Assessment of genetic variation in black poplar in Ireland using microsatellites

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Abstract

Ireland represents the most northwesterly limit of the distribution range of black poplar (*Populus nigra* L). The objective of this study was to increase the understanding of genetic variation in the species in Ireland by using a species-specific marker and seven microsatellite markers to determine i) the accuracy with which morphological methods can distinguish between black poplar and the introduced hybrid poplar, *P. x euramericana* and ii) the degree of clonal reproduction which has occurred in the past. In addition, hairiness of the leaf petiole was assessed to determine whether the Irish black poplar trees sampled were members of the subspecies *betulifolia*.

A total of 117 black poplar trees were sampled in spring 2003 by taking distal sections of twigs, preferably with healthy flowering buds, from the crowns and standing them in water in the lab until bud burst. Samples were taken from trees believed to be black poplar from counties Dublin, Kildare, Offaly, Tipperary and Galway. The species-specific molecular marker indicated that almost a third of the sampled trees were *P. x euramericana* which had been misidentified as *P. nigra*. There was considerable clonal duplication such that only nine genotypes were present in the 80 *P. nigra* samples that were DNA tested. These results indicate that considerable vegetative propagation had occurred in the past. The majority of the black poplar belonged to the sub-species *betulifolia*.

Keywords: *Populus nigra, Populus* x *euramericana,* black poplar, species-specific genetic marker.

Introduction

Black poplar (*Populus nigra* L.) is not grown as a commercial species in Ireland; interest in its occurrence is due to its endangered status, both in Ireland and elsewhere in Europe.

The species's natural range extends from the Iberian Peninsula, across southern, southeastern, central and eastern Europe, as far as western Siberia. It also occurs in North Africa, Asia Minor and the Caucasus (Zsuffa 1974, Bialobok 1973). Its occurrence in Ireland represents the northwestern limit of its natural range, although its status remains uncertain. It was not considered native by Colgan and Scully (1889), while Elwes and Henry (1913) were also doubtful of its native status. In contrast, Hobson (1991) believed the tree to be native, on the basis of its close association with river valleys and flood plains and its local abundance in areas such

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as Kildare and Lough Derg. Many Irish botanists believe it was introduced to Ireland by Anglo-Irish settlers. (Smyth (1997) explains that as a result of a series of parliamentary acts between 1698 and 1791, many tree species, including poplar, were planted. However there is no information available on the species of poplar used.) Black poplar, in common with other members of the genus, roots readily from cuttings, which have been used for centuries to propagate the species.

Black poplar has been recorded in four botanical surveys in Ireland during the past fifteen years. The first of these, conducted by the Botanical Society of the British Isles (BSBI), concentrated on black poplar in England and Wales (Milne-Redhead 1990), but a small number of records were included from Ireland, with two from Co Kildare and one from Co Cork. Later, a survey of southern and central Ireland, carried out by Hobson (1991), located 210 trees. Hobson later extended his work (1993), so that a total of 373 trees covering a wide age range were eventually recorded. High levels of occurrence were recorded along the river Shannon, and in the headwaters of the Liffey and Barrow rivers (both located in the east of Ireland). The most recent survey was conducted in 2000 by the first author of this paper. It formed the basis for the sampling described here.

It is difficult on the basis of morphology to distinguish black poplar from its hybrid *P. x euramericana* (crosses with North American poplar species, usually *P. deltoides*). Hence, there are concerns that the surveys may have overestimated the number of black poplar by erroneously including *P. x euramericana*. However, the development of a DNA based species specific-marker by Heinze (1997), based on primers developed by Bradshaw et al. (1994), has provided a molecular method for separating *P. x euramericana* from *P. nigra*, and this has been followed in this study. The species specific marker (win3) amplifies a single band of about 265 base pairs (bp) for *P. deltoides* and one or two bands or a smear of about 165-210 bp for *P. nigra.* The *P. x euramericana* hybrid produces a banding pattern which is a combination of the two parental species (Heinze 1997).

P. nigra comprises two sub-species: *nigra* (or *typica*) and *betulifolia*. Both subsp. *nigra* and *P. x euramericana* have non-hairy petioles, whereas subsp. *betulifolia* is characterised by the presence of hairs on the petioles of young leaves. Also, *betulifolia* has burrs that grow on the trunks and lower branches of mature trees (Bialobok 1973, Elwes and Henry 1913). These do not occur in subspecies *nigra.* Furthermore, *betulifolia* has a largely western distribution; almost all black poplar which were examined from *ex-situ* genebanks of trees growing in England and Wales had the hairy petioles characteristic of this subspecies (Cottrell et al. 2002). There has been no equivalent morphological assessment of putative Irish black poplar, so it is not known to which sub-species they belong. However, the fact that Hobson (1993) observed that fifty per cent of the Irish trees do not possess stem burrs suggests that they are either subsp. *nigra*, or that they have been misidentified as *P. x euramericana.*

Molecular tools have, however, recently become available which enable the clonal identity of individual trees to be determined. Microsatellites, or simple sequence repeats are highly variable regions of DNA which provide effective

markers for the clonal identification of poplar material (Storme et al. 2004). This technique was applied to black poplar from *ex-situ* clone banks containing samples from England and Wales; only 16 genotypes were detected in the 66 trees that were analysed (Storme et al. 2004). There has therefore been a great deal of vegetative propagation of this material in the past.

This paper documents genetic variation found in Irish black poplar populations in counties Dublin, Kildare, Offaly, Tipperary and Galway. This was done by surveying the geographical distribution of the species, examining whether putative trees belonged to the species, using the species-specific DNA marker (win3), examining petiole hairiness to determine if trees belonged to the subspecies *betulifolia* and using microsatellite markers to establish the number of unique genotypes present in the populations.

Materials and methods

Survey and collection of cuttings

A survey of *P. nigra* in Ireland carried out by the principal author in the summer of 2000 verified the findings of earlier surveys of Hobson (1991, 1993). This most recent survey was restricted to Dublin, Kildare, Offaly, Tipperary and Galway. The survey used Hobson's directions to locate the trees. Additional putative black poplar found en-route were surveyed, recorded and included in the study. The grid reference location of each tree was recorded using a Trimble ProXRS Global Positioning System. This consists of an Easting and a Northing, measured in meters and logged to 5 decimal places. In addition, tree height, diameter at breast height, sex, general health condition, silvicultural/management activities and general landscape character were recorded for most of the trees visited. The geographic and attribute data for each tree were exported to Arc View 3.1, and maps of the distribution of the species were generated. In all 117 trees were recorded.

Just prior to bud burst, dormant twigs, 15-25 cm long, were collected from each tree and were placed in sealed plastic bags and dispatched to the Forest Research laboratory, at Roslin, near Edinburgh, for molecular analysis. On arrival, twigs were removed from the bags and stood in water. Buds on 105 samples eventually flushed sufficiently for a single leaf disc (0.8 cm diameter) to be taken from the twig. The leaf discs were placed in plastic microtubes and stored at –80°C. Petioles of the youngest expanding leaves were examined and their hairiness recorded. If flowers were present the sex was noted. On very tall trees it was only possible to sample epicormic twigs; these did not flower, and therefore their sex could not be determined.

DNA extraction

DNA was extracted from young leaves using QIAGEN plant DNeasy™ mini kits according to the manufacturer's instructions. The Polymerase Chain Reaction (PCR) and electrophoresis conditions for the species-specific marker were the same as those described by Heinze (1997). The seven microsatellites reported by Storme et al.

(2004) were used to provide a multilocus genotype for the 105 trees which provided leaf samples. The microsatellite locus known as PMGC014 was developed in *P. trichocarpa* and is listed in the Poplar Modular Genetics Co-operative (PMGC) database (http://www.poplar2.cfr.washington.edu/pmgc). The other six microsatellite loci were developed in *P. nigra* by van der Schoot et al. (2000) and Smulders et al. (2001). Details of the primer pair used for the species specific marker and the seven

Table 1. *Primers used and the species in which they were developed, primer sequences and the PCR conditions.*

a) Species specific marker (Heinze 1997 and Bradshaw et al. 1994)

Primer	***Species in which primer was developed***	***Forward Primer 5'>3'***	***Reverse Primer 5'>3'***	***Cycle***	***Annealing temperature °C***
Win3	*P. deltoides and P. trichocarpa*	*CCCGAAGTGTC CAGAGC*	*CCCACTCAAAT AGTCTAC*	*See Heinze (1997)*	*55*

b) Microsatellite markers used for clonal identification

Primer	***Species in which primer was developed***	***Forward Primer 5'>3'***	***Reverse Primer 5'>3'***	***Cycle***	***Annealing temperature °C***
PMGC014	*P. trichocarpa*	*TTCAGAATGTG CATGATGG*	*GTGATGATCTC ACCGTTTG*	*NP*	*50*
PMS09	*P. nigra*	*CTGCTTGCTAC CGTGGAACA*	*AAGCAATTTGG GTCTGAGTATC TG*	*LP*	*60*
PMS12	*P. nigra*	*TTTTTCGTATTC TTATCTATCC*	*CACTACTCTGA CAAAACCATC*	*NP*	*50*
PMS14	*P. nigra*	*CAGCCGCAGC CACTGAGAAAT C*	*GCCTGCTGAG AAGACTGCCTT GAC*	*LP*	*60*
PMS16	*P. nigra*	*CTCGTACTATTT CCGATGATGAC C*	*AGATTATTAGG TGGGCCAAGG ACT*	*LP*	*55*
PMS18	*P. nigra*	*CTTCACATAGG ACATAGCAGCA TC*	*CACCAGAGTC ATCACCAGTTA TTG*	*LP*	*60*
PMS20	*P. nigra*	*GTGCGCACATC TATGACTATCG*	*ATCTTGTAATT CTCCGGGGCA TCT*	*NP*	*60*

primer pairs that were used to provide a multilocus genotype for each tree in the collection are presented in Table 1 (a and b respectively).

The PCR reaction for the microsatellites was as follows: 5 µl template DNA, 2 µl x 10 PCR buffer (GibcoBRL), 2 µl of both 200 µM primers, 0.8 µl dNTP Mix (Perkin-Elmer, 10 mM), 0.4 U Taq polymerase (Gibco BRL) made up to a final volume of 20 µl using water. Amplification was carried out in a PE 9700 thermal cycler using either a short reaction time cycle (NP) or a long reaction time cycle (LP). The NP programme had an initial denaturing step of 3 minutes at 94°C, followed by 30 cycles of: 5 seconds at 94°C, 15 seconds at the annealing temperature and 60 seconds at 72°C, followed by a final elongation step of 10 minutes at 72°C. LP programmes had an initial denaturing step of 3 minutes at 94°C, then 30 cycles of; 45 seconds at 94°C, 45 seconds at the annealing temperature and 105 seconds at 72°C, followed by a final elongation step of 10 minutes at 72°C. The annealing temperature used for each microsatellite locus is listed in Table 1.

PCR products for the microsatellite analysis were denatured and resolved on 6% denaturing polyacrylamide gels using a Licor DNA sequencer. Gels were scored with the aid of standard ladders.

Results

Hybrid status

Results were obtained using the species-specific marker for the 105 samples from which DNA was extracted. Twenty five samples produced the banding pattern typical of *P. x euramericana*. All the sampled trees from the townland of Pallas proved to be *P. x euramericana* with the exception of Pallas West.

In the remaining 80 samples the banding pattern was typical of *P. nigra,* 76 produced a single band and four samples (Birr Caravan, Birr Boyds, Abbey West and Ardchroney layby) produced a double band. Both these banding patterns have been described by Heinze (1997) and are typical of *P. nigra*. The reason for this double banding pattern is not understood as the fragments have not been sequenced.

Petiole hairiness

All the samples classified as *P. x euramericana* on the basis of the species specific marker, had young leaves with non-hairy petioles with the single exception of Looscaun Church North (Appendix Table 1). In five of the *P. x euramericana* samples it was not possible to assess petiole hairiness because the buds did not open sufficiently. Of the 80 trees classified as *P. nigra* on the basis of the species specific marker, 65 had hairy petioles and only four samples had no hairs on the petioles of young leaves (Ballinderry 5/4, Sawnagh Mid 6, Birr Boyds and Ardchroney layby). The leaves produced by the first two of these did not expand fully and it was difficult to be entirely certain that no hairs were present. In eleven of the *P. nigra* samples it was not possible to assess petiole hairiness.

Sex

The eleven *P. x euramericana* samples that flowered were males; the remaining 14 did not flower. Of the 41 *P. nigra* which flowered, 36 were males and only five were females. The remaining 39 *P. nigra* samples could not be sexed because they did not flower.

Microsatellites

Number of alleles per locus

In the *P. nigra* samples locus PMS14 was the most diverse, with five alleles. There were four alleles in the following four microsatellite loci: PGMC14, PMS09, PMS18 and PMS20. Locus PMS16 had three alleles and locus PMS12 had two alleles. The average number of alleles per locus was 3.7.

Number of genotypes

According to the multilocus genotype based on the seven microsatellite loci there were three distinct clones in the 25 *P. x euramericana* trees. Of these, clone 1H was only represented by a single tree; eight trees belonged to clone 2H. The remaining 16 *P. x euramericana* trees were all members of clone 3H.

The 80 *P. nigra* samples consisted of nine distinct clones. Three clones were particularly common and over 85% of the *P. nigra* trees that were tested belonged to one of these. The *P. nigra* clone 6 was the most common and was represented by 37 of the 80 samples. Eleven trees of this clone came from the Ballinderry 15 group and all eight of the trees from the Blackrock group were members of clone 6. Although there was a tendency for several trees from a single location to be of the same clone there was usually some clonal variation present at most sites. For example, the twelve trees from the Ballinderry 15 group consisted of two genotypes. None of the clones in Ballinderry 15 were unique to that location. The three trees from the Ballinderry 5 location consisted of two clones, one of which was unique to that location and one which was also present in the Ballinderry 15 site. When the two Ballinderry sites were taken as one, the clonal composition was as follows, clone 3 (3 trees), clone 4 (1 tree), clone 6 (11 trees). In contrast, the eight trees at Blackrock, which is about 150 km to the east, consisted of only a single clone; clone 6. Some locations, such as Aughameelick, and Shannon Park consisted of both *P. x euramericana* and *P. nigra* trees.

Only two *P. nigra* female clones were detected, clone 2 consisted of a single female representative from Abbey West and clone 8 which was represented by female trees from Terryglass South, Drumcooley East 4, Drumcooley East 5 and Aughameelick Mid South. Clone 8 consisted of another eight trees which, although they were not sexed, were likely, on the basis of their common genotype, to be female. Three of these unsexed trees representing clone 8 resided at Aughameelick, four at Drumcooley, and one at Kilcormack. The Terryglass female grew in the same vicinity as *P. nigra* male clone 3. The Aughameelick female grew near a male (clone 3H). The Drumcooley female grew in the same region as a *P. x euramericana* male

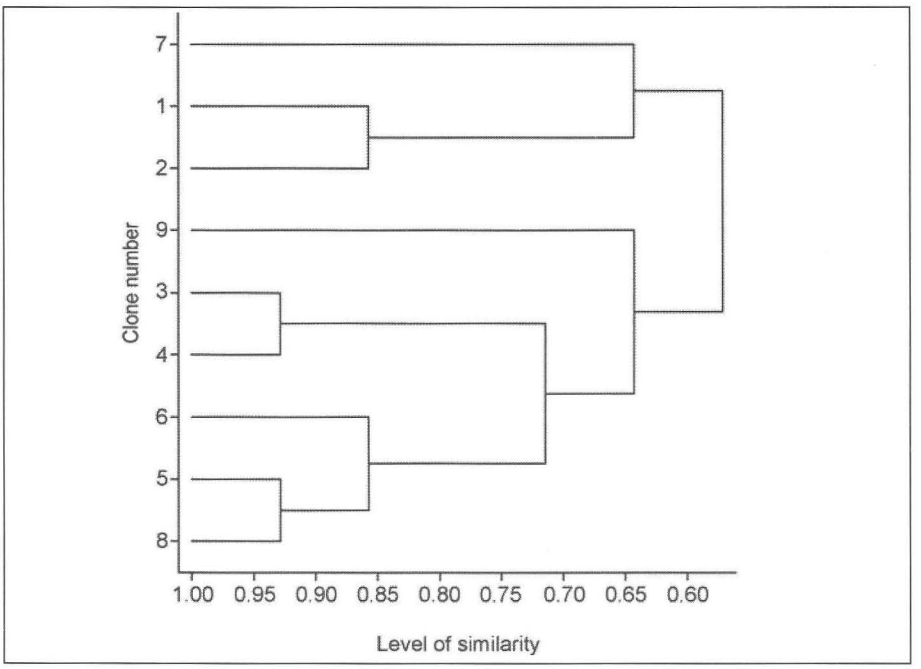

Figure 1. *Dendrogram illustrating the similarity of the clones based on microsatellite analysis.*

(clone 3H) and the only recorded sample of *P. nigra* clone 5 of undetermined sex.

The four *P. nigra* trees which produced the unique double banded pattern with the species specific marker also had rare alleles at several microsatellite loci. These samples were the sole representatives of clones 1, 2 and 7. The two of these which flushed had hairless petioles, which indicates that although they are *P. nigra*, they do not belong to sub-species *betulifolia*. The similarity dendrogram, calculated using the program Popgene (http://www.ualberta.ca/~fyeh/), demonstrates that the microsatellite fingerprints of these three genotypes are very different from those of the other *P. nigra* clones investigated in this study (Figure 1).

Geographic distribution

Using Hobson's early surveys, together with the more recent survey carried out in connection with this work, the number of trees that are now positively identified as *P. nigra* with a heretofore unprecedented level of confidence is 85 in the counties of Dublin, Kildare, Offaly, Tipperary and part of Galway. The geographical distribution of the individuals from which DNA was extracted and subsequently positively identified as *P. nigra* is shown in Figure 2.

By using Hobson's unpublished records of *P. nigra* it is apparent that approximately 40 trees cannot now be located. The disappearance of these trees may have resulted in a loss of genetic diversity. The status of approximately 200 trees

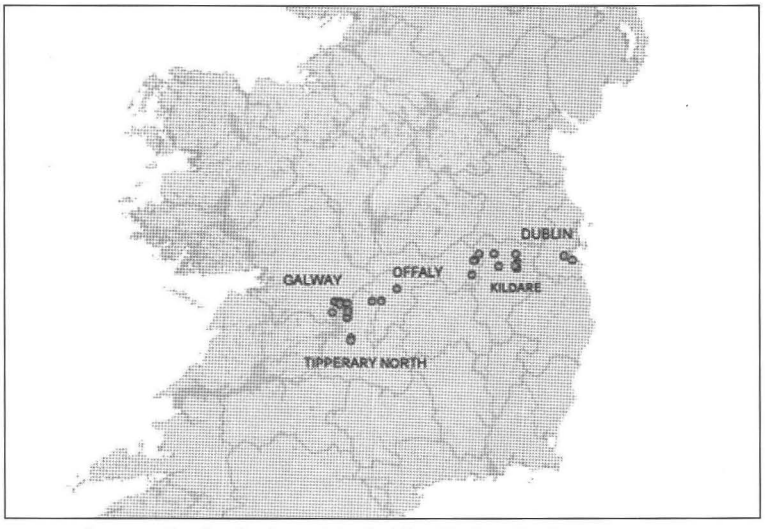

Figure 2. *Distribution of individuals positively identified as P. nigra.*

surveyed by Hobson in the counties Leitrim, Limerick, and the remainder in Galway, Clare, Cork and Wexford is not known.

The distribution of the three most prevalent genotypes (clones 3, 6 and 8), is wide (Appendix Table 1). For example, clone number 3 is present in northwest Co Kildare as Timahoe-Carbury, but it also occurs at Ardchroney Garden in North Tipperary. The distance between these trees is about 95 km. The distance between the trees in Blackrock (all clone 6) and the majority of the Ballinderry 15 group of trees (also clone 6) is approximately 140 km. Most members of clone 8 are located around east Co Offaly. However one individual; Terryglass South 1 is present in north Co Tipperary, which is at least 77 km from its nearest counterpart in Co Offaly.

Discussion

There was a high level of misidentification in the current study, with almost a quarter of the trees being *P. x euramericana* and not *P. nigra* as originally thought. This is due to the difficulties associated with distinguishing *Populus* species based on morphological traits and crown structure. This identification problem has led to the rarity of *P. nigra* being underestimated (Milne-Redhead 1990).

The development of the species specific marker by Heinze (1997) is valuable because it offers a tool that allows first generation *P. x euramericana* to be securely distinguished from *P. nigra* trees. The fact that the majority of the *P. nigra* trees in our sample had young leaves with hairy petioles compared with the *P. x euramericana* which were mostly non-hairy is also a useful morphological character with which to distinguish *P. nigra* trees in Ireland. The presence of hairs on the petioles confirms that, like the English and Welsh black poplar, the majority of the Irish trees in the sample belong to the subspecies *betulifolia*. This subspecies

develops burrs on the trunk, and Hobson's (1993) observation that such trees only comprise 45% of the Irish population is a further indication that there may, in the past, have been cases where *P. x euramericana* (without burrs) were misidentified as *P. nigra*. This, accompanied by the fact that a large number of the trees surveyed by Hobson are no longer present today, indicates that black poplar is even rarer than it was thought to be in Ireland.

The Irish sample also resembles the English and Welsh trees in that male trees are more common than females. In a sample from England and Wales, the males outnumbered the females by a ratio of nearly 4 to 1 (Cottrell et al. 2002). This compares with a ratio of 9:1 in the sample from Ireland. This may be the result of active selection against females because they produce unsightly seed fluff which is undesirable in amenity areas (Tabbush 1996).

Many of the clones in the Irish sample were represented several times. This was also the case in the English and Welsh sample. On average, each *P. nigra* clone was represented by 9 trees in the Irish sample. This means that the number of clones in Ireland is even lower than had been anticipated. If Hobson (1993) located the majority of black poplar in Ireland then the 373 trees found in his survey are likely to represent less than 41 clones. If misidentified *P. x euramericana* trees are also taken into account this number could be even lower. Clones were not repeated to the same extent as the sample from England and Wales, where each clone occurred five times in the total sample (Cottrell et al. 2002, Storme et al. 2004). This suggests that the English and Welsh population may have experienced less human interference than in the Irish case. Also, the trees sampled in England and Wales had a wider geographic distribution and this may be a reason why there was less clonal duplication in this than the Irish sampling. There was a tendency for several of the trees growing in close proximity in Ireland to belong to the same clone, although there was usually some diversity at a given site. The opportunity for sexual reproduction however, is low because of the small number of female trees. The fact that some of the females grow in close proximity to *P. x euramericana* trees is of some concern, although Tabbener and Cottrell (2003) found no evidence of interspecific crossing between *P. nigra* and *P. x euramericana* in their study of seedling paternity. Other studies (Fossati et al. 2003, van den Broeck et al. 2002, and van den Broeck et al. 2004) confirm that interspecific crossing is confined to situations where there are only *P. x euramericana* males within pollinating distance of *P. nigra* females. The absence of suitable sites for seed germination in Ireland also means that they are unlikely to become established so that any interspecific crossing events are unlikely to have any lasting effect.

These results do not allow the question of the native status of *P. nigra* in Ireland to be addressed. However, the fact that three clones were very different from the other six clones in terms of their microsatellite fingerprints suggests that there may have been at least some importation of material from abroad. These three clones (Clones 1, 2 and 7) were also different in that they did not have hairy petioles in those samples where the leaves were present, and they produced the double rather than the single banded pattern with the species specific marker. An analysis based on the

maternally inherited chloroplast DNA markers (Cottrell et al. 2005) might provide more insight into the geographic origin of these clones.

It is reasonable to presume, based on the distribution of the different clones throughout the countryside that the population of *P. nigra* in Ireland has been very heavily influenced by human activity, i.e. the propagation and establishment of cuttings from existing trees. Nevertheless, the abundance of the species around south east Co Galway merits further molecular investigation and survey work. There is a need to complete the geographic and molecular survey of the trees in the remaining counties so that an informed conservation policy can be developed for the species in Ireland.

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Appendix Table 1. *Tree name and location, sex and the hairiness of the young expanding leaves of the sample. Trees are classified as P. x euramericana or P. nigra on the basis of the species specific marker and as a clone number (based on the multilocus genotype produced by the seven microsatellite loci.*

Tree name and Watsonian Vice-County	Sex	Petiole hairs a: absent p: present	Heinze marker	Clone number	Tree name and Watsonian Vice county	Sex	Petiole hairs a: absent p: present	Heinze marker	Clone number
Birr Caravan, Offaly			P. nigra (double band)	1	Looscaum Church middle, SE. Galway	m	p	P. nigra	6
Ardchroney layby, N. Tipperary		a	P. nigra (double band)	1	Looscaum Church South, SE. Galway	m	p	P. nigra	6
Abbey West, SE. Galway	f		P. nigra (double band)	2	Millisent House, Kildare	m	p	P. nigra	6
Ardchroney Garden, N. Tipperary	m	p	P. nigra	3	Millisent Quarry, Kildare	m	p	P. nigra	6
Ballinderry 15/1, N. Tipperary		p	P. nigra	3	Millisent Track, Kildare	m	p	P. nigra	6
Ballinderry 5/2,N. Tipperary		p	P. nigra	3	Sawnagh House, SE. Galway	m	p	P. nigra	6
Ballinderry 5/4, N. Tipperary		a	P. nigra	3	Shannon Park 1, SE. Galway	m		P. nigra	6
Baltracey X, Kildare		p	P. nigra	3	Shannon Park 2, SE. Galway		p	P. nigra	6
Riverstown 1, N. Tipperary	m		P. nigra	3	Shannon Park 4, SE. Galway		p	P. nigra	6
Riverstown 2, N. Tipperary	m		P. nigra	3	Shannon Park 5, SE. Galway		p	P. nigra	6
Sawnagh mid 1, SE .Galway	m	p	P. nigra	3	Shannon Park North, SE. Galway		p	P. nigra	6
Sawnagh mid 2, SE Galway	m	p	P. nigra	3	Stoney Island, SE. Galway	m		P. nigra	6
Sawnagh mid 3, SE. Galway	m	p	P. nigra	3	Loughanroe West, SE. Galway			P. nigra	6
Sawnagh mid 4, SE. Galway	m	p	P. nigra	3	Birr Boyds, Offaly		a	P. nigra-(double band)	7
Sawnagh mid 5, SE. Galway	m		P. nigra	3	Terryglass South, 1 N. Tipperary	f	p	P. nigra	8
Sawnagh mid 6, SE. Galway	m	a	P. nigra	3	Kilcormack, E. Offaly		p	P. nigra	8
Sawnagh mid 7, SE. Galway	m	p	P. nigra	3	Drumcooley East 5, Offaly	f	p	P. nigra	8
Terryglass North 1, N. Tipperary	m	p	P. nigra	3	Drumcooley East 2, Offaly		p	P. nigra	8
Terryglass North 2, N. Tipperary	m	p	P. nigra	3	Drumcooley East 1, Offaly		p	P. nigra	8
Terryglass North 3, N. Tipperary		p	P. nigra	3	Drumcooley West, Offaly		p	P. nigra	8
Terryglass North 4, N. Tipperary	m	p	P. nigra	3	Drumcooley East 4, Offaly	f	p	P. nigra	8
Terryglass North 5, N. Tipperary	m	p	P. nigra	3	Agameelick South, Offaly		p	P. nigra	8
Terryglass South 2, N. Tipperary	m	p	P. nigra	3	Agameelick North, Offaly		p	P. nigra	8
Timahoe-Carbury, Kildare		p	P. nigra	3	Agameelick mid, South Offaly	f	p	P. nigra	8
Edenderry East 1, Kildare	m	p	P. nigra	3	Agameelick mid, North Offaly		p	P. nigra	8
Edenderry East 2, Kildare	m	p	P. nigra	3	Drumcooley East 3, Offaly		p	P. nigra	8
Pallas West, SE. Galway	m	p	P. nigra	3	Mount Pleasant, Dublin		p	P. nigra	9

Ballinderry 5/3, N. Tipperary	m	p	P. nigra	4
Drumcooley far East South, Offaly		p	P. nigra	5
Allenwood Canal, Kildare		p	P. nigra	6
Ballinderry 15/10, N. Tipperary		p	P. nigra	6
Ballinderry 15/11, N. Tipperary		p	P. nigra	6
Ballinderry 15/14, N. Tipperary		p	P. nigra	6
Ballinderry 15/2, N. Tipperary		p	P. nigra	6
Ballinderry 15/3, N. Tipperary			P. nigra	6
Ballinderry 15/5, N. Tipperary		p	P. nigra	6
Ballinderry 15/4, N. Tipperary		p	P. nigra	6
Ballinderry 15/6, N. Tipperary		p	P. nigra	6
Ballinderry 15/7, N. Tipperary	m	p	P. nigra	6
Ballinderry 15/8, N. Tipperary		p	P. nigra	6
Ballynderry15/13, N. Tipperary			P. nigra	6
Blackrock 1, Dublin		p	P. nigra	6
Blackrock 2, Dublin	m	p	P. nigra	6
Blackrock 3, Dublin			P. nigra	6
Blackrock 4, Dublin		p	P. nigra	6
Blackrock 5, Dublin	m	p	P. nigra	6
Blackrock 6, Dublin	m	p	P. nigra	6
Blackrock 7, Dublin	m	p	P. nigra	6
Blackrock 8, Dublin		p	P. nigra	6
Claggernagh East North, SE. Galway	m	p	P. nigra	6
Claggernagh West1, SE. Galway	m	p	P. nigra	6
Claggernagh West 2, SE. Galway	m	p	P. nigra	6
Clane Dublin Road, Kildare	m	p	P. nigra	6
Castle Park South, Dublin		a	P. x euramericana	1H
Looscaum Church North, SE. Galway	m	p	P. x euramericana	2H
Pallas South 1, SE. Galway	m		P. x euramericana	2H
Pallas South 2, SE. Galway		a	P. x euramericana	2H
Pallas South 3, SE .Galway	m	a	P. x euramericana	2H
Sawnagh South East, SE. Galway		a	P. x euramericana	2H
Sawnagh South West, SE. Galway		a	P. x euramericana	2H
Shannon Park 3, SE. Galway	m		P. x euramericana	2H
Kylemore West, SE. Galway			P. x euramericana	2H
Aughameelick South East, Offaly	m	a	P. x euramericana	3H
Booth Road, Dublin		a	P. x euramericana	3H
Carrigahorrig 1, N. Tipperary		a	P. x euramericana	3H
Carrigahorrig 3, N. Tipperary		a	P. x euramericana	3H
Carrigahorrig 5, N. Tipperary		a	P. x euramericana	3H
Castle Park North, W. Dublin		a	P. x euramericana	3H
Drumcooley far East North, Offaly	m		P. x euramericana	3H
Fair English River, Kildare		a	P. x euramericana	3H
Pallas Hill, SE. Galway		a	P. x euramericana	3H
Pallas South 4, SE. Galway	m	a	P. x euramericana	3H
Pallas South 5, SE. Galway	m	a	P. x euramericana	3H
Pallas South 6, SE. Galway	m	a	P. x euramericana	3H
Pallas South 7, SE. Galway	m	a	P. x euramericana	3H
Riverstown 3, N. Tipperary	m		P. x euramericana	3H
Riverstown 4, N. Tipperary		a	P. x euramericana	3H
Monastery Gate Copse, Dublin		a	P. x euramericana	3H