Title: Genetic diversity and structure of endangered native yew *Taxus baccata* in remnant populations in Belgium

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ABSTRACT

Background – The European yew *Taxus baccata* L. is a conifer tree species for which the autochthonous populations have become rare and endangered in many European countries after a long history of human overexploitation. The general objective of this study was to perform a population genetic analysis of rediscovered and putative autochthonous relict populations of *T. baccata* in Belgium.

Material and methods – We genotyped 223 individuals from eight relict populations in Wallonia, using seven microsatellite loci. The retrieved genetic data was used to assess the allelic richness without and with correction for the population size (A and A_C), the observed and expected heterozygosity (H_O and H_E), level of inbreeding (F_{IS}), and pairwise genetic differentiation among populations (F_{ST}). The spatial genetic structure within populations (SGS) was quantified using kinship coefficients (F_{ij}). Principal coordinates analyses (PCoA) and a Bayesian clustering analysis were performed to assess the relatedness among populations.

Results – We found high levels of genetic diversity within the relict populations, but also high levels of inbreeding. Furthermore, the results indicated moderate to high levels of isolation and limited gene flow among populations. The Bayesian clustering analysis indicated the presence of four distinct genetic clusters, showing only a weak relatedness among most of the larger relict populations. These results can be explained by long-term fragmentation and isolation of these likely autochthonous populations, possibly due to historical exploitation and interference.

Conclusion – The loss of genetic diversity through inbreeding and limited gene flow among populations may currently compromise the long-term survival of wild European yew in Belgium. The high levels of inbreeding could be addressed, for example, by assisted exchange of germplasm between these populations.

KEYWORDS (up to 10 keywords, ordered alphabetically)

Autochthony, European yew, inbreeding, long-term isolation, native species, population genetics, remnant populations

1. INTRODUCTION

Trees form the foundation of forest ecosystems which provide vital ecosystem services, including carbon sequestration (Rivers et al. 2019), regulation of rainfall patterns (Ellison et al. 2017), and provision of materials and medicines (de Vries et al. 2015; Messier et al. 2022). However, tree species often function as keystone species in other habitats as well; supporting biodiversity, ecosystems, and their services (Rivers et al. 2019). Tree species diversity in forests has been recognized as an important driver of healthy forest ecosystems, enhancing forest ecosystem functioning and the provisioning of ecosystem services. Furthermore, tree diversity also promotes forest resilience against environmental and climatic changes (Messier et al. 2022). Yet, the conservation status of many European forest tree species is worrying (Flensted et al. 2016). According to the latest report from the IUCN on the European Red List of Trees, between 37.1% and 49.6% of the tree species that are native to Europe are considered to be threatened (Rivers et al. 2019).

One European tree species with a long history of decline is the European yew (*Taxus baccata* L., hereafter "European yew" or "yew") (Dubreuil et al. 2008). This evergreen conifer tree species is characterized by its longevity and shade tolerance (Thomas and Polwart 2003). Despite the presence of toxins in most parts of the yew, several animal species feed on the yew, especially during winter (Mysterud and Østbye 1995; Thomas and Polwart 2003). Furthermore, the European yew is an important source of the anti-cancer drug taxol (a secondary metabolite of *T. baccata* that inhibits cell proliferation; Malik et al. 2011) and can be considered as cultural heritage, given its symbolism in both the early European heathen religions as well as Christianity, and its long history of usage in medicine and crafting (Thomas and Polwart 2003).

A wide variety of causes have been attributed to the general decline of *T. baccata* throughout Europe, a decline that started from the late Sub-boreal onwards (around 3,500 BP). One of the causes that has been put forward, is climatic change that occurred during this period, resulting in a generally warmer climate (Thomas and Polwart 2003; Deforce and Bastiaens 2007; Uzquiano et al. 2015). This may have been especially important in the Mediterranean region, where it resulted in increasingly smaller relict populations currently confined to the cooler, moister Mediterranean mountains (Thomas and Polwart 2003; De Beaulieu et al. 2006; Uzquiano et al. 2015). The changing climate also resulted in a secondary effect, the colonisation of European forests by European beech (*Fagus sylvatica* L.*)*. Several authors have stated that due to the rapid growth and colonisation of beech, the natural regeneration of yew decreased, as the slow-growing yew seedlings that require light for survival could not keep up with the fast-growing beech and thus became overshadowed (Thomas and Polwart 2003; Deforce and Bastiaens 2007; Uzquiano et al. 2015). However, other studies have shown that European yew can grow relatively well under a canopy of deciduous trees, suggesting that the climatic change and vegetation changes were not the only reasons of the decline of the yew throughout temperate Europe (Deforce and Bastiaens 2007).

A third, and likely more important factor explaining the decline of the European yew is human exploitation. Because of its durability and elasticity, yew wood has been used for the construction of bows and other tools ever since Prehistoric times (Voliotis 1986; Thomas and Polwart 2003; Deforce and Bastiaens 2007; Uzquiano et al. 2015). However, the impact of humans on the occurrence of yew became more substantial during the Middle Ages, when European yew was used for the production of longbows, and massively traded with England.

This impact was further enhanced through the demand for yew wood for construction and the manufacturing of other tools, and through the felling of yew trees to prevent cattle poisoning (Thomas and Polwart 2003; Deforce and Bastiaens 2007). All this ultimately led to the overexploitation of *T. baccata* and the decline of its population sizes and range (Voliotis 1986; Thomas and Polwart 2003; Deforce and Bastiaens 2007; Uzquiano et al. 2015).

T. baccata is one of only three native coniferous tree species that occur in Belgium (the other two being common juniper *Juniperus communis* and Scots pine *Pinus sylvestris*). However, before the locations of the putative relict populations were discovered for this study, hardly any knowledge was present regarding the occurrence of wild populations of the European yew in Belgium (Lejeune and Courtois 1836; Duvigneaud 1965; Saintenoy-Simon 2006; Deforce and Bastiaens 2007). During this research, larger natural populations of the European yew were found in Wallonia, where the species is considered endangered and protected (Deforce and Bastiaens 2007; OFFH 2021). Given the discussed long history of human use of the yew in Europe, the fossil remains of yew found in Belgium (Deforce and Bastiaens 2007), and the occurrence of very old individuals at certain locations, like castle parks, *Taxus baccata* can be considered a native species in Belgium. Interestingly, the effects of the historical overexploitation of the European yew are still visible today within the Belgian populations, which are mainly confined to the steep slopes of river valleys, like those of the rivers Sambre and Meuse (pers. obs.). These slopes likely served as refuge, as they were too steep for wood harvesting, cattle grazing or any other type of agricultural activity, and the trees thus were spared from cutting. Similar mechanisms of long-term survival of indigenous tree species in refugia have been observed elsewhere too, for instance in church forests and other sacred groves (Aerts et al. 2016) and in high mountain forest patches (Thijs et al. 2015).

The general objectives of this study were to get insight in the genetic diversity and composition of rediscovered remnant populations of *Taxus baccata* L. in Belgium, and to formulate recommendations for conservation and management.

The following specific research questions were formulated:

- What is the degree of natural regeneration within the relict populations of *Taxus baccata* L.?
- What is the level of genetic diversity present within the relict populations and are there indications of inbreeding?
- Is there spatial genetic structure within and between these populations, and does it concur with the assumption of autochthony?
- Is there genetic similarity between the natural populations and yew trees occurring in castle parks, and what does this imply regarding the origin of the planted yew trees?

2. MATERIALS AND METHODS

2.1. Study species

Taxus baccata L. is an extremely long-lived and shade-tolerant evergreen conifer; with several known adult trees growing over 1,000 years old (Thomas and Polwart 2003). Marked by a slow growth, sexual maturity is generally reached after about 70 years. Yew is dioecious, although on rare occasions, monoecious individuals can occur. Adult individuals can reproduce vegetatively; when their branches reach the soil, they can develop adventitious roots, which are able to survive even if the main tree dies off (Thomas and Polwart 2003). Pollination occurs through anemophily, and the seeds are mainly dispersed by birds, but some mammals also consume its fruit and can act as dispersers (Thomas and Polwart 2003; Farris and Filigheddu 2008). However, the number of mammal species feeding on the fruits of *T.*

baccata is relatively limited, as the seeds contain a poisonous combination of alkaloids known as taxins (Thomas and Polwart 2003). Taxins also occur in other parts of the tree and several cases of fatal poisoning in humans have been reported (e.g. Grobosch et al. 2013; Brooks-Lim et al. 2022); nevertheless, a variety of herbivores are known to feed on any part of the tree (Thomas and Polwart 2003; Farris and Filigheddu 2008). This is possible as the yew is not equally poisonous to all mammals (Thomas and Polwart 2003) and may even be only toxic to adult animals that have never browsed on yew before (Farris and Filigheddu 2008; Uzquiano et al. 2015).

T. baccata grows best in moist, mild temperate climatic conditions, and is restricted by severe cold and frost, drought and high temperatures (Voliotis 1986). This is reflected in its distribution throughout Europe: the borders of the natural distribution of European yew occur in Norway, Sweden, and the Baltic States (limitation by cold temperatures and frost); in Turkey and the Caucasus Mountains (dry continental climate); and in North Africa (high temperatures) (Thomas and Polwart 2003; Benham et al. 2016). Its altitudinal range, on the other hand, spans from the European plains in the north to heights up to 2,500 metres in the south, with an average altitude of 1,500 metres. The slope gradient can vary widely from moderate to very steep, and the yew can even be found growing on cliffs (Thomas and Polwart 2003). As for the substratum, European yew generally occurs on humus-rich neutral to alkaline soils and limestones (Thomas and Polwart 2003; Benham et al. 2016). However, yew individuals can grow on nearly any soil, including sandy soils and silicate rocks (Voliotis 1986; Thomas and Polwart 2003).

A recent comprehensive genetic study on all *Taxus* spp. has shed light on the origin and historical diversification of the genus *Taxus*, tracing down the origin to North-America during the Late Cretaceous, followed by several intra- and intercontinental migrations, as well as multiple hybridisation events, especially in south-eastern Asia (Möller et al. 2020). The

European yew, *Taxus baccata*, is thought to have colonised Europe from east to west (Mayol et al. 2015). During the Quaternary period, two genetically distinct clusters emerged: a western and an eastern cluster. These two clusters may have found their origin in the retreat of *T. baccata* in two allopatric refugia in Europe during glacial periods, and may have further differentiated through adaptation to the locally occurring environments and climate during interglacial periods (especially differences in temperature regimes) (Mayol et al. 2015).

2.2. Study area

For this study, eight natural populations of *Taxus baccata* L. were sampled, all of which are thought to be autochthonous. To identify these populations, several sources were used: old floras of Belgium (Lejeune and Courtois 1836; Lawalrée 1952), scientific publications (Duvigneaud 1965; Saintenoy-Simon 2006), databases (Van Rompaey and Delvosalle 1979; observations.be 2021), and targeted expeditions in ancient woodlands on steep calcareous slopes. Based on these sources, populations were found at the following localities: Marche-les-Dames, Ben-Ahin, Lompret, Jamioulx, Barbençon, Devant-Bouvignes, Yvoir and Trooz (Fig. 1). These locations were situated within three agro-ecological regions: Condroz, Fagne-Famenne, and Pays de Herve. All populations occurred between 80 m and 400 m above sea level, and were mostly found on very steep slopes, often difficult to access. Furthermore, the relict populations all occurred on loamy soils with rocky substrates, either limestone, schist or sandstone. The naturally occurring forests were mostly remnants of ancient broadleaved and mixed forests that have been continuously present since the topographic map of the Austrian Netherlands dating from 1770-1780 (Alderweireld et al. 2015; Koninklijke Bibiliotheek van België (KBR) 2022).

A plant species is considered to be autochthonous when it has been present in a region since the last Ice Age, after establishing there spontaneously, thus without any human interference. Such species have always regenerated naturally, or have been artificially expanded using only local material (Maes 2002). All sampled populations met at least some of the requirements to be considered as an autochthonous population. More specifically, they occur on soil types and in habitat types in which yew naturally grows, and inside natural areas. Some of the larger populations contained old individuals that likely have been around for over a century (pers. obs.). These old specimens have likely survived in these locations because of the steep slopes on which they grow (pers. obs.), rendering those locations inaccessible for harvesting or browsing by domestic animals. Another argument for the autochthony of at least some of the relict populations is their location in ancient forest, defined as forests continuously present since the maps of De Ferraris (which is the case for the populations in Lompret, Yvoir, Marche-les-Dames, Ben-Ahin, and Trooz; this could not be verified for the population in Barbençon) (SPW 2020; KBR 2022).

Apart from the natural populations, we sampled 14 yew trees from two castle parks (Arenberg and Hamme-Mille) to serve as an outgroup, and to investigate whether genetic similarities occur between park populations and the relict populations (Fig. 1).

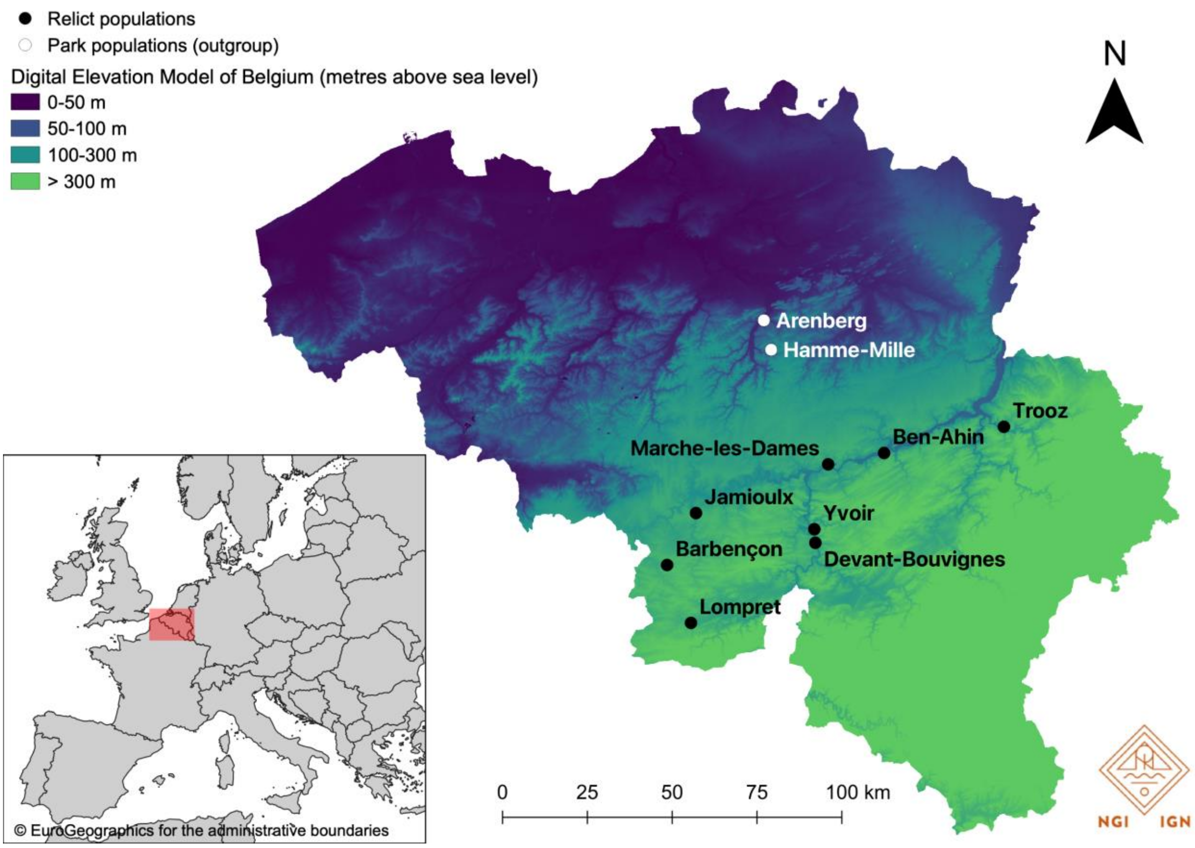

Figure 1. Digital Elevation Model of Belgium showing the locations of the sampled natural relict populations all located in the provenance area "lower Meuse Plateau" (black dots) and the two additional populations in castle parks (white dots) in the provenance area "north of Sambre and Meuse". The inset map shows the location of Belgium in Europe.

2.3. Sampling method and laboratory protocol

In all populations, a minimum of 25 randomly selected individuals were sampled, with the exception of the populations in Jamioulx, Yvoir, and Devant-Bouvignes, as they contained fewer individuals (Appendix A, table A1). Population sizes were approximated by the area of the sampling sites, instead of the number of individuals. These areas were computed in QGIS v3.6 for MacOS (QGIS.org 2022). Young leaves were removed from each tree and stored in labelled paper bags. For each sampled tree, the circumference was measured at breast height with a measuring tape. When multiple trunks occurred, the circumference was taken of either the thickest trunk or right under the onset of the branching of the trunk (when present). Sex was visually determined when possible. Individuals with a circumference smaller than eight centimetres were regarded as seedlings, those with a circumference between 8 and 15 centimetres were considered saplings. All samples were immediately stored in a refrigerator at 4 °C. Total genomic DNA was extracted from 50-100 mg of fresh leaf material during the days following sampling, using a Norgen's Plant/Fungi DNA Isolation Kit (Norgen Biotek Corp., Thorold, Ontario, Canada). The eluted DNA samples were labelled and concentrations were determined for all samples using NanoDrop™ 2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA), after which the samples were stored at -20 °C until further processing.

Following the extractions, the DNA samples were amplified using PCR, for which a Biometra TAdvanced Thermocycler® (Analytik Jena GmbH+Co., Jena, Germany) was used. For this purpose, nine species-specific Simple Sequence Repeat (SSR) microsatellite primers were selected (Tax23, Tax26, Tax31, Tax36, Tax60, Tax86, Tax92, TS09 and ABRII-TB1) (Maroso et al. 2021). Multiplex PCRs were performed, which included an initial activation step of five minutes at 95 °C, during which the HotStarTaq Plus DNA Polymerase was activated; followed by a three-step cycle: first denaturation for 30 seconds at 95 °C, followed by an annealing step of 90 seconds at 57 °C, and finally an elongation step for 30 seconds at 72 °C. This cycle was repeated 30 times, after which a final elongation step took place for 30 minutes at 68 °C. The amplified fragments were then detected using a Capillary DNA Sequencer ABI3500 from Applied Biosystems® (Thermo Fisher Scientific Inc., Waltham, MA, USA). Allele scoring was performed using GeneMapper® v5.0 (Thermo Fisher Scientific Inc., Waltham, MA, USA). During the allele scoring, two loci (Tax31 and Tax60) showed high levels of non-specificity and these two loci were excluded from the data.

2.4. Data analysis

Frequency distributions of the circumferences at breast height were made. The genetic data was tested on deviation from the Hardy-Weinberg equilibrium (HWE) and the presence of null alleles in R v4.2.3 using the R-package PopGenReport v.2.0 (Adamack and Gruber 2014). To assess the genetic diversity within populations, the observed heterozygosity (H_O) and expected heterozygosity (H_E) were quantified for all sampled populations, using GenAlEx v6.5 (Peakall and Smouse 2006, 2012). A correlation was made between the area of the sampling sites, H_O, and H_E. The mean number of alleles per population (allelic richness, A) and with correction for the population size (A_C) were calculated following Helsen et al. (2015).

An Analysis of Molecular Variance (AMOVA) was used to calculate the F-statistics: the inbreeding coefficient (F_{IS}) of all populations was quantified, and the genetic diversity among populations was assessed by calculating the pairwise genetic differentiation among populations (F_{ST}) in GenAlEx. Additionally, to investigate the effect of the presence of null alleles on the results, the F_{ST} was calculated a second time, both including and excluding null alleles using FreeNA (Chapuis and Estoup 2007; Chapuis et al. 2008), following the method of Komárková et al. (2022). Isolation-by-distance was evaluated with a Mantel test with 999 permutations in GenAlEx. Finally, GenAlEx was also used to perform two principal coordinates analyses (PCoA): one for the genetic distance between all samples, and one for the average genetic distance between all populations (Aerts et al. 2013; Helsen et al. 2015).

In order to assess the spatial genetic structure within populations, SPAGeDi v1.4 was used (Hardy and Vekemans 2002). The average kinship coefficient (F_{ij}) of Loiselle et al. (1995) was calculated per distance interval. Eight distance intervals were chosen with fixed maximum distances (10 metres, 20 m, 30 m, 40 m, 50 m, 100 m, 150 m, and 200 m). The maximum distance of the final distance interval (200 m) was based on the spatial extent of the smallest population, to allow for comparison among populations (Helsen et al. 2015). Ninetyfive percent confidence intervals and the significance of the kinship coefficients were determined through the comparison of the observed F_{ij} and those obtained by performing 10,000 permutations of individuals among different locations (Helsen et al. 2015).

A Bayesian clustering analysis was then performed using STRUCTURE v3.2.1 (Pritchard et al. 2000). For this analysis, an admixture model was used with correlated alleles, implementing 10,000 burn-ins and 100,000 Markov Chain Monte Carlo (MCMC) iterations. *K* varied between one and ten, and 20 iterations were performed for every value of *K* (Aerts et al. 2013; Helsen et al. 2015). The true value of *K* (the optimal number of clusters) was then obtained using Structure Harvester (Earl and vonHoldt 2012).

3. RESULTS

3.1. Sex determination and frequency distributions of circumferences at breast height

Due to the difficulty of sexing individuals in the field, apart from fruit bearing females, no analyses were performed related to the sex ratio of the yew populations.

The frequency distributions of the circumference at breast height were relatively similar among the larger populations (Fig. 2). There was a moderate number of sampled seedlings (circumference < 8 cm, 28 samples) and saplings (circumference 8-15 cm, 24 samples) (Fig. 2). Most of the sampled individuals had a circumference lower than 100 cm (185 of 237 individuals including outgroups, 182 out of 223 individuals of the relict populations, respectively). The circumference range of 25-50 cm accounted for the largest fraction of sampled individuals (58 in total).

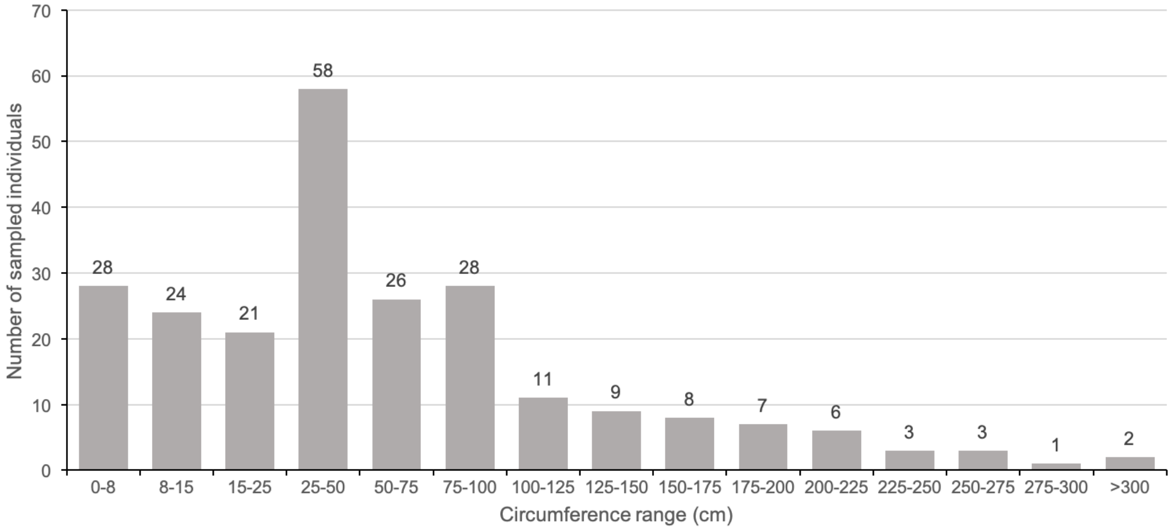

Figure 2. Frequency distribution of the circumferences at breast height (in cm), divided into ranges, of 237 randomly sampled *Taxus baccata* trees within eight relict populations (N = 223) and two castle park (N = 14) in Belgium.

3.2. Genetic diversity, differentiation and inbreeding

The deviation from the HWE varied strongly between combinations of loci and populations, with some combinations showing no or a small deviation from the equilibrium (deviation < 0.1 for most populations), and other combinations showing a (very) strong deviation (up to a deviation of 1). On the other hand, except for the locus ABRII-TB1, null alleles were present in all loci, with a frequency varying between 0.1 and 0.6 (Appendix B, figure B1). This is a commonly reported issue of microsatellite markers (Huang et al. 2016; Komárková et al. 2022). All seven loci showed polymorphism, and the number of alleles varied between 9 and 27 (mean: 19.29; SD: 5.50). The mean number of alleles per population (A) varied between 3.57 and 11.14, with an overall mean of 7.29 (SD: 3.03). The mean total allelic richness with correction for the population size (A_C) was 0.39 (SD: 0.18). For the genetic diversity within the populations, the mean observed heterozygosity (H_O) equalled 0.496 (SE: 0.038), and H_O proved to be consistently lower than the expected heterozygosity (H_E; mean: 0.676; SE:

0.022) for all populations (Table 1). No significant correlation could be found between heterozygosity and population size.

Table 1. Observed heterozygosity (H_O) and expected heterozygosity (H_E) for each relict population of the European yew in Wallonia, as well as their overall average.

Population	H_O	H_E
Lompret	0.423 ± 0.078	0.664 ± 0.056
Barbençon	0.465 ± 0.093	0.644 ± 0.041
Jamioulx	0.500 ± 0.121	0.650 ± 0.059
Yvoir	0.486 ± 0.127	0.666 ± 0.071
Devant-Bouvignes	0.524 ± 0.160	0.587 ± 0.108
Marche-les-Dames	0.548 ± 0.079	0.750 ± 0.039
Ben-Ahin	0.500 ± 0.101	0.709 ± 0.068
Trooz	0.519 ± 0.120	0.741 ± 0.020
Overall average	0.496 ± 0.038	0.676 ± 0.022

The AMOVA indicated that the mean inbreeding coefficient (F_{IS}) was 0.358 and the pairwise genetic differentiation among populations (F_{ST}) was moderate to high (0.163). The total genetic variation could mostly be explained by the genetic variation within individuals (54%), followed by that among individuals (30%) and among populations (16%). All variables were significant ($p < 0.001$). To explore the effects of the null alleles on the robustness of our results, the F_{ST} values were recalculated with FreeNA, which showed limited divergence between the results with and without correction for null alleles (0.176 vs. 0.143 averaged across all loci for all populations) (Fig. 3), suggesting that the presence of null alleles only has a small effect on genetic differentiation.

Table 2. Genetic differentiation (F_{ST}) between all relict populations of the European yew in Wallonia, with and without correction for the presence of null alleles, computed with FreeNA.

F_{ST} without correction for null alleles

Population	Lompret	Marche-les-Dames	Ben Ahin	Trooz	Barbençon	Jamioulx	Yvoir
Marche-les-Dames	0.165						
Ben Ahin	0.180	0.137					
Trooz	0.220	0.139	0.182				
Barbençon	0.221	0.144	0.192	0.243			
Jamioulx	0.178	0.084	0.082	0.169	0.126		
Yvoir	0.177	0.068	0.154	0.181	0.070	0.068	
Devant-Bouvignes	0.205	0.090	0.120	0.174	0.170	0.055	0.055

F_{ST} with correction for null alleles

Population	Lompret	Marche-les-Dames	Ben Ahin	Trooz	Barbençon	Jamioulx	Yvoir
Marche-les-Dames	0.125						
Ben Ahin	0.144	0.099					
Trooz	0.189	0.115	0.146				
Barbençon	0.184	0.114	0.146	0.205			
Jamioulx	0.172	0.068	0.075	0.156	0.100		
Yvoir	0.139	0.060	0.105	0.154	0.065	0.059	
Devant-Bouvignes	0.173	0.066	0.080	0.141	0.143	0.063	0.044

Figure 3 shows the principal coordinates analysis (PCoA) for the genetic distance between all samples. In general, the populations seem to show a gradient in relatedness that more or less matches the geographical west-east gradient of the populations. Only the Lompret population shows a relatively strong genetic differentiation from the other populations. A similar gradient in relatedness can be noticed on the map showing the genetic relatedness among populations (Appendix C, figure C1). Finally, the Mantel test showed a small but significant positive correlation between the genetic and geographic distances of the samples (R = 0.363, P-value: 0.001).

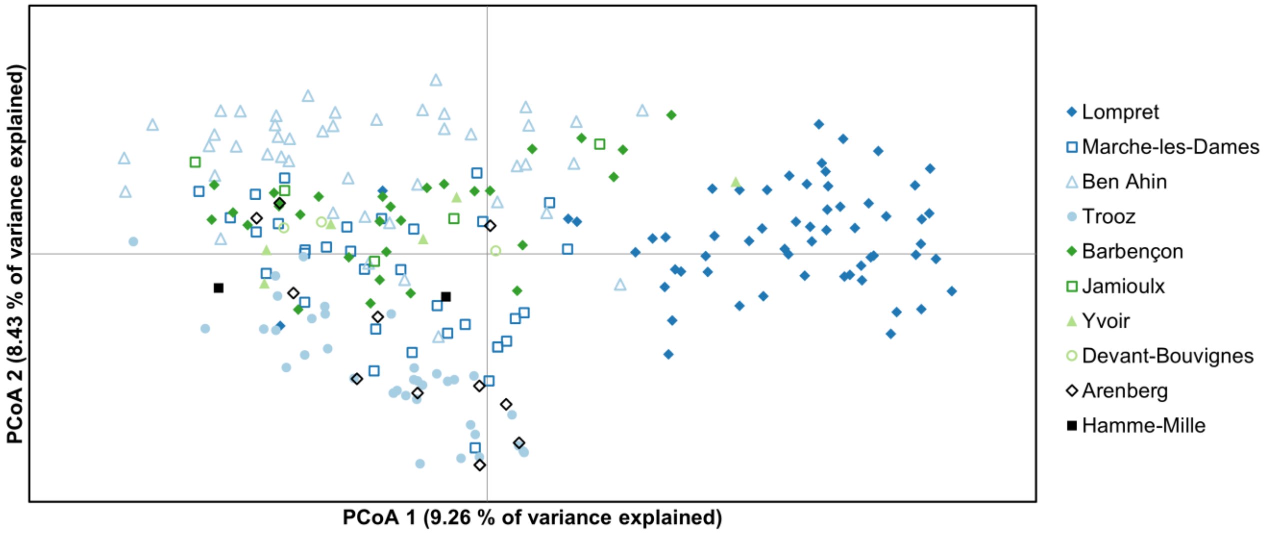

Figure 3. Principal coordinates analysis based on the genetic distances between all sampled European yew trees within the eight Walloon relict populations, as well as the two outgroups. The outgroups are indicated in black.

3.3. Bayesian clustering analysis and spatial genetic structure

The admixture model in STRUCTURE indicated the presence of four genetic clusters (*K* = 4) (Fig. 4; Appendix D, figure D1). There was again a strong genetic distinction between the population in Lompret and the other populations. The population in Ben-Ahin also appeared to form a second genetic cluster on its own. Furthermore, the populations of Trooz and Arenberg showed a similar genetic composition, which seemed to occur in the PCoA as well. The population in Marche-les-Dames, on the other hand, showed the strongest intermixture of the four genetic clusters.

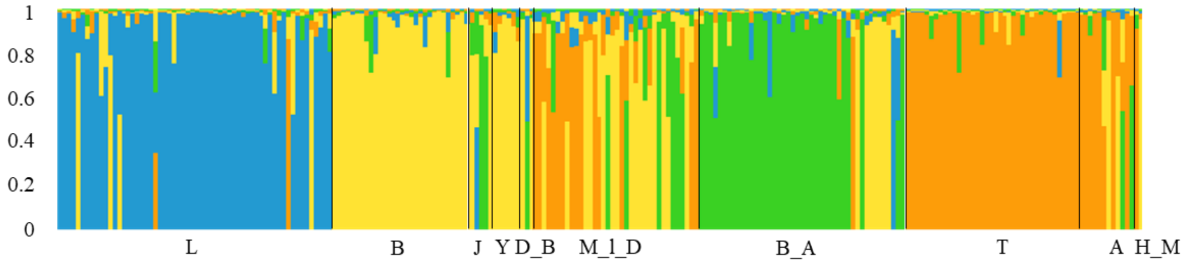

Figure 4. Results of the Bayesian clustering analysis for all sampled European yew populations in Belgium. The remnant populations are arranged according to their geographical locations from west to east. Four distinct genetic clusters are shown. Each column represents an individual; the colours show which average proportion of their genome is assigned to the different clusters. The populations are delineated by thin black lines; the names of the populations are represented by the first letter of the location where they occur (L: Lompret; B: Barbençon; J: Jamioulx; Y: Yvoir; D_B: Devant-Bouvignes; M_l_D: Marche-les-Dames; B_A: Ben-Ahin; T: Trooz; A: Arenberg; H_M: Hamme-Mille).

The P-values of the spatial autocorrelation analyses of the different distance intervals across populations varied widely (P-values between 0 and 0.995); indicating that the spatial genetic structures were not significant for any of the populations. The spatial genetic structure of one relict population (Devant-Bouvignes) was omitted because all samples occurred within the same distance interval smaller than 200 m (Appendix E, figure E1). Because the kinship coefficients remained positive within the selected geographical distance of 200 m within all populations, the spatial extent of the spatial genetic structure could not be quantified. However, in the Lompret population, F_{ij} had a small negative value for the distance interval of 10 m, and the highest value of the kinship coefficient was reached only at the distance interval of 40 m. The average numbers of pairs of individuals for each distance class are given in Appendix F, table F1.

4. DISCUSSION

4.1. Age distribution within populations

When using the circumference at breast height as a proxy for the age distribution within the populations, it becomes clear that the majority of the sampled individuals are relatively young.

Recruitment levels seem to vary strongly between populations. In the populations of Jamioulx, Yvoir, and Devant-Bouvignes, very few older trees could be found, all of them having a circumference smaller than 100 cm. These trees are likely descendants from either nearby planted individuals, or have established there through zoochory from either planted or wild parental trees that are located further away. Conversely, the larger relict populations show high variation in recruitment levels. In the literature, the most often discussed variables thought to affect regeneration and recruitment in European yew populations are light availability, the presence of *Fagus* sp., and herbivory (Svenning and Magård 1999; Klumpp and Dhar 2011; Litkowiec et al. 2018; Jensen and Svenning 2021). During our fieldwork, seedlings and saplings were observed to grow under and near beech trees, and under closed canopy. Indications of herbivory were also relatively scarce in most populations. Only in one population, multiple clear indications of herbivory by roe deer were present. As has been discussed earlier, all populations grow on similar types of soil; therefore, this factor is also unlikely to be the cause of the variation in recruitment.

4.2. Population genetic variation

The Belgian populations of European yew seem to be characterized by an overall high genetic diversity. Similar results were found by, for example, Litkowiec et al. (2018) for the populations of *Taxus baccata* in Poland, by Gargiulo et al. (2019) for the yew populations in Britain, as well as by Dubreuil et al. (2010) for the *T. baccata* populations that occur in the

Montseny Mountains in north-eastern Spain. This is in line with the idea that high levels of genetic diversity are typical for temperate tree species that are anemophilous and dioecious with obligatory outcrossing (Litkowiec et al. 2018; Gargiulo et al. 2019). It has been assumed that plant species with such life history traits are less affected by habitat fragmentation and small population sizes, in terms of loss of genetic diversity (Vranckx et al. 2011). However, the meta-analysis conducted by Vranckx et al. (2011) has shown that woody species can suffer just as much from habitat fragmentation as herbaceous plants, and that wind-pollinated trees can be affected by limited gene flow due to fragmentation, resulting in a decline in expected heterozygosity. However, the mean value of H_E found in this study is quite high. This deviation from the findings of Vranckx et al. (2011) may be associated with the obligatory outcrossing of yew on the one hand, and its longevity on the other hand, which could be the cause of a time lag between habitat fragmentation and loss of genetic diversity within populations (Fuller and Doyle 2018). This time lag may represent an extinction debt, and may conceal the actual loss of genetic variation that is currently present within the relict populations (Vellend et al. 2006; Vranckx et al. 2011).

We observed high levels of inbreeding possibly caused by the occurrence of historical genetic bottlenecks (Dubreuil et al. 2010; Litkowiec et al. 2018; Gargiulo et al. 2019). Similar results were found in other studies on the genetic composition of *T. baccata* populations in other European countries, for example by Dubreuil et al. (2010) in Spain, Klumpp and Dhar (2011) in Austria, Litkowiec et al. (2018) in Poland, and Gargiulo et al. (2019) in Britain. The overexploitation of the European yew likely has been an important driver, resulting in small, isolated populations which consisted of only a few individuals, and which were able to survive only at inaccessible places like steep valley slopes (Voliotis 1986; Thomas and Polwart 2003; DeLong and Prange 2006; Deforce and Bastiaens 2007).

Secondly, the small population sizes may have resulted in an imbalance in sex ratio within the populations. Given that the European yew is an obligatory out-crossing species, sex ratios are an important determinant for reproduction (Klumpp and Dhar 2011; Rosche et al. 2018). However, in populations that are characterized by small population sizes, there is a relatively high probability that the sex ratio is either female- or male-biased, resulting in decreased effective population sizes (Rosche et al. 2018). Consequently, this results in a limited number of potential mates within a population, which can lead to diminished sexual reproduction (Rosche et al. 2018).

The AMOVA indicated a moderate to high level of population isolation and limited gene flow between them (F_{ST}: 0.163). Significant spatial isolation of the sampled populations was also indicated by the results of the Mantel test. Very similar results have been reported for the yew populations in Poland (Litkowiec et al. 2018). The populations of *T. baccata* in the Montseny Mountains in Spain, on the other hand, showed very high levels of genetic differentiation among populations and therefore a strong population isolation (Dubreuil et al. 2010), while conversely, the yew populations in Britain as well as those in the Austrian Alps showed low levels of isolation and high levels of gene flow among populations (Klumpp and Dhar 2011; Gargiulo et al. 2019). Even though wind dispersal of pollen and especially bird-mediated seed dispersal are thought to enhance gene flow between populations, small population sizes and strong fragmentation can counteract these mitigating effects (Klumpp and Dhar 2011; Vranckx et al. 2011; Litkowiec et al. 2018). Additionally, the small population sizes and strong isolation of the majority of the Belgian populations very likely limit both the number of fruits consumed by birds and the bird-mediated exchange of seeds – and thus genetic material – between populations.

4.3. Spatial genetic structure within populations

As F_{ij} remained positive over all distance intervals for almost all populations, genetic structuring within populations appears to be moderate. However, it has to be noted that most of the values for F_{ij} were high, often extremely so for the distance intervals up to 50 m. This validates the high levels of inbreeding detected within the populations, as well as the limited gene flow among populations, and can be considered as a strong indication for the presence of a genetic bottleneck (Litkowiec et al. 2018). Furthermore, it also explains the results of the Bayesian clustering analysis, showing strong genetic structuring among populations.

4.4. The relatedness and autochthony of the relict populations

The STRUCTURE analysis indicated that most of the larger relict populations of the European yew seem to act as the core of a genetic cluster, and therefore show little kinship in relation to the other larger yew populations. The map showing the genetic relatedness between the populations showed similar results, only indicating low to medium levels of relatedness among most of the larger relic populations. On the other hand, the principal coordinates analyses showed that only the genetic composition of the population in Lompret differs significantly from the other populations. All populations, except for the one in Lompret, seem to be genetically related to one another, following a geographical west-east gradient. Similar results as the ones obtained from the Bayesian clustering analysis were observed for the *Taxus baccata* populations in the Montseny Mountains in Spain, where each studied population seemed to form a genetic cluster on its own, also resulting in four genetic clusters (Dubreuil et al. 2010). As an explanation for the occurrence of four isolated genetic clusters, Dubreuil et al. (2010) discussed the historical degradation and chronic fragmentation of the forest patches covering the Montseny Mountains. A second possibility brought forward by them, is the establishment of dispersed seeds originating from planted individuals, which would, in combination with the fragmentation of the forest area, have resulted in four different genetic clusters over time (Dubreuil et al. 2010).

Accordingly, we propose two hypotheses that could explain the presence of four genetic clusters within the relict populations of *Taxus baccata* in Wallonia. The first one is based on long-term historical isolation of the larger relict populations. However, given that all populations except Lompret show at least some level of intermixture in the Bayesian clustering analysis, and that overlap is occurring among most populations in the PCoA, it suggests that the populations have not always been isolated, and are not completely isolated at present. The De Ferraris maps do show a strong forest fragmentation in Wallonia between 1770-1780 (KBR 2022). The Belgian forests were probably somewhat less fragmented during the Middle-Ages and the centuries before that, given that the forest cover in Belgium reached an all-time low in the 1800s (Alderweireld et al. 2015; McGrath et al. 2015). Nevertheless, considering that the European yew is a slow-growing and long-living understorey tree species (Thomas and Polwart 2003), it is likely that even when the Belgian forests were less fragmented, the yew populations were still quite isolated from each other by distance. Therefore, population isolation is likely a major cause of the occurrence of four genetic clusters. However, there may also be a second reason for this genetic divergence.

A second, less likely hypothesis revolves around the presence of planted individuals in castle parks, graveyards, and other historical locations that occur in the vicinity of the relict populations. The European yew has always been a popular ornamental plant species, and can often be found in historical castle parks (Turi-Farkas and Kovács 2016). Additionally, there were strong ties between the yew and the religious culture of the early European inhabitants, which also seems to have taken hold in Christianity, serving as a symbol for resurrection and immortality (Thomas and Polwart 2003; DeLong and Prange 2006; Uzquiano et al. 2015).

This is why this tree can still be found today, for example, in graveyards. Given that the specimens found in churchyards and graveyards are often very old, it is also very likely that the yew trees growing on holy ground were protected, and were not allowed to be cut (DeLong and Prange 2006) (as in the sacred groves mentioned earlier). Although we did not include trees from such locations in this study, it is possible that some of the small relict populations contain descendants from escapees from castle parks, graveyards, or other historical locations occurring in the vicinity of these populations.

However, most of the relict populations show indications of autochthony. While it can be difficult to determine whether a population is truly autochthonous given the possibility of historical introductions and plantations, Maes (2002) developed some criteria to determine whether a population is potentially autochthonous, based on taxonomic factors, forest history, and cultivation history. All sampled relict populations meet at least some of the criteria of an autochthonous population. Except for one population, all populations seem to show a gradient of relatedness, as can be seen in the PCoA and on the relatedness map. When considering the locations of the populations, the gradient seems to occur from west to east. This gradient of relatedness can be considered as a sign of autochthony of the relict populations. Additionally, the relatedness that occurs among the castle park populations and the relict populations may indicate that the originally planted individuals in the castle parks originated from autochthonous plant material. However, to further ascertain the autochthony of the populations, future research should compare the genetic composition of the Belgian yew populations with that of foreign populations. Nevertheless, given the different indications of autochthony that have been observed in the relict populations, like the gradient in relatedness, the Belgian populations of the European yew can currently very likely be considered as autochthonous.

4.5. Recommendations for conservation and restoration

A major threat to the long-term conservation of the European yew in Belgium, is the very high relatedness and high levels of inbreeding found within these populations. This could affect the resilience of the yew populations, and their ability to adapt to changing environmental conditions, endangering their long-term survival (Litkowiec et al. 2018). The best way to address this issue, is by improving the gene flow among populations. Yet, as the yew populations in Wallonia are not really located closely to one another, the establishment of forest corridors may prove difficult. Therefore, a better option may be to artificially expand the populations by planting seed mixtures from other autochthonous populations in Belgium, as they all show relatively high levels of genetic diversity. This will ensure genetic diversity within the populations, but will also weaken the distinction between the genetic clusters. However, the genetic diversity represented by the four genetic clusters could be preserved through the establishment of a seed bank before the manual exchange of genetic material among the yew populations. The divergent population of Lompret could be excluded from this operation.

Another potential threat to the conservation of the native European yew populations and their genetic composition is introgression with cultivars that contain foreign genetic material. To address this issue, special conservation areas should be created to preserve the larger relict populations *in situ*, which should ideally be combined with *ex situ* conservation, for example through the establishment of a seed bank or seed orchard derived from cuttings (EUFORGEN 2021). Currently, only one population in Belgium occurs in an area designated as a nature reserve for preservation of the European yew (Barbençon). However, this area is not listed as a genetic conservation unit for *Taxus baccata* in the database of EUFORGEN (European Forests Genetic Resources Programme) (EUFORGEN 2022). Therefore, after the creation of special conservation areas for each of the larger yew populations in Wallonia, it is recommended to recognise each of them as conservation areas for forest genetic resources, and to add them to the EUFORGEN list of genetic conservation units for *Taxus baccata*. To counteract introgression, it is recommended to replace ornamental yew trees recently planted in the vicinity of the natural populations by specimens propagated from local material. To assess whether some naturally occurring yew individuals or populations contain foreign genetic material, further research is required to determine the origin of the relict populations in Wallonia. One option for a follow-up research study could be to compare the genetic data assembled for this study with the data of other studies examining naturally occurring *T. baccata* populations in Europe, and that used the same microsatellite markers; for example, the Polish yew populations studied by Litkowiec et al. (2018). A strong genetic differentiation between the Belgian and foreign yew populations in a follow-up research study can further prove their autochthony; while a strong genetic resemblance between some of the Belgian populations and other European populations could be considered as a sign of relatedness between those populations, implying that these Belgian populations may contain foreign genetic material, and/or vice versa.

5. CONCLUSION

This study indicated that the discovered Belgian relict populations are characterized by high levels of genetic diversity, but also by high levels of inbreeding and spatial isolation. Similar results for diversity and inbreeding have been found in several other yew populations across Europe. Natural regeneration within the Belgian populations varied strongly between populations, but seemed to be moderate overall. A relatively strong genetic differentiation was found between the populations, which was confirmed by the presence of four genetic clusters. The occurrence of four genetic clusters may be caused by long-term spatial isolation of the populations and possibly by a genetic bottleneck.

Currently, the high levels of inbreeding within the populations pose a major threat to the long-term survival of the European yew in Belgium, and should therefore be addressed. An option would be to enhance the gene flow among populations to reduce inbreeding levels and promote genetic diversity within populations. This could be achieved, for example, by manually intermixing and expanding populations with genetic material stemming from the other autochthonous populations occurring in Belgium. When choosing this option, the genetic diversity of the four clusters can be conserved through the establishment of a seed bank or seed orchard.

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8. APPENDICES

Appendix A. Overview of sampled remnant populations of *Taxus baccata* in Wallonia.

Table A1. Table providing an overview of the locations of the sampled remnant populations of the European yew in Wallonia, their coordinates, the number of sampled individuals per population, the number of seedlings, saplings, and adults in each population, the area of the sampling sites, and the observed (H_O) and expected heterozygosity (H_E).

Location	**Coordinates**	**No. of sampled trees**	**No. of seedlings (circum-ference: 0-7.99 cm)**	**No. of saplings (circum-ference: 8-15 cm)**	**No. of adults (circum-ference: >15 cm)**	**Area sampling site (m^2)**	H_O	H_E
Lompret	50° 03’ 50” N – 4° 22’ 42” O	59	3	5	51	424,713.6	0.423	0.664
Marche-les-Dames	50° 29’ 10” N – 4° 57’ 36” O	37	6	2	29	190,596.9	0.548	0.750
Ben-Ahin	50° 30’ 07” N – 5° 11’ 06” O	45	6	7	32	87,391.6	0.500	0.709

Trooz	50° 34’ 43” N – 5° 40’ 23” O	38	3	1	34	209,201.1	0.519	0.741
Barbençon	50° 13’ 17” N – 4° 17’ 03” O	30	6	5	19	18,182.2	0.465	0.644
Jamioulx	50° 21’ 10” N – 4° 24’ 45” O	5	0	0	5	13,143.1	0.500	0.650
Yvoir	50° 19’ 39” N – 4° 52’ 42” O	6	3	3	0	16,423.3	0.486	0.666
Devant-Bouvignes	50° 16’ 11” N – 4° 54’ 27” O	3	1	1	1	11,001.4	0.524	0.587

Appendix B. Overview of the null allele frequencies for all loci.

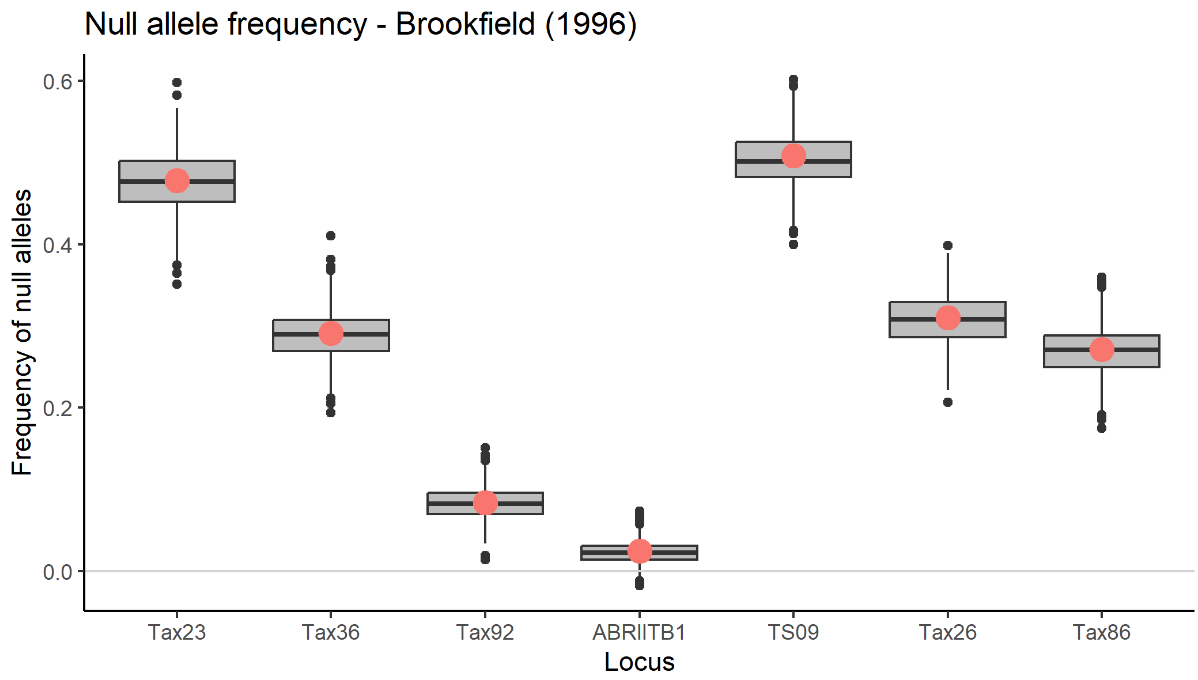


Figure B1. Graph showing the range of the null allele frequencies for each locus according to Brookfield (1996). The boxplots show the bootstrap distribution of the allele frequencies for each locus. The lower and upper edge of the boxes represent the 25th and 75th percentiles of the distribution; the solid line in the boxes represents the 50th percentile. The whiskers show 1.5 * the inter-quartile range; the solid black dots indicate outliers while red dots indicate the observed average allele frequencies.

Appendix C. Overview of the relatedness between all sampled European yew populations.

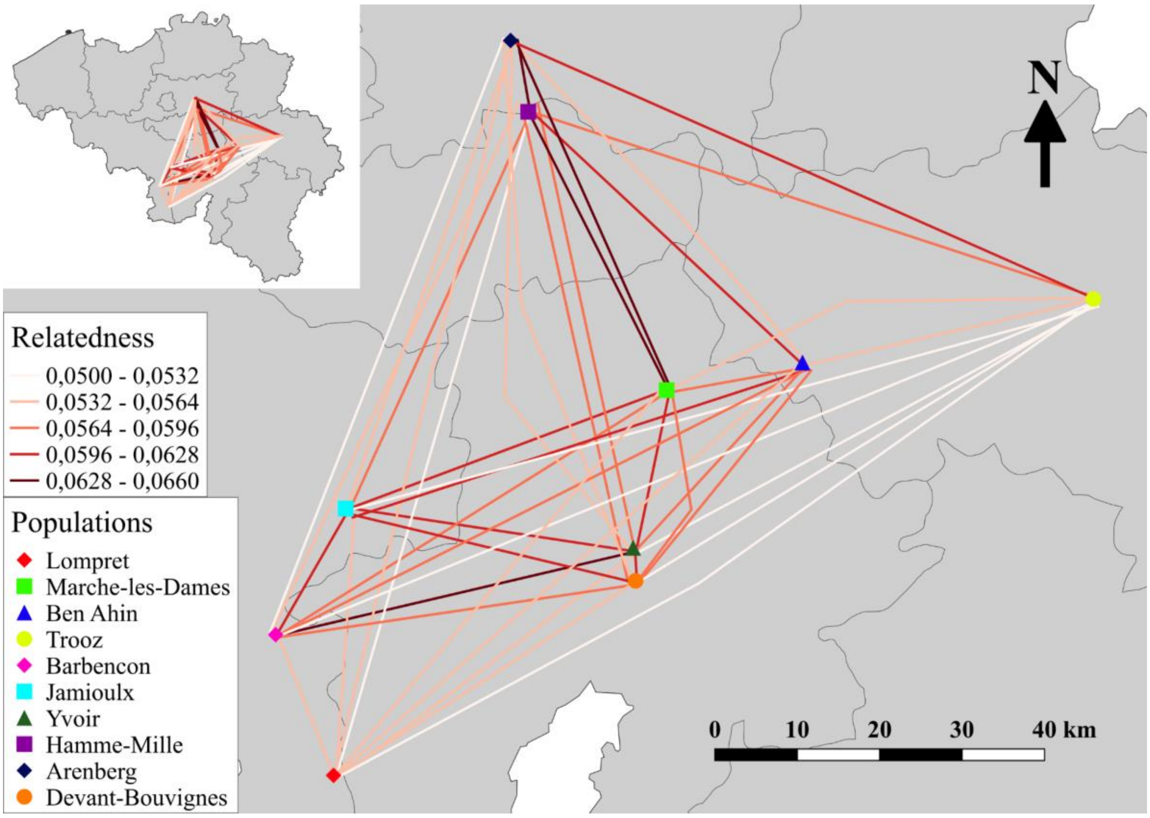


Figure C1. Map showing the genetic relatedness between all sampled populations of the European yew in Belgium.

Appendix D. Graph showing the results for the true value of *K*.

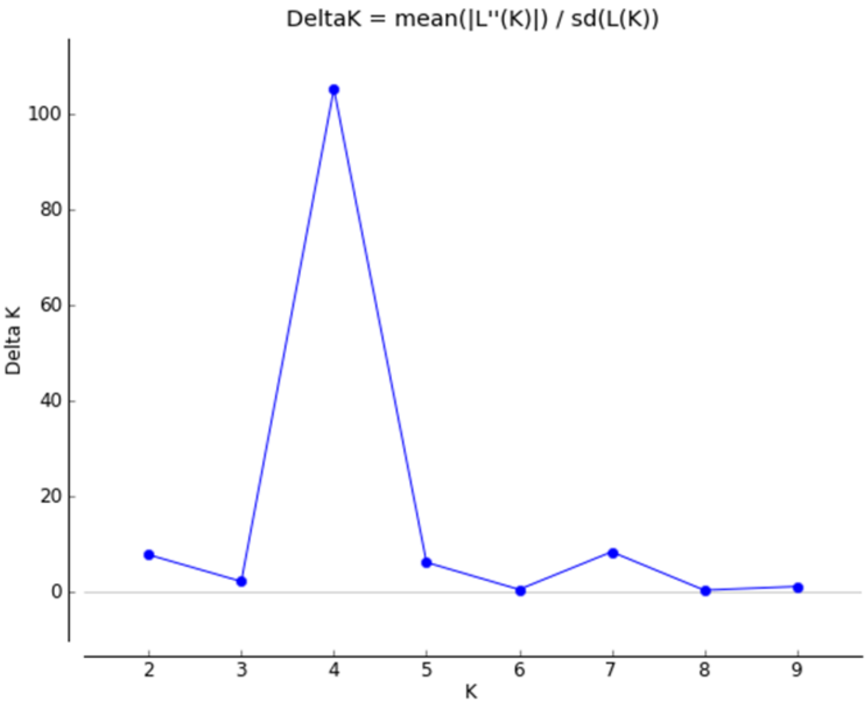
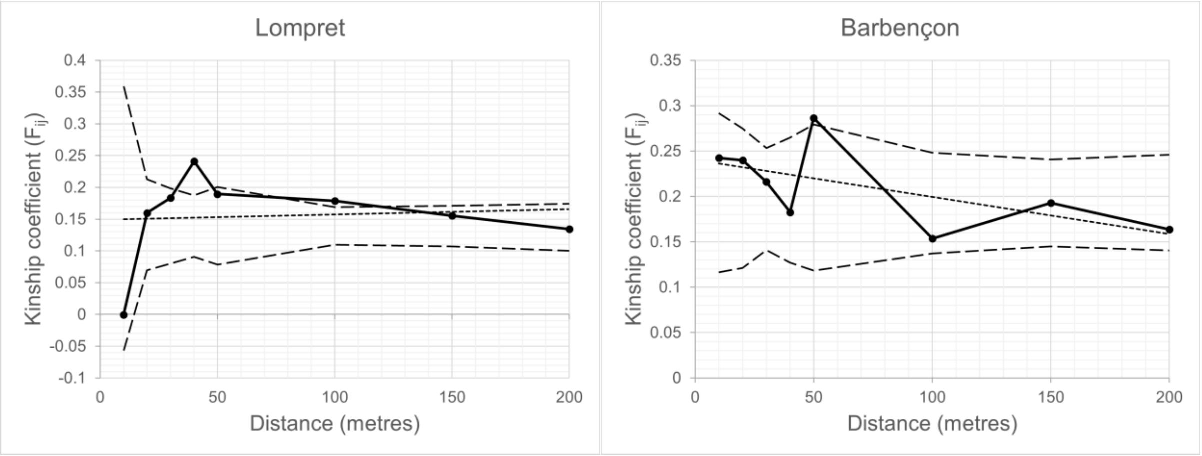


Figure D1. Graphical representation of the optimal value for *K* after performing 20 iterations for every value of *K* between 1 and 10.

Appendix E. Overview of the spatial genetic structure occurring in the sampled relict populations.



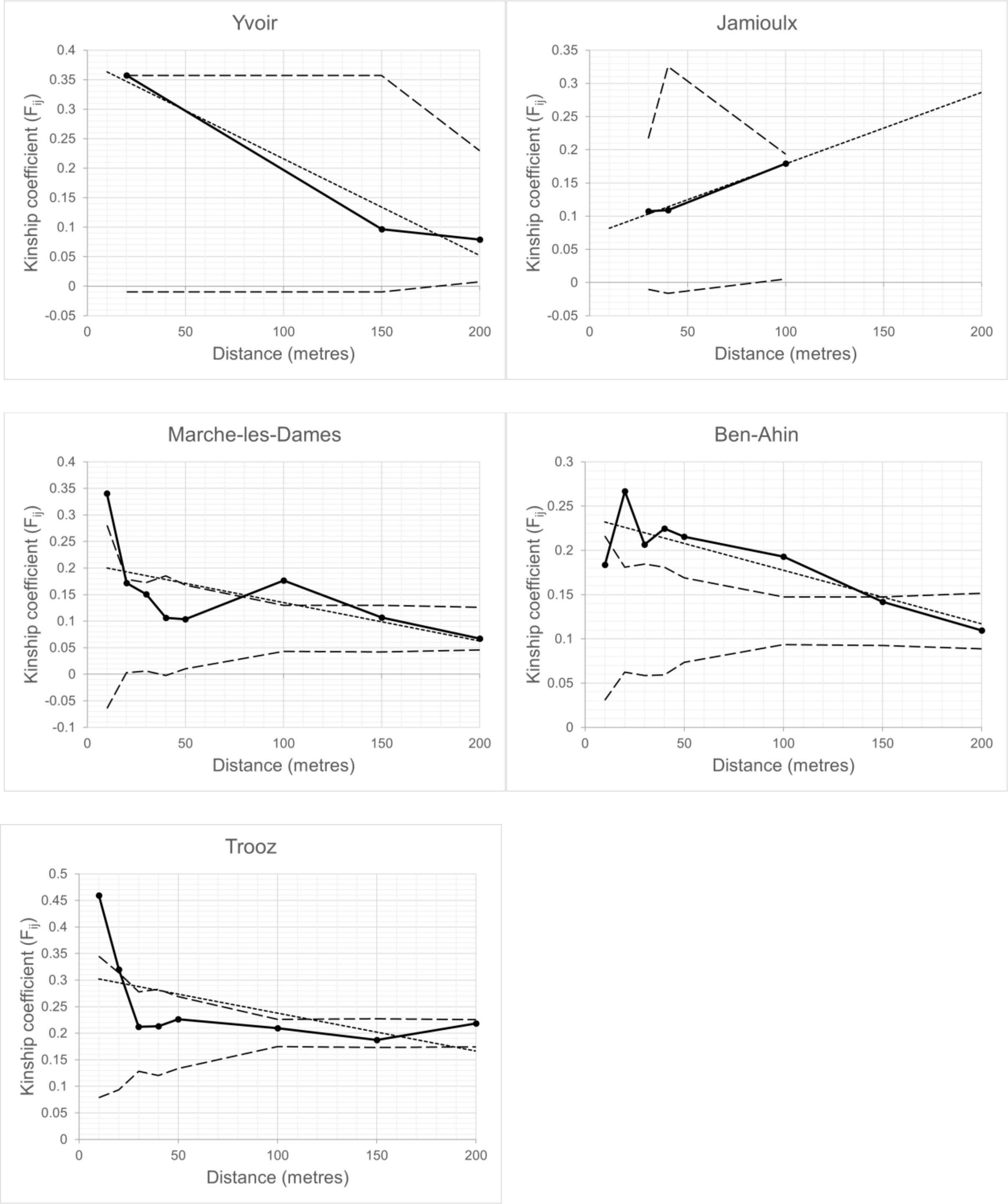


Figure E1. Graphs showing the spatial genetic structure within the relict populations of the European yew in Wallonia, represented by the change in kinship coefficient (F_{ij}) with geographic distance between individuals within the populations. The dashed lines represent the upper and lower values of the 95% confidence intervals. Trend lines (dotted black lines) are shown for correlation assessment.

Appendix F. Overview of the average numbers of pairs of individuals for each distance class in the spatial autocorrelation analyses.

Table F1. Table giving an overview of the average numbers of pairs of individuals for all distance classes and all sampled yew populations in Wallonia.

Popul.	**1 (10 m)**	**2 (20 m)**	**3 (30 m)**	**4 (40 m)**	**5 (50 m)**	**6 (100 m)**	**7 (150 m)**	**8 (200 m)**
Lompret	2	18	25	34	25	134	132	113
Marche-les-Dames	3	13	14	11	15	61	71	74
Ben-Ahin	13	31	28	39	56	216	201	147
Trooz	5	7	16	13	19	114	119	116
Barbençon	21	28	46	33	32	81	95	94
Jamioulx	0	0	2	1	0	3	0	0
Yvoir	0	1	0	0	0	0	1	3
Devant-Bouvignes	0	0	0	0	1	0	0	0