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1 **Interspecific hybridisation and interaction with cultivars affect the genetic variation of**
2 ***Ulmus minor* and *U. glabra* in Flanders**

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13

1 **Abstract**

2 Interspecific hybridisation and gene flow from cultivated plants may have profound effects on the
3 evolution of wild species. Considering the cultural history and past use of *U. minor* and *U. glabra* trees
4 in Flanders (northern Belgium), we investigated the extent of human impact on the genetic variation of
5 the remaining, supposedly indigenous elm populations. We therefore examined the rate of
6 interspecific hybridisation, which is expected to be higher under human influence, the occurrence of
7 clones within and among locations, the presence of cultivars and their possible offspring. Based on
8 results produced using 385 Amplified Fragment Length Polymorphic (AFLP) markers, 46% of the 106
9 investigated Flemish elms appeared to be F1 hybrids or backcrosses to one of the parent species,
10 while no F2 hybrids (F1 x F1 progeny) were found. Clonality was mainly found among *U. minor* and
11 hybrids, which are more likely to form root suckers or sprouts as opposed to *U. glabra*. The majority of
12 the studied locations (76% of the locations with multiple samples) showed evidence of clonal
13 reproduction. Several, sometimes distant, locations shared a multilocus lineage. We also found
14 indications of gene flow from cultivated elms into native species. It is conceivable that reproductive
15 material has been moved around extensively, obscuring the natural genetic structure of the elm
16 populations. The results help guide the Flemish elm genetic resources conservation program.

17 Key words: *Ulmus minor*, *Ulmus glabra*, hybridisation, elm cultivars, clonal reproduction, human-
18 mediated disturbance

1 Introduction

2 *Ulmus minor* Mill. (Field elm) and *U. glabra* Huds. (Wych elm) are native deciduous trees in western
3 Europe. Especially *U. minor* and *U. x hollandica* (i.e. the hybrid between *U. minor* and *U. glabra*) have
4 been widely planted over the ages, in the cities as well as in the countryside, on roadsides and along
5 property borders, in hedges, near manors and farmsteads, as standards as well as coppice. Planting
6 stock was sometimes obtained from relatively distant provenances (Heybroek et al. 2009). In a forest
7 in The Hague (the Netherlands), for example, elms from Flanders (northern Belgium) and Utrecht (the
8 Netherlands) were planted in the 16th century (van Dissel 1912). Richens (1983) speculated that even
9 prehistoric tribes carried live elms on their wanderings. But typically local material from surrounding
10 forests, seedlings or root suckers, would have been used for plantings. Elm timber was greatly
11 appreciated for many purposes, e.g. coppice produced firewood and pliable twigs. Consequently,
12 elms have a long history of cultivation. Local cultivars were known since the 17th century and
13 probably earlier. In the Mediterranean, but also in areas in western Europe where the Romans
14 introduced their viticulture, elms were raised to act as vine supports, while the elm leaves served as
15 an excellent fodder for cows and sheep (Forster and Heffner 1954; Fuentes-Utrilla et al. 2004). For
16 this purpose, they brought a special cultivar, the 'Atinia' (Gil et al. 2004). More recent cultivars are
17 often non-European elms or hybrids between European and non-European elms, such as *U.*
18 *wallichiana* Planch. (Himalayan elm), *U. japonica* (Rehder) Sarg. (or *U. davidiana* var. *japonica*
19 (Rehder) Nakai; Japanese elm) and *U. pumila* L. (Siberian elm).

20 In addition to habitat loss and fragmentation due to changes in land use, the Dutch Elm Disease
21 (DED) severely reduced the number of elm trees starting from around 1910. DED is caused by the
22 non-native fungi, *Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier, which are spread by
23 bark beetles of *Scolytus* Geoffroy (Coleoptera, Scolitidae; Brasier 2001). The main vectors in western
24 Europe are *S. scolytus* and *S. multistriatus* (Webber 2004). Considering the severe threats European
25 elms are under, national actions were taken to conserve the remaining germplasm. In turn, this has
26 led to the initiative within the European Forest Genetic Resources (EUFORGEN) cooperative program
27 to realise a conservation plan on a European level (Collin 2002). Furthermore, an EU project on the
28 "Co-ordination for conservation, characterisation, collection and utilisation of genetic resources of
29 European elms" ran from 1997 till 2001 (RESGEN CT96-78). In Flanders, our study area, new clones
30 of native elms are still being added to the *ex situ* collections, initiated within the RESGEN project. Still,
31 limited knowledge is available on the level of genetic variation residing in elm populations and to what
32 extent this was influenced by human activities.

33 *U. minor* and *U. glabra* co-occur in Flanders, but are also able to hybridise with each other (*U. x*
34 *hollandica* Mill.), making their taxonomy extremely difficult (Goodall-Copestake et al. 2005). Although
35 hybridisation between *U. minor* and *U. glabra* is a natural process, it can also be human-mediated,
36 through habitat modification, species introduction or translocation. Human activities can therefore
37 encourage hybridisation (Ellstrand and Schierenbeck 2000; Rhymer and Simberloff 1996). For
38 example, *U. pumila* was introduced in Spain and Italy. Its hybrid with the native *U. minor* is now
39 common in both countries (Brunet et al. 2013; Cogolludo-Agustin et al. 2000). Natural interspecific

1 hybridisation as well as gene flow from cultivated elms to their native relatives are processes with
2 significant evolutionary consequences. A potential concern is outbreeding depression when
3 coadapted gene-complexes are broken up, causing a decrease in fitness (*Eucalyptus* spp.: Potts et
4 al. 2003; Edmands 2007; Laikre et al. 2010; Lynch 1991). Also, asymmetric gene flow could occur
5 when one species is less abundant than the other, with females of the rare species receiving a high
6 amount of heterospecific pollen, causing a higher risk of mate recognition errors (Mayr 1963; Wirtz
7 1999). Moreover, first generation backcrossing will more likely happen with the more common
8 species, leading to asymmetric introgression (Rieseberg 1997). For instance, species abundance
9 influenced the direction of introgression in oak populations (Lepais et al. 2009) as well as between
10 two *Morus* spp. (Burgess et al. 2005). Interspecific hybridisation may, however, also result in the
11 addition of genetic variability that facilitates habitat expansion (Arnold 1997). Conversely, the
12 commercial release of cultivated trees (or cultivars) can result in homogenisation of the genetic
13 composition and structure of the wild populations through introgression. These genetic risks are of
14 particular concern when the planted trees represent a narrow gene pool spread on a wide scale and
15 the related wild species is highly threatened by habitat reduction or diseases (e.g. *Populus* spp.:
16 Santos-del-Blanco et al. 2013; Vanden-Broeck et al. 2012; Smulders et al. 2008; *Malus* spp.: Kron
17 and Husband 2009; *Coffea arabica*: Aerts et al. 2013; *Prunus avium*: Mariette et al. 2010).

18 Considering the cultural history and past use of *U. minor* and *U. glabra* trees in Flanders, we
19 investigated if the genetic variation of the remaining, supposedly indigenous elm populations is
20 influenced by human activities. The answer to this research question could help guide the Flemish
21 elm genetic resources conservation program. We therefore examined 1) the rate of interspecific
22 hybridisation, which is expected to be elevated due to human activities, 2) the occurrence of ramets
23 (i.e. genetically identical individuals originating vegetatively from a single ancestor) within and among
24 locations and 3) the presence of cultivars and their offspring in Flemish indigenous elm populations.

25 **Material and methods**

26 *Samples and DNA extraction*

27 Three sets of samples were used in this study. Between 2007 and 2009 leaves of adult *Ulmus minor*,
28 *U. glabra* trees and their hybrids were collected in Flanders in northern Belgium for the first set (Table
29 1, Fig. 1). The leaves were collected in a gene bank containing ramets of the original trees (i.e. ortets)
30 except for the samples in the location coded BESP (Table 1). The indigenous ortets were at least 3 m
31 apart from each other to diminish the risk of sampling ramets. The census number of elm trees was
32 lower than 30, except at location BERI (n > 50) (Table 1). We limited the number of samples per
33 location to address the research questions on the regional scale (i.e. Flanders). The species of the
34 elm trees was identified morphologically according to Lambinon et al. (1998). This set of samples
35 ('dataset 1') entails the focus of our study and is therefore used in every analysis.

36 The second set ('dataset 2') comprises samples of *Ulmus minor* and *U. glabra*, which are used as
37 references for these three pure species in the hybrid detection analyses (Table 1). They were
38 obtained from the *ex situ* collection of the RESGEN CT96-78 project (indicated with 1 to 6 in

1 superscript in Table 1). They are originally from Belgium (location BETO), France, Italy, Germany and
2 Greece, and were collected mainly in forests and screened genetically by Goodall-Copstake et al.
3 (2005). Also, a few samples from six localities in France were added (locations FRAU, FRCB, FRLR,
4 FRLV, FRSP and FRBL), as well as one sample of *U. procera* Salisb. (English or Atinian elm)
5 collected in Kallstadt, Germany.

6 The third set of samples ('dataset 3') contains cultivars which were mainly collected in the
7 Netherlands, except for 'Klemmer', which was provided by Irstea (France) and originated from
8 Wakehurst Place (UK) (Table 2). The Dutch locations of elm cultivars are well known by Heybroek et
9 al. (2009); several recent cultivars in this study were even selected and released by H. Heybroek.
10 Some of the cultivars are hybrids with *U. wallichiana*, *U. japonica* or *U. pumila*. The cultivars were
11 included in clone and parentage analyses to investigate their influence on supposedly indigenous
12 individuals of *U. minor*, *U. glabra* and their interspecific hybrids. Well-known cultivars 'C. Buisman' (*U.*
13 *minor*), 'Sarniensis' (*U. minor*) and 'Horizontalis' (*U. glabra*) were also used as reference samples
14 representing pure *U. minor* and *U. glabra* in hybrid detection analyses.

15 The leaves were stored on silica gel. Total DNA was extracted from ground leaf samples, partly with
16 QuickPick™ SML Plant DNA purification kit in combination with the PickPen 8-M magnetic tool or the
17 MagRo 8-M robotic workstation (Isogen Life Science) on 5 mg of dried leaf tissue, and partly with
18 DNeasy Plant Mini Kit (Qiagen) on 20 mg of dried leaf tissue. The integrity of the DNA was assessed
19 on 1.5% agarose gels, and the purity and concentration with the ND-1000 Nano-Drop
20 spectrophotometer (NanoDrop Technologies).

21 *AFLP analysis*

22 Amplified Fragment Length Polymorphism (AFLP) fingerprints were generated according to Vos et al.
23 (1995), but with restriction and ligation conducted in one single step. Initially, 21 primer combinations
24 (EcoRI/MseI) were tested on 16 samples (14 individuals and 2 replicates). The following two primer
25 combinations were selected for the selective amplifications: EcoRI-AGC(ned)/MseI-CTG and EcoRI-
26 ACC(ned)/MseI-CTG. We used the RawGeno v 2.0 R CRAN package (Arrigo et al. 2009) for
27 automated scoring, which resulted in 385 polymorphic loci.

28 There were seven samples with poor quality AFLP electropherograms for one or both primer
29 combinations. To avoid series of missing values, we discarded these samples from further analysis,
30 leaving a total of 177 samples of which 34 (19%) were randomly replicated to assess reproducibility.
31 Mean typing error reached 4% (following Bonin et al. 2004) and mean Dice similarity between pairs of
32 replicates was 0.95. The polymorphism information content (PIC) of each locus was calculated
33 (Anderson et al. 1993).

34 *Data analysis*

35 *Linkage disequilibrium*

36 The hybrid detection analysis and the sibship analysis were executed under the assumption of
37 unlinked markers (see below). We therefore assessed linkage disequilibrium (LD) among AFLP loci

1 by performing logistic regressions between pairs of loci (e.g. Poncet et al. 2010). To correct for
2 multiple testing, we used the false discovery rate approach (FDR; Benjamini and Hochberg 1995) with
3 R package brainwaver (Achard 2012). The maximum FDR was set at 5%. Computations were done
4 with the statistical software platform R version 2.15 (R Development Core Team 2010). Another
5 method to detect linkage disequilibrium by Haubold et al. (1998) was used, implemented in LIAN 3.6
6 (Haubold and Hudson 2000). The standardized index of association (I_A^S) was calculated, based on
7 10,000 permutations. Values for dataset 1, with 309 polymorphic markers, were produced, as well as
8 for three subsets of dataset 1: *U. glabra* samples (199 polymorphic markers), *U. minor* samples (183
9 polymorphic markers) and their hybrids (232 polymorphic markers). This subdivision was based on
10 the results of the hybrid detection analyses. It is expected that *U. minor* and the hybrids will show
11 higher LD because of their ability to reproduce asexually. The subsets of dataset 1 were reanalysed
12 with duplicate ramets excluded, resulting in 197 polymorphic markers for the *U. glabra* samples, 172
13 for *U. minor* and 222 for hybrid samples. Recent hybridization between taxa derived from two gene
14 pools with different allele frequencies, should exhibit strong LD. Still, associations among loosely
15 linked markers decay rapidly with subsequent generations of hybrids, resulting in a reduced level of
16 LD (Goodman et al. 1999).

17 *Hybrid identification*

18 In order to investigate the actual extent of interspecific hybridisation among Flemish elms, we used
19 the Bayesian method implemented in BAPS v 5.4 (Corander et al. 2008). An admixture analysis
20 based on pre-defined groups was performed using 100 iterations. We restricted the analyses to
21 samples identified as *U. minor*, *U. glabra* and their hybrids of all three datasets (i.e. 164 samples of
22 182). The samples screened genetically by Goodall-Copestake et al. (2005), were used as reference
23 samples for *U. minor* and *U. glabra* (i.e. of the second dataset and the sample of location BETO from
24 dataset 1). Also the well-known cultivars 'C. Buisman' (*U. minor*), 'Sarniensis' (*U. minor*) and
25 'Horizontalis' (*U. glabra*) were treated as reference samples for both pure species. Individuals with
26 admixture values (q_i) ≥ 0.90 for either of the two groups were assigned to purebreds. In other cases
27 they were considered to be admixed (Vähä and Primmer 2006).

28 Another Bayesian approach, NewHybrids v1.1b3 (Anderson and Thompson 2002), was used on the
29 same dataset. The program was run with the default parameters for the six genotype class
30 frequencies (pure *U. minor*, pure *U. glabra*, F1 hybrids and F2 progeny (of F1 hybrid parents) and
31 backcrossed F1 hybrids), uniform priors and two runs with a burn-in phase of 10,000 steps and
32 100,000 MCMC iterations. The same reference samples as in BAPS were used to represent the pure
33 species. The samples used as priors were specified as sampled separately from the rest of the
34 mixture of interest, except the sample of BETO located in Flanders. Individuals were considered to
35 belong to one of the predefined classes if $P \geq 0.90$ for that genotypic class (Vähä and Primmer 2006).

36 It must be noted that the model underlying NewHybrids relies on the assumption that the genetic
37 markers in the dataset are unlinked (Anderson 2008), which also applies for the BAPS analysis.
38 Although we found significant LD among loci of the Flemish samples (see results), this concerned
39 only a very small number of loci.

1 *Clone assignment*

2 Ramets (or clone mates) belonging to the same clonal lineage bear the same multilocus genotype or,
3 if somatic mutations have occurred, the same multilocus lineage (MLL). To identify MLLs among the
4 samples, we used GenoType (Meirmans and Van Tienderen 2004) on the three datasets combined.
5 First, the discriminative power of the AFLP markers was assessed by plotting the number of loci
6 versus genotypic richness (G:N; i.e. the ratio of the number of MLLs and number of samples) for an
7 increasing number of loci. For the calculations of genotypic richness we first used a set of two highly
8 informative markers successively adding sets of 16 markers with decreasing information content,
9 resulting in calculations based on two, 18, 34, 50, 66, etc. markers, respectively. For each set of
10 markers, the number of MLLs was obtained using several thresholds, ranging from 0.90 to 0.99 Dice
11 similarity, to account for differences in actual thresholds between different sets of loci. When
12 genotypic richness reaches a plateau, the markers are considered to possess enough resolution to
13 reliably assign clonal lineages (Arnaud-Haond et al. 2005). The Dice similarity index was used as a
14 distance measure between all pairs of individuals. As scoring errors and mutations can cause a
15 genetic distance between members of the same MLL to be higher than zero, a threshold needs to be
16 chosen to account for these differences, which are indicated by a multimodal frequency distribution of
17 genetic distances (Arnaud-Haond et al. 2005; Douhovnikoff and Dodd 2003; Meirmans and Van
18 Tienderen 2004). Consequently, the frequency distribution of the distances and the mean Dice
19 similarity of the pairs of replicated samples were used to define an appropriate threshold.

20 Whether members of a MLL were the result of sexual reproduction, was evaluated through the clonal
21 population structure test implemented in GENODIVE v2.0b17 (Meirmans and Van Tienderen 2004).
22 Here, the observed clonal diversity is compared with the diversity achieved by randomising alleles
23 over individuals (1,000 permutations). Clonal diversity is expected to be lower under clonal
24 reproduction than under sexual reproduction (Gómez and Carvalho 2000). This test includes the
25 threshold used for assigning MLLs. Nei's corrected diversity index (Nei 1978) was used as test
26 statistic and the alleles were randomised over all individuals of the Flemish region (i.e. dataset 1). The
27 same calculations were performed for the Flemish *U. minor*, *U. glaba* and hybrid trees separately. It
28 should be noted that similar genotypes could also be the result of selfing, biparental inbreeding and
29 population genetic structure (Halkett et al. 2005).

30 *Parentage and sibship analysis*

31 To evaluate past or present influence of elm cultivars on the putative indigenous elm populations of
32 Flanders, we conducted a sibship and parentage analysis with Colony v2.0.1.9 (Jones and Wang
33 2009). In this program, a maximum likelihood method is used. Offspring are clustered into paternal
34 and maternal families using a simulated annealing approach to maximise the group likelihood value.
35 Then, candidate parents are assigned to the clusters at a 95% confidence level. If no candidate
36 parents seem available, the program reconstructs parental genotypes. It can also deal with scoring
37 errors. We excluded monomorphic loci, resulting in a set of 355 polymorphic markers. The offspring
38 dataset contained unique ramets of dataset 1, while the cultivars of dataset 3 were set as the potential
39 parents, considering their release dates are often quite old (18th – early 19th century) and/or their

1 widespread use, especially of 'Belgica', 'Major', 'Klemmer' and 'Vegeta'. The more recent cultivars,
2 mostly parented by non-native elms, were included as 'negative controls' in the candidate parents set.
3 We found some linkage among markers (see results). This could lower the precision of the analysis,
4 but is probably compensated by the high number of informative markers (Wang and Santure 2009).
5 We did not consider tree size or any other measure of tree age in the analysis. Because many trees in
6 our study are part of old hedges, coppice trees or root suckers of trees that succumbed to DED, age
7 estimates are highly unreliable. The results from this parentage and sibship analysis will therefore
8 only highlight close relations, rather than exact parent-offspring relationships.

9 **Results**

10 *Marker information and independence*

11 The AFLP loci are only moderately polymorphic (Table 3), with a comparable mean PIC for *U. minor*,
12 *U. glabra* and their hybrids (0.17-0.22). However, when considering loci with a band presence
13 between 5% and 95%, a minimum of 145 loci still remained with a substantially higher mean PIC
14 between 0.26 and 0.30.

15 In dataset 1, 3.7% of the pairwise logistic regressions were significant. Although I_A^S was low (0.0174)
16 it appeared significant ($P < 0.0001$). The proportion of significant logistic regressions decreased when
17 only *U. minor* (0.6%) or hybrids (0.4%) were considered, while I_A^S slightly increased (0.0182 and
18 0.0175 with $P < 0.0001$, respectively). Retaining only unique ramets further rendered insignificant
19 logistic regressions for *U. minor* and only one significant pairwise comparison (0.004%) for hybrids.
20 The I_A^S value stayed the same for *U. minor*, but decreased to 0.0111 ($P < 0.0001$) for the hybrid
21 group. For the set of Flemish *U. glabra* samples, no significant logistic regressions were detected,
22 while I_A^S stayed significant with even lower values (0.0064 and 0.0049 ($P < 0.0001$), with and without
23 duplicate ramets, respectively).

24 *Hybrid detection*

25 Out of 106 trees sampled in Flanders, only 28 were assigned as pure *U. glabra* and 29 as pure *U.*
26 *minor* with $q_i = 1$ using BAPS (Fig. 2). Three out of eight samples that were identified as hybrids in the
27 field, appeared to be pure species (one *U. glabra* and two *U. minor*). So, 49 (46%) of the Flemish
28 samples seemed genetically to be hybrids between these two species, of which 38 (78%) were
29 identified morphologically as a pure species. Most of the samples were assigned with high
30 probabilities using NewHybrids (80% with $P \geq 0.90$). Eight Flemish samples showed probabilities
31 between 0.80 and 0.90. Only 12 Flemish samples had maximum probabilities below 0.80 with seven
32 below 0.70. These individuals with $P < 0.90$ were all classified as hybrids using BAPS, except for two
33 of location BERI and one sample of BEGE. Their maximum probabilities ($0.78 < P < 0.89$) given by
34 NewHybrids also suggested them to be purebreds. Furthermore, two reference samples of *U. minor* in
35 dataset 2, one of FRGS and one of GELD, were classified by NewHybrids as backcrosses to *U.*
36 *minor*, however with probabilities of 0.52 and 0.55, respectively. BAPS did not reclassify these
37 reference samples. No F2 hybrids were found, based on the NewHybrids results. As for the cultivars,
38 the analyses identified 'Groeneveld' and 'Vegeta' as (multiple generation) backcrosses to *U. minor*

1 and 'Dampieri' as a cultivar of *U. minor*. Furthermore, both replicates starting from leaf tissue of
2 'Major' appeared to be pure *U. minor*, but the cultivar is claimed to be a hybrid. The other cultivars
3 involving solely *U. minor* and/or *U. glabra* and the remaining samples of dataset 2 were assigned to
4 their predetermined group.

5 For all the following analyses, samples were assigned to the species class given by NewHybrids and
6 BAPS results.

7 *Clonality*

8 The resolution of the markers appeared high enough to reliably detect clones. The relationship
9 between number of markers and genotypic richness is asymptotic, reaching a plateau at
10 approximately 96 markers (Fig. 3). The frequency distribution of pairwise Dice similarity values was
11 more or less bimodal, with a small peak towards lower distances. Fig. 4 shows this small peak.
12 Although the valley between the first and second peak is considered a probable threshold (Meirmans
13 and Van Tienderen 2004), which is at a Dice similarity of 0.90, this seemed questionable. At this level
14 of similarity, three French samples of two locations separated by more than 100 km, appeared to be
15 clones. Although this is not impossible through human mediation, this seems a less likely scenario.
16 We therefore resided to select a more conservative threshold of 0.94 Dice similarity, which is slightly
17 lower than the mean Dice similarity of 0.95 calculated for the duplicate samples to account for somatic
18 mutations and which coincides with the first small drop in frequency in Fig. 4. In addition, three ramets
19 of 'Lobel' and two of 'Plantyn', each collected at a different locality, were assigned to the correct MLL
20 at this threshold.

21 Fig. 2 indicates the identified MLLs with multiple ramets for the Flemish locations. Clones were
22 detected at 12 of the 16 Flemish sampling locations and at four French locations, where more than
23 one tree was sampled. They were mostly found among *U. minor* and hybrids, mainly with higher
24 admixture levels for *U. minor* genes, but also a few individuals of *U. glabra* appeared to be ramets.
25 Two MLLs with each two ramets of *U. glabra* were detected at location BERI (MLL '9' and '10'; Fig. 2).
26 The *U. glabra* ramets of MLL '9' are neighbouring trees.

27 Certain MLLs are present on more than one location (Euclidean distances between locations): MLL
28 'A' at locations BEDI, BEME2 and BESC (16 to 39 km), MLL 'B' at BEDM2 and BEHO (4.5 km), MLL
29 'C' at BEEG and BERI (70 km), and MLL 'E' at BEMA and BESC (4.5 km) (Fig. 1). Interestingly, one
30 individual of BEGE and all samples except one of BEME1 belong to the same MLL as the 'Klemmer'
31 cultivar.

32 Clonal diversity was significantly lower than diversity under random mating for dataset 1, as well as
33 for each separate species in Flanders ($P < 0.001$).

34 Also in France, some neighbouring *U. minor* trees at FRAU, FRLV, FRCB and FRLR, respectively,
35 had a MLL in common. Furthermore, a common MLL was found at locations FRLV and FRAU with a
36 distance of 16 km between them.

1 *Parentage and sibship analysis*

2 After retaining only unique ramets, dataset 1 contained 64 Flemish samples defined as offspring in
3 the analysis. As expected, no recent cultivars were inferred as parents of our Flemish samples. On
4 the other hand, 'Klemmer', 'Belgica' and 'Major' came up several times as potential parents with high
5 probabilities. These cultivars are at least 130 years old and known to have been widely planted in
6 Flanders. 'Klemmer' was inferred as a parent of a sample of BEGE and of BEME1, where also ramets
7 of 'Klemmer' were found (see the clonality results). 'Major' was found to be a potential parent of
8 individuals at the locations BEOO, BESP, BEEN and BEDI. Additionally, the BEDI location contained
9 another individual, possibly parented by 'Belgica'. This individual is a ramet of MLL 'A', which was
10 also present in BEME2 and BESC. Furthermore, MLL 'E' found in BEMA and BESC has 'Belgica' as a
11 potential parent. In four (50%) of these parent-offspring combinations, the admixture proportions of
12 the parents are different from those of the offspring. This suggests that a possible clonal relationship
13 as opposed to a parent-offspring relationship is less likely.

14 According to the results of Colony, many of the samples were found to be full-sibs or half-sibs (Fig. S1
15 in Supplementary Material). Only one of 569 pairwise full-sib relationships between individuals (of
16 BEOO and BEEN) had a probability of 0.848, while other probabilities equalled one. Also, 1 of a total
17 of 89 of inferred pairwise half-sib relationships had a probability of 0.943 and the remaining
18 combinations received the maximum probability value. Location BERI holds many full-sibs of which a
19 few were identified as backcrosses, while the majority seemed to be pure *U. glabra*. Also, a few
20 peculiar full-sib and half-sib combinations could be detected between samples of distant locations,
21 such as BERI, BEGE, BEEG and BEMA. Here, the full-sib relationships are often between potential
22 pure Wych elms and hybrids (F1 or backcrosses to *U. glabra*). The majority of the full-sib
23 combinations between pure *U. glabra* and hybrids, including backcrosses, can possibly be attributed
24 by the uncertainty of the species assignment of several samples using NewHybrids. In fact, 90% of
25 the 234 full-sib relationships between different species classes, involved samples that were classified
26 as F1 hybrids or backcrosses to probabilities below 0.90, of which 50% was below 0.70. Taking
27 admixture proportions of these individuals given by BAPS into account, did not change these unlikely
28 combinations. The remaining full-sibs are mainly within particular locations (e.g. BEBR: *U. minor*) or
29 between locations quite near to each other (e.g. BERI and BETO: *U. glabra*).

30 As stated before, because we were not able to estimate the age of the Flemish elms, they could just
31 as well be a mixture of parents and offspring instead of pure offspring, potentially causing full-sib
32 associations between individuals of different admixture classes. In addition, the cultivars assigned as
33 potential parents, may be close relatives but not the parents of the alleged offspring. The results of
34 the sibship analysis are therefore only indicative of family relations.

35 **Discussion**

36 *Occurrence of interspecific hybrids*

37 Our results indicate that the formation of first-generation hybrids (F1) between *U. minor* and *U. glabra*
38 and backcrosses in either direction is common in Flanders. This suggests that no strong pre- or post-

1 fertilisation barriers between these two species exist. Former morphological studies already
2 suggested that in Flanders and the Netherlands, the full spectrum of the *U. minor-U. glabra* complex
3 is present (e.g. Touw 1963). Still, the hybrids do not seem to be recent, since the level of LD appears
4 to be low and advanced generation hybrids are predominant among hybrids (Fig. 2). Because of the
5 high number of hybrids, hybridisation could have been facilitated by human-mediated habitat
6 disturbance (Ellstrand and Schierenbeck 2000; Rhymer and Simberloff 1996). Hybrid clone mates
7 and full-sibs were often found among different locations, indicating human influence through
8 movement of elm planting material and the establishment of cultivars, often hybrids, near natural elm
9 populations (see below).

10 It seems that F1 elm hybrids are not sterile, judging by the backcrosses that were found. However, F2
11 offspring of F1 hybrids were not identified among the sampled trees. A possible reason for failing to
12 detect F2 hybrids, could be an insufficient discriminative power of the AFLP markers, partly because
13 of their dominant character. Still, assignment probabilities to the F2 genotype class never exceeded
14 0.15. A lack of F2 generations might suggest selection against offspring containing homozygous loci
15 from either species. A loss in fitness might not arise until the second or later hybrid generations
16 because of the increasing severity of homozygous incompatibilities according to the (Bateson-
17)Dobzhansky-Muller model (Turelli and Orr 2000) or because of outbreeding depression (Edmands
18 2007; Lynch 1991). Zalapa et al. (2009) also did not find second-generation hybrids between *U.*
19 *pumila* and *U. rubra*. They suggested pollen–stigma incompatibilities between F1 hybrid parents as a
20 possible cause, since F1 hybrids would contain S-alleles from both parents and thus be less likely to
21 crossbreed successfully with another F1 hybrid. On the other hand, Brunet et al. (2013) did find F2
22 hybrids of *U. pumila* and *U. minor*, which they attributed to the relatively self-compatible nature of *U.*
23 *minor*, described in the paper of Mittempergher and La Porta (1991). Based on many controlled
24 selfings and crosses between several species and hybrids, including *U. minor* and *U. glabra*, Heybroek
25 (1993) detected, however, a high degree of self-incompatibility. Over a period of ten years, producing
26 24,149 seedlings out of 2,378 pollination bags, 770 crosses and 196 selfings, he found that cross
27 pollination gave 23 times more viable seeds and seedlings than selfing.

28 Out of 87 samples taken throughout Italy and in some locations in France, Brunet et al. (2013)
29 identified 13 trees (15%) as F2 with a posterior probability of 0.90 or greater using NewHybrids, or
30 35% of the total number of hybrids (37). Bearing in mind these relatively high percentages and our
31 more intensive sampling (i.e. on average the same amount of trees was sampled within a smaller
32 region), missing an F2 hybrid seems less plausible, unless they are extremely rare.

33 *Natural and artificial clonal propagation*

34 Evidence of clonality was mostly found among *U. minor* and hybrids with a higher representation of *U.*
35 *minor* genes. Although clonal reproduction seemed mainly to occur within locations, several ramets of
36 the same MLL were found at different locations in Flanders, with distances of 4.5 km to more than 60
37 km. This suggests translocation of elm planting material, preferably root suckers, from one location to
38 the other. Ramets of a genotype in BEHO (*U. minor*), of MLL 'C' in BERI and BEEG (*U. x hollandica*),
39 and of MLL '3' in BEBR (*U. minor*) were even detected in the Netherlands using microsatellites (J.

1 Buiteveld, unpublished data). More pronounced examples of clonal spread through human activity
2 were found for *Populus alba* in Sardinia (Brundu et al. 2008) and in Malta (Fussi et al. 2012).
3 Furthermore, many elms in Flanders were found in old hedges (of *U. minor*; Table 1), indicating past
4 human activity. One of the MLLs identified among the investigated elms appeared to be the cultivar
5 'Klemmer', confirming human involvement in elm establishment in Flanders. 'Klemmer' originates from
6 Belgium (Ypres) (Elwes et al. 1906). It dates back from 1877 or maybe 1789. It was always rare in the
7 Netherlands and common in Belgium and the north of France in the beginning of the 20th century.
8 However, it is now considered to be rare in general (Heybroek et al. 2009).

9 As mentioned before, it is difficult to infer an exact threshold to determine which of the samples
10 belong to the same MLL. It seemed highly unlikely that the inferred clone mates resulted from random
11 mating ($P < 0.001$). Even though we cannot entirely rule out the occurrence of selfing, the elm species
12 in question are basically deemed self-incompatible (Heybroek 1993; Nielsen and Kjaer 2010;
13 Townsend 1975). Nearly identical genotypes can also be produced due to extensive inbreeding.
14 Nevertheless, we also found such similar genotypes among distant locations and mainly among *U.*
15 *minor* and hybrids which can easily be propagated vegetatively using root suckers. We are therefore
16 quite confident about the identified MLLs and the threshold used.

17 A few samples of *U. glabra*, of BERI, were detected as ramets of the same MLL. *U. glabra* is not
18 known to regenerate through sprouting, except on trunks of young trees, nor through root suckering.
19 The two closest clones in BERI could therefore be remnants of an old coppice tree, although no
20 apparent evidence pointing towards this hypothesis was found in the field. Planting, on the other
21 hand, is very plausible as cultivation of the species in Flanders has occurred since the 17th century
22 (Maes 2006). This could also explain the presence of related genotypes of *U. glabra* in BERI, BEGE
23 and BEEG.

24 Although it is feasible that, within locations, the clones among *U. minor* and its hybrids with *U. glabra*
25 were the products of natural clonal reproduction, they could just as well be planted. Even the ortet
26 trees could have originated from another location, most probably from the same region. It is therefore
27 difficult to assess human interference on this level.

28 *Influence of cultivars*

29 Besides the samples with the same MLL as 'Klemmer' found in BEME1 and BEGE, this cultivar could
30 also be the parent of at least two other trees growing in the same locations. Moreover, 'Belgica' and
31 'Major' were found as potential parents of several sampled trees. Consequently, the three cultivars
32 have influenced the natural elm populations, either through planting and possibly clonal reproduction,
33 and through hybridisation. Like 'Klemmer', cultivars 'Major' and 'Belgica' are old. 'Major' is probably
34 the oldest and dates from around 1600 or even before that (Heybroek et al. 2009). Its origin is
35 uncertain, but seemed to be brought to England in 1689 by Willem III from the Netherlands. It was
36 planted frequently in the Netherlands until 'Belgica' became more popular mid-19th century. The latter
37 was thought to originate from Belgium in the 18th century, in the Abbey of Dunes with nurseries in
38 Furnes and Bruges (Elwes et al. 1906). It was extensively planted starting from 1850. Almost all elm

1 plantings in Belgium and the Netherlands consisted of this cultivar until 1928. In addition, for a long
2 time, 'Belgica' was used as rootstock. Consequently, their age and frequent use in the past make the
3 three cultivars feasible parents of some of the sampled elms. Due to the Flemish origin of at least
4 'Klemmer' and 'Belgica', with potentially close relatives still present in Flanders, family connections
5 other than parent-offspring relationships are quite possible and should be taken into consideration. An
6 alternative explanation for the assignment of cultivars as parents could be that some elms are in fact
7 ramets of the cultivars in question, suggesting that more somatic mutations have occurred than
8 accounted for by the current threshold for clone assignment. Nonetheless, half of these parent-
9 offspring combinations displayed different admixture levels between parent and offspring, whereas
10 the opposite would be expected among clones.

11 *Consequences for conservation*

12 Our results suggest high human impact on elm populations obscuring their natural genetic structure.
13 A high number of (advanced generation) hybrids were detected, probably facilitated by human
14 activities such as translocation of trees. This was supported by the ramets found among locations. In
15 addition, cultivar 'Klemmer' was identified among the investigated elms, as well as several individuals
16 showing potential introgression from cultivars 'Major', 'Belgica' and 'Klemmer'.

17 LD among loci was low but significant and can partly be attributed to clonal reproduction (de Meeûs
18 and Balloux 2004; Tibayrenc et al. 1991). The potential problem of mate or pollen limitation that
19 comes with clonal reproduction could lead to a decrease in fertility or an increase in self-compatibility
20 (Honnay and Jacquemyn 2008; Vallejo-Marín et al. 2010), of which examples can be found in
21 domesticated crop plants (McKey et al. 2010). Linkage was still present when potential clone mates
22 were excluded, which could be the result of a bottleneck in the elm populations (Flint-Garcia et al.
23 2003) caused by DED and habitat loss. In addition, the investigated elms seemed often highly related,
24 which might suggest non-random mating. Non-random mating is likely to occur in small, fragmented
25 woodland populations such as the Flemish elm populations. This can cause genetic drift and reduced
26 genetic variation (Jump and Peñuelas 2006). Given the fact that biparental inbreeding is likely (e.g.
27 Nielsen and Kjaer 2010), population differentiation might increase as a result of genetic drift and
28 continuing losses of genotypes due to disease and additional loss of habitat. In addition, the spread of
29 cultivars has probably influenced the remaining genetic resources of *U. minor* and *U. glabra*,
30 potentially further altering the genetic variation. Steps taken to conserve the remaining elms in a field
31 gene bank are therefore no wasted effort. The genetic markers and methodology used in this study
32 will help in distinguishing clones, pure species, hybrids and cultivars among elms in the ex situ
33 collection.

34 Since sexual reproduction appears to be rare in some locations in Flanders, especially in *U. minor*,
35 energy put into the formation of F1 hybrids might be considered as wasted reproductive effort of the
36 pure species, because F1 hybrids do not seem to succeed in creating F2 hybrids. The elm hybrids
37 occupy, however, a great part of the ecological niches of both pure elm species in Flanders and
38 therefore partly fulfil the ecological role of the parent taxa. Although the hybrids appear to be mainly

1 human induced, the focus should be on conserving their ecological function. In turn, this will help
2 maintain the structure of plant communities also containing (at one point) pure elms (Stronen and
3 Paquet 2013). Moreover, because of the abundance of clones and highly related individuals, the
4 residual gene pool, especially of the pure species, appears small and could become even smaller as
5 mentioned above. Introgression can be a source of genetic variation in the elm populations and may
6 promote adaptation (Grant and Grant 1994). Consequently, conserving the overall genetic variation is
7 warranted. Nonetheless, further research is required to determine whether elm hybrids in Flanders
8 have a competitive advantage over pure elm species, especially in regard to DED.

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14 their insightful comments.

15 **Supplementary Material**

16 **Fig. S1** Best maximum likelihood sibship assignment plot of the sibship structure for the unique
17 ramets of Flemish multilocus lineages of the *U. minor-U. glabra* complex obtained with Colony
18 v2.0.1.9. Above diagonal (black dots): full-sibs; below diagonal (grey dots): half-sibs. On both axes,
19 the location code of each individual is given

20 **Data Archiving Statement**

21 *AFLP data: data will be deposited at Dryad*

22

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Figures

Fig. 1 Map depicting the locations of the elm samples of Flanders (northern Belgium). The codes are given in Table 1.

Fig. 2 Posterior probabilities of genotype frequency classes given by NewHybrids (a) and admixture proportions given by BAPS (b) for Flemish elm samples and cultivars of *U. glabra*, *U. minor* or *U. x hollandica*. The first half of the results is given above, the second half below. Samples are indicated with their location code (Table 1) or cultivar name (Table 2). Ramets of the same multilocus lineage (MLL) are indicated with the same number before the location code or a capital letter in case of a MLL found among locations. UM: *U. minor*; UG: *U. glabra*; F1: first generation hybrid; F2: offspring of F1 hybrids; BC to UM/UG: backcross to *U. minor/U. glabra*.

Fig. 3 Plot of number of loci versus genotypic richness (G:N) based consecutively on two highly polymorphic markers and sets of multiples of 16 markers with decreasing information content. For each set of loci, a range of G:N values (vertical lines), which were calculated using a different thresholds (0.90 to 0.99 Dice similarity). The line goes through mean G:N values.

Fig. 4 Frequency distribution of pairwise Dice similarity index values with a maximum of 0.80.

Tables

Table 1 List of sampled locations of *Ulmus minor*, *U. glabra*, their hybrids and *U. procera*. N: number of sampled individuals; N AFLP: number of samples successfully analysed with AFLP; Nc: estimate of total number of individuals present on a location; Type LE: type of landscape element; UM: *U. minor*; UG: *U. glabra*; UH: *U. x hollandica*; UPR: *U. procera*; NA: not available.

Dataset 1: Flemish samples (with species identification based on morphology)									
Location	Country	City	Species (number of trees)	Lon	Lat	N	N AFLP	Nc	Type LE
BEBR	Belgium	Brugge (Sint-Pieters)	UM	3.1728	51.2387	8	7	20	Old hedge
BEDI	Belgium	Dilbeek	UG	4.2530	50.8536	2	2	10	Forest
BEDM1	Belgium	Diksmuide	UM	NA	NA	2	2	NA	NA
BEDM2	Belgium	Diksmuide	UM	2.9040	51.0095	4	4	5 - 10	Old hedge
BEEG	Belgium	Heverlee	UG	4.6662	50.8541	8	6	20 - 30	Forest
BEEN	Belgium	Oudenaarde (Ename)	UM	3.6593	50.8616	3	3	20 - 30	Wooded banks
BEGE	Belgium	Geraardsbergen	UG (7), UH (1)	3.9290	50.7700	8	8	20	Forest
BEHE	Belgium	Heusden	UM	3.8159	51.0099	2	2	10 - 20	Old hedge
BEHO	Belgium	Houthulst	UM	2.8720	50.9770	4	4	10 - 20	Old hedge
BELE	Belgium	Lemberge	UM	3.7693	50.9788	5	5	10 - 20	Old hedge
BEMA	Belgium	Maarkedal	UM (1), UG (5), UH (1)	3.6520	50.8247	7	7	10 - 15	Wooded bank
BEME1	Belgium	Merelbeke	UM (5), UH (1)	3.7198	50.9519	6	6	10	Forest
BEME2	Belgium	Merelbeke	UH	3.7508	50.9739	3	3	10	Forest
BEOO	Belgium	Oosterzele	UM	3.8065	50.9350	1	1	10	Forest edge
BERI	Belgium	Riemst	UM (4), UG (29), UH (3)	5.6857	50.8058	38	36	50 - 100	Forest
BESC	Belgium	Schorisse	UM (1), UG (4), UH (2)	3.7065	50.8028	7	7	20 - 30	Forest, wooded bank
BESP	Belgium	Sint-Pieters-Kapelle (Herne)	NA	3.9795	50.6930	2	2	NA	NA
BETO ^a	Belgium	Tongeren	UG	5.4908	50.8035	1	1	NA	Private park
Dataset 2: reference samples									
Location	Country	City	Species	Lon	Lat	N	N AFLP		
FRAM ^b	France	Amplier	UM	2.4010	50.1352	1	1		
FRAR ^b	France	Argentan	UM	-0.0187	48.7402	1	1		
FRAU	France	Aunay	UM	0.6307	49.0205	2	2		

FRBB ^b	France	Bourg-Blanc	UM	-4.5017	48.5005	1	1
FRBL	France	Blismes	UM	3.8202	47.1315	1	1
FRCB	France	La Chapelle-Bâton	UM	0.3297	46.4746	3	3
FRCM ^b	France	Saint-Martin-de-Ré	UM	-1.3593	46.2027	1	1
FRCU ^b	France	Cucq	UM	1.6207	50.4742	1	1
FRGO ^b	France	Godewaersvelde	UM	2.6380	50.7898	1	1
FRGS ^b	France	Grande-Synthe	UM	2.2897	51.0087	1	1
FRIL ^b	France	Illkirch-Graffenstaden	UM	7.7185	48.5243	1	1
FRLR	France	Le Rheu	UM	1.7954	48.1011	2	2
FRLV	France	Le Vey	UM	0.4701	48.9175	5	5
FRLW ^b	France	La-Wantzenau	UM	7.8222	48.6575	1	1
FRMA ^b	France	Magnicourt-en-Comte	UM	2.4877	50.4018	1	1
FRME ^b	France	Meteren	UM	2.6880	50.7383	2	2
FRMQ ^b	France	Mecquignies	UM	3.7890	50.2738	1	1
FROS ^b	France	Ostwald	UM	7.7058	48.5403	1	1
FRSP	France	Saint-Pé-de-Bigorre	UG	0.1552	43.0737	3	2
FRST ^b	France	Strasbourg	UM	7.7537	48.5845	1	1
GEGO1 ^c	Germany	Göttingen	UG	9.1500	51.3333	3	2
GEGO2 ^c	Germany	Göttingen	UM	9.9557	51.6502	1	1
GEKA	Germany	Pfalz	UPR	NA	NA	1	1
GELD ^c	Germany	Lüchow-Dannenberg	UM	10.8833	53.2667	3	3
GRIR ^d	Greece	Iraklion	UM	24.8062	35.3878	1	1
GRTH ^d	Greece	Thessaloniki	UM	23.7340	40.7523	1	1
ITBO ^e	Italy	Bolzano	UG	11.1167	46.6167	1	1
ITCA ^e	Italy	Catanzaro	UM	16.7500	38.8833	1	1
ITFV ^e	Italy	Fiume Veneto	UM	12.6833	45.9167	1	1
ITLA ^e	Italy	Latina	UM	13.0000	41.4667	1	1
ITMO ^e	Italy	Monfalcone	UM	13.5333	45.8000	1	1
ITNI ^e	Italy	Nimis	UM	13.2500	46.2167	1	1
ITSE ^e	Italy	Sesto Al Reghena	UM	12.7833	45.8667	1	1
ITTA ^e	Italy	Tamai	UM	12.5667	45.9333	2	2

ITTR ^e	Italy	Trieste	UM	13.7000	45.7167	1	1
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Samples from the RESGEN collection provided by the following institutes:

^a Research Institute for Nature and Forest (INBO), Belgium

^b Institut national de recherche en sciences et technologies pour l'environnement et l'agriculture (Irstea), France

^c Niedersächsische Forstliche Versuchsanstalt (NFV), Germany

^d National Agricultural Research Foundation (NAGREF), Greece

^e Consiglio Nazionale delle Ricerche (CNR), Italy

Table 2 List of sampled elm cultivars included in the study (i.e. dataset 3).

Cultivars	Parents/ species
Lobel (3 ramets)	clone 202 (<i>U. glabra</i> 'Exoniensis' x <i>U. wallichiana</i>) x clone 336 ('Bea Schwarz' ^a , selfed)
Clusius	clone 202 (<i>U. glabra</i> 'Exoniensis' x <i>U. wallichiana</i>) x clone 336 ('Bea Schwarz' ^a , selfed)
Sapporo Autumn Gold	<i>U. pumila</i> x <i>U. japonica</i>
73P	<i>U. pumila</i> (mother tree of 'Sapporo Autumn Gold') x ? (open pollinated)
2P	<i>U. japonica</i>
Klemmer	<i>U. x hollandica</i> or <i>U. minor</i>
Dodoens	Selfed seedling of clone 202 (<i>U. glabra</i> 'Exoniensis' x <i>U. wallichiana</i>)
Groeneveld	clone 49 (<i>U. glabra</i> or <i>U. x hollandica</i>) x clone 1 (<i>U. minor</i>)
Commelin	<i>U. x hollandica</i> 'Vegeta' x clone 1 (<i>U. minor</i>)
Plantyn (2 ramets)	Clone 202 (<i>U. glabra</i> 'Exoniensis' x <i>U. wallichiana</i>) x clone 302 (<i>U. minor</i> '1' x <i>U. minor</i> '28')
Christine Buisman	<i>U. minor</i>
Vegeta	<i>U. x hollandica</i>
Major	<i>U. x hollandica</i>
Belgica	<i>U. x hollandica</i>
Horizontalis	<i>U. glabra</i>
Dampieri	<i>U. x hollandica</i> or <i>U. minor</i>
Den Haag	<i>U. pumila</i> x <i>U. x hollandica</i> 'Belgica'
Columella	Probably selfed seedling of Plantyn
Sarniensis	<i>U. minor</i>

^a 'Bea Schwarz' is an *U. minor* or *U. x hollandica*.

Table 3 Information on the variation of the AFLP loci. N: number of samples; NPL: number of polymorphic loci; BF: mean band frequency; PIC: mean polymorphic information content; NPL_{0.05-0.95}: number of polymorphic loci with a band frequency between 0.05 and 0.95; PIC_{0.05-0.95}: mean polymorphic information content of loci with a band frequency between 0.05 and 0.95.

Primer combinations	N ^a	NPL	BF (st. dev.)	PIC (st. dev.)	NPL _{0.05-0.95}	PIC _{0.05-0.95} (st. dev.)
EcoRI-AGC/MseI-CTG	174	169	0.35 (0.35)	0.21 (0.17)	110	0.30 (0.14)
EcoRI-ACC/MseI-CTG	174	216	0.26 (0.35)	0.15 (0.15)	100	0.27 (0.14)
Species group	N ^a	NPL	BF (st. dev.)	PIC (st. dev.)	NPL _{0.05-0.95}	PIC _{0.05-0.95} (st. dev.)
<i>Ulmus minor</i>	77	314	0.31 (0.36)	0.17 (0.14)	187	0.26 (0.13)
<i>Ulmus glabra</i>	34	222	0.35 (0.35)	0.22 (0.15)	163	0.27 (0.14)
Hybrids <i>U. minor</i> – <i>U. glabra</i>	53	249	0.35 (0.36)	0.20 (0.16)	145	0.30 (0.13)
remaining samples	10	248	0.39 (0.27)	0.33 (0.12)	248	0.33 (0.12)

^a Excl. duplicate ramets of Lobel and Plantyn.





