forest edge, hedge rows, floodplains	25	75
De Manteling	51º34′N/3º31′E	mature stand, coppice, innerdunes	25	50
Maasheggen	51º37′N/6º00′E	forest edge, hedgerows, floodplains	41	180
Limburg	50º53′N/5º48′E	mature scattered in terrace forests, coppice	26	40

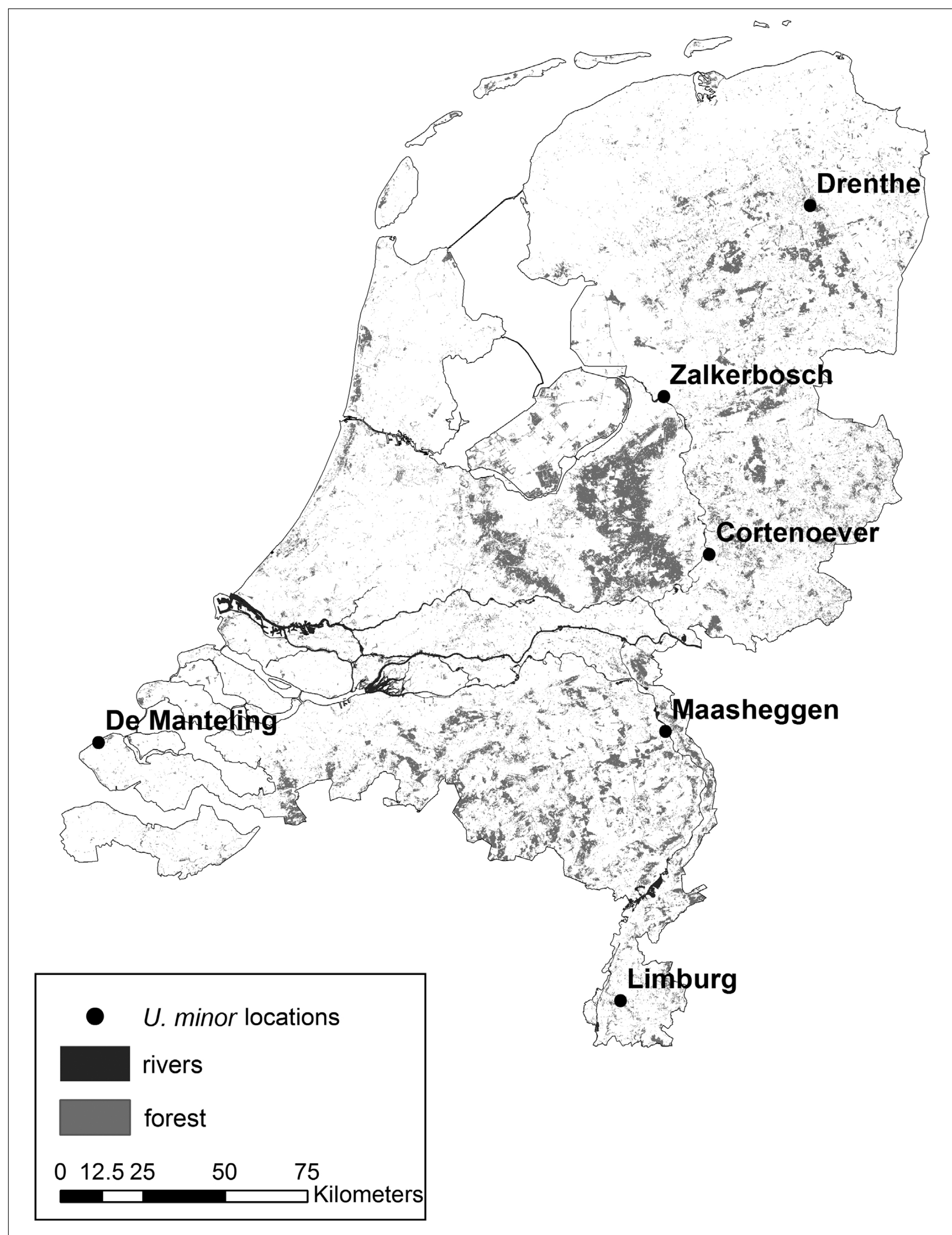

Figure 1 – Map of the Netherlands showing the location of the sampled populations.

coordinates of the sampled trees were mapped with GPS. The trees were morphologically identified as *U. minor* species during collection using the following characteristics: (1) flower and fruit peduncle (no stalks); (2) leaf form, (oval and oblong); (3) leaf base (leaf lips do not fall over the petiole); (4) leaf hairiness (smooth) and (5) twigs (smooth, sometimes corky) (Maes 2006). Fresh leaf material was collected for DNA extraction and stored at -20°C.

To investigate if the Dutch samples are genetically distinct from *U. minor* material outside the Netherlands additional material was obtained: one gene bank collection of *U. minor* from the Research Institute for Nature and Forest Research (INBO) including 23 samples (Belgian material collected from seven locations in the Flemish part of Belgium) and a second collection of *U. minor* with gene bank and nursery material including 32 *U. minor* specimens from France (see electronic appendix 1 for maps depicting the sampling locations of the elm collection material of Flanders (northern Belgium) and France).

Additionally well-known cultivars were included as reference samples representing pure *U. minor* (‘Sarniensis’ and ‘Hoersholmiensis’) and *U. glabra* (‘Nana’, ‘Horizontalis’ and ‘Camperdownii’) and the hybrid *U.* × *hollandica* (‘Belgica’) to investigate the occurrence of putative hybrid trees in sampled locations by a principal coordinate analysis (PCoA). Samples of these cultivars were derived from the Vermeerderingstuinen (Propagation Nurseries The Netherlands), except ‘Horizontalis’, which was collected in Wassenaar, The Netherlands by H. Heybroek.

SSR analysis

Total DNA from leaf material from the Dutch populations and reference samples was extracted using a DNeasy Plant mini Kit (Qiagen). Twenty three primer pairs of SSR loci characterized in *U. minor* (Collada et al. 2004), *U. laevis* (Whiteley et al. 2003) and *U. rubra* (Zalapa et al. 2008) were examined in an initial screening in a subset of the material. Only ten loci (Ulm2, Ulm3, Ulm8, Ulmi1-21, Ulmi1-98, UR123, UR138, UR175, UR158 and UR188a) showed successfully polymorphic amplification products and were subsequently used for this study.

Amplification with Ulm8, Ulmi1-21 and Ulmi1-98 was performed according to the PCR profile b as described in Collada et al. (2004), Ulm 2 and Ulm3 according to Whiteley et al. (2003) and UR123, UR138, UR175, UR158 and UR188a following the protocol of Zalapa et al. (2008). Optimized annealing temperatures were 55°C for UR175, UR158, UR188a, 60°C for UR123, UR138, 63°C for Ulm2, Ulm3, Ulm8 and 50°C for the other primers Ulmi1-21 and Ulmi1-98. PCR products were analysed and visualized with a 4300 IR2 DNA analyser (LiCOR).

Data analysis

To investigate the occurrence of clones in the populations, we calculated P_{gen} and P_{sex} (Parks & Werth 1993). P_{gen} was calculated as the probability that individuals with the same multilocus genotype (MLG) were derived via sexual reproduction, given the allele frequencies in the population. For each repeated MLG in each population, also P_{sex} was calculated as the probability for a given multi locus genotype to be observed in N samples as a consequence of two different sexual reproductive events.

After clonal assignment a number of estimates was calculated to describe the clonal diversity and structure using: G, the number of distinct multilocus genotypes (MLGs); R = the genotypic diversity, calculated as

$$R = \frac{(G-1)}{(N-1)}$$

where N is the total number of ramets sampled (Dorken & Eckert 2001); the adapted Simpson index for genotypic diversity

$$D^* = 1 - \Sigma\left[\frac{N_i\,(N_i-1)}{N\,(N-1)}\right]$$

(Pielou 1969), where N_i is the number of ramets with genotype i and the corresponding evenness index

$$ED^* = \frac{(D - D_{min})}{(D_{max} - D_{min})}$$

where

$$D_{min} = \left\{(2N-G)x\left(\frac{G-1}{N^2}\right)\right\} x \frac{(N)}{(N-1)}$$

and

$$D_{max} = \frac{(G-1)}{(G)} x \frac{N}{(N-1)}$$

To test for existence of spatial aggregation of ramets belonging to the same MLG, the spatial aggregation index (A_c) was estimated according to Arnaud-Haond & Belkhir (2007). This index compares the average probability of clonal identity of all ramet pairs with the average probability of clonal identity among pairwise nearest neighbours. This index ranges from 0 (ramets belonging to the same MLG are dispersed within the population) to 1 (situation where ramets of the same clone are spatially aggregated). Significance of A_c was tested by 10,000 permutations.

When slightly distinct MLG’s occur this may be the result of somatic mutations or genotyping errors in the database. To screen for these a frequency distribution (%) of the pairwise number of allele differences between MLGs of all samples was computed. All these calculations were performed using the Software package GenClone 2.0 (Arnaud-Haond & Belkhir 2007). As GenClone does not allow missing values clonal diversity estimates were calculated for the 159 fully genotyped trees. After the number of MLGs was determined in each population, the range of clone sizes in each of the Dutch populations was calculated using the GenAlEx 6.5 package (Peakall & Smouse 2006, 2012).

For subsequent analyses a reduced dataset with only one ramet per MLG per population was used. Two different methods were used to understand the genetic structure in the populations. First, the clustering algorithm in STRUCTURE 2.3.4 (Pritchard et al. 2000) was run to assess the genetic structure of the data set for each K ranging from 1 to

Table 2 – Clonal diversity measures for the six Dutch locations.
N = the number of individuals fully genotyped; G = the number of distinct multilocus genotypes (MLGs); Gs = the number of shared genotypes; R = the genotypic richness; D* = Simpson index for genotypic diversity; ED* = genotypic evenness; A_c = aggregation index.* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; other values not significant. G_h = the number of genotypes with putative hybrid origin based on genetic profiles.

Population	N	G	G_s	G_h	R	D*	ED*	A_c	Range of clone sizes (m)
Drenthe	17	2	2	1	0.06	0.44	0.78	1.0***	4–4059
Zalkerbosch	28	18	1	4	0.63	0.93	0.59	0.19**	98–907
Cortenoever	25	24	0	0	0.96	1.00	0.00	0.12***	16
De Manteling	23	6	2	2	0.23	0.65	0.53	0.26	132–1273
Maasheggen	40	15	4	0	0.36	0.82	0.64	0.48***	51–10831
Limburg	26	8	3	5	0.28	0.85	0.85	0.68***	39–13251

10 using 50,000 Markov chain Monte Carlo iterations with a burn-in of 50,000 and 5 replicates per run. The admixture model was used and allowed correlation of allele frequencies among clusters. The approach by Evanno et al. (2005) in STRUCTURE HARVESTER v0.6.94 (Earl & vonHoldt 2012) was used for selecting the most appropriate K clusters. The STRUCTURE analysis was performed including all samples (Dutch, Belgian and French) and only the Dutch samples. The STRUCTURE results identified K = 2 as the most appropriate genetic clustering in both analyses, separating 14 individuals from the rest of the samples ($q > 0.8$). These fourteen individuals were derived from the Dutch populations (six from Limburg, two from De Manteling, five from Zalkerbosch and one individual from Drenthe). Knowing that hybridization between *U. minor* and *U. glabra* is possible and that *U. hollandica* specimens might be present at the sampled locations a principal coordinate analysis (PCoA) was performed on the genetic distances among individuals in GenAlEx 6.5 (Peakall & Smouse 2006, 2012). The analysis was run on the individuals assigned to the genetic clusters as inferred from the STRUCTURE results and six reference samples/cultivars with known species origin (*U. glabra* 'Nana', 'Horizontalis' and 'Camperdownii', *U. minor* 'Sarniensis' and 'Hoersholmiensis' and the *U. × hollandica* cultivar 'Belgica').

On the basis of these analyses, twelve individuals of putative hybrid origin were omitted from further analyses. A STRUCTURE analysis was rerun on the remaining 100 samples (Dutch, French and Belgian) and again a PCoA was used to examine the genetic structure.

The degree of genetic differentiation among populations was also estimated using F_{ST}, calculated across all Dutch populations as well as on a population pairwise basis using the software FSTAT version 2.9.3.2 (Goudet 1995). Pairwise comparisons of F_{ST} values were tested for significance and critical values were adjusted for multiple tests with the Bonferroni correction. Finally, standard measures of genetic diversity were calculated using GenAlEx 6.5 (Peakall & Smouse 2006, 2012): number of alleles, number of private alleles (A_p), observed heterozygosity (H_o) and expected heterozygosity (H_e). Allelic richness (A_r), which corrects for sample size using a rarefaction index following Petit et al. (1998) was determined using FSTAT V2.9.3.2 (Goudet 1995). Wright's fixation index (F_{IS}), averaged over all loci, was calculated and deviation from Hardy-Weinberg expectations was determined using GENEPOP 4.2 (Raymond & Rousset 1995). In these analyses Drenthe was omitted as only one genotype was remained after omitting the twelve individuals of putative hybrid origin in the data set.

RESULTS

Clonal diversity

All P_{gen} values were < 0.001 in our data set. In all cases, the P_{sex} for repeated MLGs within populations was less than 0.05 (the highest value in a population varied from 0.001 to 1.5×10^{-6}) and therefore, the duplicated multilocus genotypes were considered clones of the same genet (i.e. products of asexual reproduction).

Of the total of 164 trees sampled in the Dutch populations, 159 were fully genotyped. Within these 159 samples 66 distinct MLGs were identified. Out of these 66 MLGs 61 were local genotypes (occurring only at one location) and five genotypes were shared among two or four populations in the Netherlands. The Drenthe population did not contain any local genotypes, but consisted of only two shared genotypes. One of these was widespread and even found in three other Dutch populations (De Manteling, Zalkerbosch and Limburg).

The extent of clonality varied strongly among the populations. In four of the six Dutch populations genotypic richness was low to moderate (R = 0.06–0.36), while in Zalkerbosch and Cortenoever R was 0.63 and 0.96 resp., indicating a leading role for sexual reproduction. In the latter population only one single clone with two ramets was detected, while other MLGs consisted of one ramet. The other measure of clonal diversity, the Simpson Index (D*), showed a comparable picture with values ranging from 0.44 (Drenthe) to 1.0 (Cortenoever), showing a high clonal diversity for all populations except Drenthe. Evenness (ED*) ranged from 0.531 (De Manteling) to 0.85 (Limburg). These moderate to high values for evenness indicate a reasonable equal abundance

of the genotypes and no dominance of a single genotype (table 2). For Cortenoever the evenness was zero.

If somatic mutations or genotyping errors are expected then the frequency distribution of genetic distances between MLGs will show high peaks towards low distances. The frequency distribution of genetic distances showed a more or less unimodal distribution, but with a gap in the distribution between one-allele differences and four-alleles differences (electronic appendix 2). In total 11 MLG pairs appeared to be different for only one allele. In eight of these pairs, this one-allele difference involved a difference in heterozygous versus homozygous state. In the other three cases it concerns a one-repeat or two-repeat unit difference. Based on these distributions these 11 MLG pairs might be slightly different due to somatic mutation or scoring errors. When these MLG pairs are recognized as belonging to the same clonal lineage (MLL), the clonal extent in the analysed material might even be higher than detected and the number of genotypes is reduced from 66 to 56.

The size and spatial arrangement of the clones varied considerably in the Dutch populations. The aggregation index (A_c) ranged from 0.12 to 1.0 and was significant in all populations except De Manteling. In most populations ramets intermingle with other clones and genotypes in the population (for example Zalkerbosch, fig. 2A). Estimating the maximum clone sizes within a location indicated that ramets are spread over the site with large distances. In particular Limburg and Maasheggen consist of clones with a wide spatial arrangement, with ramets of a single clone dispersed up to 13 km apart from each other (table 2, fig. 2B).

Genetic structure

Using STRUCTURE two genetic clusters best explained the genetic diversity in *U. minor* individuals (in both datasets: only Dutch and all samples with only one MLG per population/collection). With K = 2 the STRUCTURE results assigned 14 individuals to a unique cluster (Cluster II, with q > 0.8) (electronic appendix 3). To check if these 14 samples were indeed *U. minor* specimens, although morphologically identified as *U. minor* in the field, a PCoA analysis was performed including six reference cultivars with known species identity, namely three *U. glabra* cultivars ('Nana', 'Horizontalis' and 'Camperdownii'), two *U. minor* cultivars ('Sarniensis' and 'Hoersholmiensis') and one *U.* × *hollandica* cultivar ('Belgica'). The PCoA results supported the results from the STRUCTURE analysis (electronic appendix 4). The first two principal coordinates, which explained 20.2% of the total genetic variation, separated *U. minor* and *U. glabra* along the first principal coordinate axis. Suspected hybrid individuals would take an intermediate position between the parental species. The PCoA illustrates that twelve samples of Cluster II cluster together with the *U.* x *hollandica* specimen apart from Cluster I (*U. minor* individuals) and the *U. glabra* specimens and might therefore have a hybrid background. Four of these putative hybrid trees are ramets of the same clone, which was wide spread and found in De Manteling, Drenthe, Limburg and Zalkerbosch. Three trees were local clones in Zalkerbosch and in Limburg. Five trees had unique genotypes located in Zalkerbosch, De Manteling en Limburg.

On the basis of these analyses, the twelve individuals that might not be pure *U. minor* were omitted from further analyses. STRUCTURE results indicated the most likely genetic clustering for K = 2 when the dataset was reanalysed excluding these twelve individuals (remaining 100 Dutch, French and Belgian samples), although K = 4 also showed a high ΔK (fig. 3). For K = 2, the first cluster is mainly formed by individuals of Maasheggen and Drenthe. Zalkerbosch, Cortenoever and France were predominated by the second cluster. Significant admixture was observed in Belgium, Limburg and De Manteling as each population was comprised of both genetic clusters. Most individuals were not admixed and had most of their ancestry in one of the two clusters, except a few individuals from Belgium and France (admixed individuals with q values between 0.4 and 0.6 for each cluster). The PCoA confirmed the genetic similarities between the Dutch material and samples from the French and Belgian reference collection, collected in Western Flanders and the North of France (fig. 4).

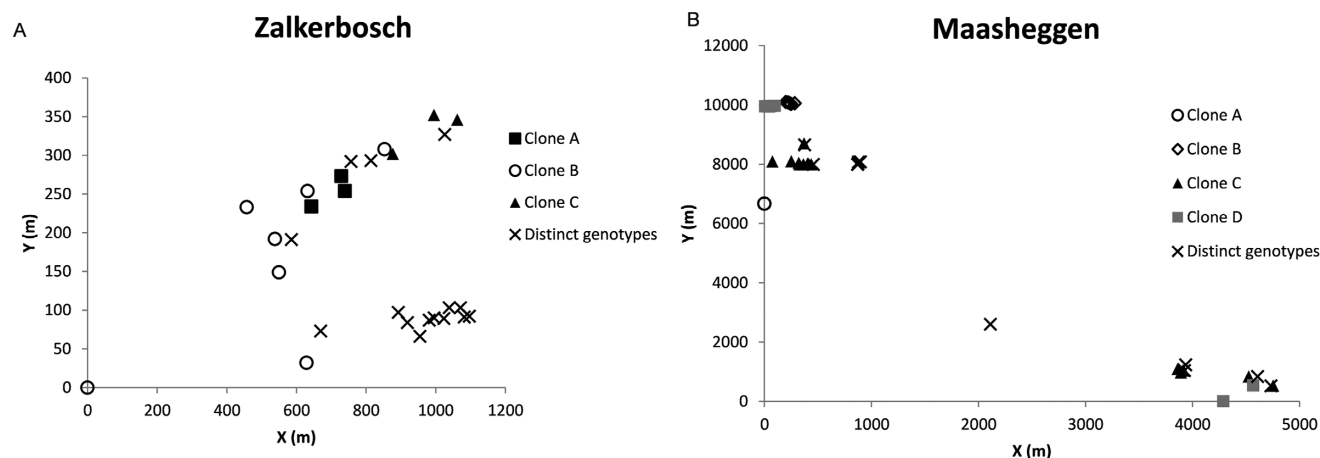

Figure 2 – Spatial distribution of the *Ulmus minor* genotypes: A, Zalkerbosch population; B, Maasheggen population. Distinct genotypes are represented by a cross.

Assessment of genetic structure among populations showed a moderate differentiation among Dutch populations (F_{ST} = 0.069, P < 0.001). Estimation of pairwise F_{ST} values (table 3) shows that the *U. minor* individuals in Maasheggen are genetically most distinct from Cortenoever and Zalkerbosch.

Genetic diversity in the Dutch populations

All ten microsatellite loci were polymorphic and the number of alleles per locus ranged from three for UR123 to 10 for UR138 for all Dutch samples. The allelic richness (A_r) ranged from 2.4 to 2.9 with the lowest values in the Maasheggen

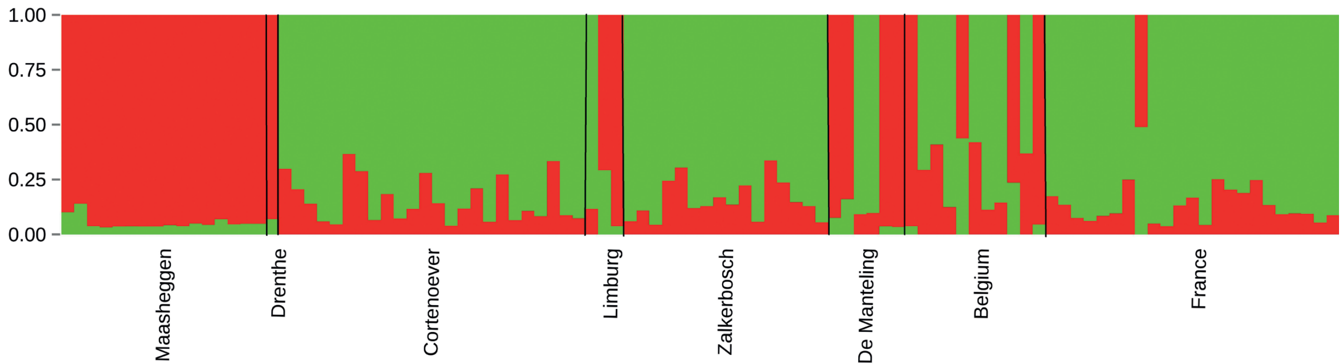

Figure 3 – STRUCTURE results of 100 individuals from six Dutch populations and two reference collections (Belgian and French) with K = 2. In the plot a column represents one MLG and the different colours illustrate the individual's estimated membership fractions to each of the two genetic clusters.

Figure 4 – Principal coordinates analysis (PCoA) based on ten microsatellite markers of the Dutch populations and two French and Belgian reference collections. Axis-1 explains 10.4% of the variation and axis-2 9.6%.

Table 3 – Pairwise genetic differentiation values (FST) for five Dutch *Ulmus minor* populations.
Calculations are based on taking only one ramet per distinguishable genotype into account.

	Cortenoever	Limburg	Zalkerbosch	De Manteling
Maasheggen	**0.113***	0.008	**0.133***	0.033
Cortenoever		0.022	**0.056***	0.**038***
Limburg			0.061	0.0
Zalkerbosch				0.036

Table 4 – Statistics of genetic diversity within the Dutch populations for ten microsatellite loci.
Calculations are based on taking only one ramet per distinguishable genotype into account. A, number of alleles; A_r, Allelic richness (corrected to account for the smallest sample size of Limburg); A_p, total number of private alleles; H_o, Observed heterozygosity; H_e, expected heterozygosity; F_{IS},Wright's fixation index; †Exact test of departure from Hardy-Weinberg genotypic proportions: *P < 0.05; **P < 0.01; ***P < 0.001; other values not significant.

Population	A	A_r	A_p	H_e	H_o	F_{IS}†
Zalkerbosch	5	2.8	5	0.579	0.537	0.118***
Cortenoever	5.4	2.8	9	0.628	0.57	0.114***
De Manteling	3.8	2.9	1	0.577	0.693	-0.112
Maasheggen	3.1	2.4	0	0.51	0.704	-0.367**
Limburg	2.8	2.6	1	0.483	0.567	0.009

population. Observed (H_o) and expected heterozygosity (H_e) ranged from 0.537 to 0.704 and from 0.483 to 0.628 respectively (table 4). Twenty-four percent of the total number of alleles (68) found was private, of which most were found in Cortenoever and Zalkerbosch. Tests of heterozygote frequencies against Hardy-Weinberg equilibrium were significant in three of the five populations. In Maasheggen a significant heterozygote excess was shown with an average F_{IS} value of -0.367. The Cortenoever and Zalkerbosch showed significant positive F_{IS} values, resp. 0.114 and 0.118.

DISCUSSION

Occurrence of clones and human influence

This study confirmed the suspected clonality in Dutch *U. minor* populations by using nuclear microsatellites. Furthermore, it revealed that clonal richness and diversity (measured as R and D*) can vary considerably between locations. Clonal growth observed in the Dutch populations might be the result of different types of clonality: (1) naturally by root suckering and sprouting; (2) 'farmer's clonality' by local layering and transplantation of root suckers to a new hedge or field; and (3) cultivar plantation. Natural root suckering, which is typical for this species might explain for a large part the clonal reproduction. Local differences in anthropological or environmental pressure e.g. differences in previous coppice culture or disease pressure between the locations, both resulting in resprouting may have contributed to variation in clonality in these Dutch populations. E.g. the disease pressure due to DED could have reduced the number of genotypes to the less susceptible ones, which can persist through clonal growth. Additionally, our results suggest that the degree of clonality might also be substantially affected by historical plantings. The presence of shared genotypes between all locations except one indicate that translocations have occurred in the past. The sharing of clones between locations at distances up to 70 km, which are not part of the same watershed might point to human intervention.

The wide spatial arrangement of ramets within locations (up to 13 km) might also suggest human influence such as local layering and/or transplantation of root suckers. Small groups of distinct ramets are expected after root suckering or resprouting. Though, the estimated clone sizes within Dutch *U. minor* were much higher than expected based on these phenomena. The distances found are quite far for natural formation of clonal structures. Although it is possible for riparian tree species to disperse (naturally) over long-distances via translocated root fragments, it is less likely for elms across terrestrial landscape. For example, in *Populus nigra* distances between ramets of the same clone of up to 19 km were found. Here, widespread asexual recruitment was possible via root fragments travelling downstream the river (Barsoum et al. 2004). Another more recent example is the widespread naturally occurrence of two *P. alba* clones in the large Douro basin (Santos-del-Blanco et al. 2013). Also long distance recruitment of *U. minor* clones is reported by Fuentes-Utrilla et al. (2014). They observed large clone sizes within populations in the Balearic islands varying from 152 m to over 1,500 m and even found the same genotypes in distant watersheds (up to 26 km apart in Majorca). They explained these phenomena by long term persistence of established clones under conditions that hinder sexual recruitment and by long-distance dispersal of root fragments. However, they could not exclude human contribution. Additionally, Cox et al. (2014) found clonal reproduction over large dis-

tances (4.5 km to more than 60 km) in *U. minor* in Flanders. They suggested that besides natural clonal reproduction the translocation of elm planting material from one location to the other might have occurred here. Though stepwise formation of patches of clones in our study locations through root suckering cannot be entirely ruled out, 13 km is quite far for such natural formations in mainly terrestrial land including fragmented, isolated habitats and these large distances might therefore also indicate human influence. Consequently, another mechanism to spread ramets throughout the population might be local layering by farmers which was quite common in coppice cultures in the Netherlands in the past.

A third type of clonality could be planting of elm cultivars. Since the 16th century field elms were frequently planted by farmers and from the late 17th century hybrid clones of *U.* × *hollandica* became popular. Some old well-known hybrid cultivars in the Netherlands are 'Major' and 'Belgica' (Heybroek et al. 2009). Although the presence of old cultivars in the *U. minor* populations was not specifically investigated in this study, the Belgica genotype was not detected in the sampled trees (data not shown).

Our results confirm that artificial establishment has played a major role in the distribution of the species and its current genetic diversity in the Netherlands. So our findings support the hypothesis of Richens (1983) that the present day geographical variation found in the *U. minor* complex is strongly influenced by human cultivation of individual clones. Apparently some genotypes within the *U. minor* complex have useful features for man and were therefore widely planted in the past. Earlier molecular studies have demonstrated this also. Hollingsworth et al. (2000) mentioned that *U. plotii* in England, a single widespread clone, occurs in localities at least 80 km apart and concluded that such a wide distribution could only occur through human plantings. Also the distribution of the Cornish elm and the 'Atinia' clone are examples of widespread clones by man (Hollingsworth et al. 2000, Gil et al. 2004).

In general clonality within locations might be under-estimated in this study. In this study we chose to sample trees with a minimum distance of 10 m to avoid sampling multi-stem individuals. This sampling design might also lead to an under-estimation of clonality. To obtain a more accurate estimate of the extent of clonality within locations all trees should have been sampled in a given area.

Genetic diversity patterns

Our results do not give evidence that Dutch populations are genetically impoverished. Despite extensive clonality in the Dutch populations, measures for genetic diversity (A_r and H_e) indicated a low to moderate level of diversity. The H_e estimates for the Dutch populations are comparable to other European field elm populations (Brunet et al. 2013: Italian collection, N = 42, H_e = 0.59; Fuentes-Utrilla et al. 2014: N = 1–29, H_e = 0.333–0.592, Bertolasi et al. 2015: N= 350, H_e = 0.671). Moreover, our findings are in agreement with reviews on clonal plant species that show that clonal species do not have reduced levels of heterozygosity (Ellstrand & Roose 1987).

Genetic diversity calculations were performed after excluding the twelve potential hybrids, however there might still be some putative hybrids or backcrosses with *U. glabra* left here. Therefore, it should be noted that genetic diversity estimates might be biased if undetected hybrids or backcrosses occur in the populations. Morphological criteria are not enough to exclude this. The morphological traits typically used to identify the species in the field did not reliably distinguish *U. minor* and hybrids. Previous work on *Ulmus* species (e.g. Zalapa et al. 2010, Brunet et al. 2013, Cox et al. 2014, Bertolasi et al. 2015) already mentioned that morphological identification is not always congruent with genetic analyses. In our case this was illustrated by the strange clustering obtained with the program STRUCTURE.

After omitting these putative hybrids the Dutch *U. minor* populations showed moderate overall differentiation. Goodall-Copestake et al. (2005) confirmed this lack of genetic differentiation within this species at the European scale. They showed that most variation (90.2%) within *U. minor* sampled from a wide geographic area within its distribution range was within regions (Spain/Portugal vs. UK. vs. Italy vs. France/Belgium). These findings might primarily indicate the role of natural gene flow in this species or historical recolonization processes. Considering the biology of European elms, wind-pollinated and wind-dispersed, reasonable high levels of genetic diversity and low levels of differentiation are expected. Moreover, high rates of clonal reproduction may decrease population differentiation (Balloux et al. 2003). However, low genetic differentiation could also suggest that ancient plantings have altered the geographical genetic structuring on a larger scale. In the clustering analysis the Dutch genotypes did not cluster separately from the Belgian and French genotypes, but partly overlapped, suggesting that the Dutch field elms are not genetically distinct from Belgian or Northern France material. Moreover, two of the genotypes found in the Dutch populations were also detected in the Belgian gene bank collection (data not shown). This sharing of genotypes between the Dutch and Belgian locations indicates that clones have been exchanged between the Netherlands and Belgium in the past and therefore supports even more the hypothesis that anthropogenic influence has altered the partitioning of genetic variation in this species. It is interesting to note that, although overall genetic differentiation is moderate among the Dutch *U. minor* populations, Maasheggen displays high levels of pairwise genetic differentiation with Cortenoever and Zalkerbosch. As also STRUCTURE keeps the Cortenoever en Zalkerbosch genotypes together and separated from Maasheggen, this grouping may reflect different ancestral origins.

Excess of heterozygotes

Maasheggen showed a negative F_{IS} value, suggesting an excess of heterozygotes in this population. Negative F_{IS} values have been reported in other partly clonal species such as *Prunus avium* (Stoeckel et al. 2006, Vaughan et. al. 2007) or *Sorbus torminalis* (Hoebee et al. 2006, Rasmussen & Kollmann 2008). However, reasons for excess of heterozygotes are still poorly understood. Stoeckel et al. (2006) mentioned a number of causes that could lead to heterozygote excess:

small reproductive population size, overdominant selection favouring heterozygote survival (heterosis), negative assortative mating for instance due to a self-incompatibility system or clonal reproduction that maintains heterozygosity. Possible explanations for the negative F_{IS} value in Maasheggen could be small populations size, human plantings (e.g. bringing together clones from genetically distinct populations), outcrossing, as self-fertilization seems to be rare in *U. minor*, suggesting self-incompatibility (Mittempherger & La Porta 1991), selective advantage of heterozygotes combined with clonal selection (survival of those genotypes with good sprouting ability or elm disease tolerance) or clonal growth. Unfortunately, our data do not allow us to test these hypothesises and therefore need further investigation. However, it is most likely that the observed heterozygote excess is partly related to extensive clonality in this population. On the other hand Cortenoever and Zalkerbosh, which showed low levels of clonality, are not characterized by heterozygosity excess. In these populations an excess of homozygotes was observed, which may be the result of genetic sub structuring (Wahlund effect) and/or inbreeding.

CONCLUSION

The importance of vegetative propagation in most of the Dutch *U. minor* populations and the significant anthropogenic influence in some populations raise the question of how we should treat *U. minor* in Dutch conservation programs. Given the DED pressure and lack of opportunities for natural seedling recruitment in Dutch climate, clonality has allowed *U. minor* to survive. However, the presence of a low number of remaining genotypes in some locations and the sharing of genotypes between locations, means that not all locations are of the same high conservation value. For example, in populations where clonal growth predominates (that is Drenthe, Maasheggen, De Manteling, Limburg), the effective number of MLGs is much lower than the census population size, which may influence the long term the viability of the population.

Gene conservation measures for *Ulmus* species follow largely the general guidelines for other Noble hardwood species proposed by Jensen et al. (1999). For *U. minor*, *ex situ* conservation is seen as the preferred tool for preserving the existing genetic diversity (Collin 2002). Based on our current knowledge regarding clonality and translocations of elms, establishing an *ex situ* collection with clones that contain all or most of the existing genetic variation seems the most justified strategy. For establishing such a gene bank, the sampling recommendations as discussed by Jensen et al. (1999) and specifically for elms by Collin (2002) can in principle be followed. Our genetic results will guide us which populations and genotypes to sample in order to maximize the genotypic diversity and to be sure that unique genotypes are included. Also the existence of hybrids and possible backcrosses, which are difficult to identify in the field, should be taken into account when planning an *ex situ* collection of pure *U.minor*. Moreover, keeping a minimum distance of 50 m between trees to sample, as Collin (2002) proposes, will probably not suffice. The visual observation of clonal patches due to root suckering or sprouting of stumps gives an inaccurate estimate of the spatial distribution of clones as widely distributed genotypes occur in the Dutch situation as well as in the Flemish situation (Cox et al. 2014). Also no large conservation efforts are needed for genotypes shared with Belgium. It is therefore recommended to operate in close cooperation with the Belgian conservation program.

Additionally, dynamic *in situ* conservation could be undertaken to promote the adaptation of elm genetic resources. However, Collin & Bozzano (2015) mentioned a lack of interest for *in situ* conservation for *U. minor* referring to the low number of Dynamic Conservation Units (only two in September 2013) in the European EUFGIS conservation network. According to them this lack of interest for *in situ* conservation of *U. minor* can be explained by three reasons: (1) conserving mature trees is difficult as trees hardly ever survive DED infection, (2) the species can be conserved naturally through root suckering and seedlings and (3) the existence of *ex situ* clone banks for this species in many European countries.

In the Netherlands candidates for *in situ* conservation might be ancient woods that contain *U. minor* trees that have been able to survive DED and that are not extremely affected by artificial plantings. In this sense not all Dutch *U. minor* locations have the same high conservation priority. For example the conservation value of Drenthe and Limburg is low due to low genotypic diversity, human translocations and the presence of putative hybrids. Zalkerbosch seems the most appropriate candidate for *in situ* preservation, as it has a higher population size and high genotypic diversity. It is part of an old elm coppice wood (alluvial forest) and a remnant of an ancient, much larger forest from the late Middle Ages. According to its management records it consists of autochthonous trees probably combined with plantings (Den Ouden et al. 1997).

However besides dynamic *in situ* conservation, Collin & Bozzano (2015) suggest to employ dynamic conservation measures in particular in areas where *U. minor* has been cultivated for centuries as an important part of the landscape. They introduced the term ‘dynamic restoration’ for this. This restoration strategy is a dynamic way of reinforcing the local gene pool with large diversity plant material from the same region. Concerning Dutch *U. minor* this could be an effective strategy for restoring in particular the old hedgerow habitats. For example, Maasheggen, which nowadays is formed by a small number of genotypes that have been able to survive, could be efficiently restored through plantings derived from an *ex situ* collection.

SUPPLEMENTARY DATA

Supplementary data are available in pdf at P*lant Ecology and Evolution*, Supplementary Data Site (http://www.ingentaconnect.com/content/botbel/plecevo/supp-data), and consist of: (1) map depicting the sampling locations of *Ulmus minor* accessions in the Belgian (Flanders) and French collections; (2) frequency distribution (%) of pairwise allele differences among all MLGs; (3) STRUCTURE results of 112 individuals from six Dutch populations and two reference collections (Belgian and French) with K = 2; and (4) Principal coordinates analysis (PCoA) based on ten microsatellite markers

of the 112 individuals (Dutch, Belgian and French) grouped according to the two genetic clusters assigned by STRUCTURE and six cultivars with known species origin.

ACKNOWLEDGMENTS

This study was financially supported by the Knowledge Base-12 Programme on Sustainable Agriculture (KB-12-005.03-002) of the Dutch Ministry of Economic Affairs. The authors would like to thank K.G. Kranenborg, J. Bovenschen, M.C. Boerwinkel, I. Laros and T. Helmink for technical assistance, B. Maes and R. van Loon for assistance with sampling the Dutch elm populations. We thank S.M.G. de Vries (CGN) and H.M. Heybroek for comments on an earlier version of the manuscript.

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Manuscript received 7 Dec. 2015; accepted in revised version 15 Feb. 2016.

Communicating Editor: Myriam Heuertz.