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Characteristics of pollen from natural triploid *Betula* hybrids

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Abstract

Birch has a key role in the Holocene vegetation history of northern Europe and in sub-arctic climates dwarf birch and tree birch co-exist. In Iceland, triploid hybrids between diploid *Betula nana* (dwarf birch) and tetraploid *B. pubescens* (downy birch) are common and therefore likely to contribute to pollen deposition. Pollen from 22 triploid trees/shrubs from ten woodlands in Iceland was examined and its size and shape compared with pollen from the parental species. The mean diameter of pollen grains from the triploid hybrids was not statistically different from that of *B. nana* pollen, but was significantly smaller than the mean value of *B. pubescens* pollen. On the contrary, the size of the vestibulum was similar to that of *B. pubescens*, which was significantly greater than that of *B. nana*, and therefore the diameter-pore depth ratio was lower than the values from either species. The pattern of size distribution within plants indicated that triploid hybrids might have produced two sizes of triporate pollen grains, but the small *B. nana* size was far more prevalent than the larger *B. pubescens* size. Several anomalies in pollen morphology were common among the hybrid pollen grains: four or more pores were the most frequent type of abnormality. Characteristics of the pollen of triploid *Betula* hybrids, especially structural anomalies, may provide a means to reveal periods of interspecific hybridisation in the analysis of sub-fossil pollen.

Keywords: Birch, *Betula nana*, *Betula pubescens*, grain diameter, hybrid pollen, pollen pore depth, pollen size, pollen morphology, triploid hybrid

Two birch species have co-existed in Iceland since the early Holocene: dwarf birch *Betula nana* L. (diploid, $2n=28$) and downy birch *B. pubescens* Ehrh. (tetraploid, $2n=56$). *Betula nana* is a prostrate shrub up to one metre in height and has its main distribution in the sub-arctic and alpine regions of the Northern Hemisphere. The species is represented by subspecies *nana* (Suk.) Hult. in Europe (including Iceland) and western Asia, and subspecies *exilis* (Suk.) Hult. in North America and central and eastern Asia (Hultén & Fries, 1986). *Betula pubescens* is a European species, represented by subspecies *pubescens* Ehrh., which may grow up to 25 m tall with single or many stems, and also by subspecies *tortuosa* (Ledeb.) Nyman, which is a shrub or low-growing tree found in the mountain regions of northern Europe (Walters, 1964). The latter subspecies, so-called mountain birch, is believed to be the result of introgressive

hybridisation with *B. nana* (reviewed in Atkinson, 1992). Due to extensive and continuous morphological variation of birch in Iceland (Thórsson et al., 2001, 2007), this tree birch species is not divided into subspecies in this study but is treated as one, *Betula pubescens sensu lato*.

Triploid plants, derived from hybridisation between diploid *B. nana* and tetraploid *B. pubescens*, are common in Iceland – they are found at an average of about 10% of birch plants when sampling at random (Thórsson et al., 2001, 2007). In controlled crosses (Anamthawat-Jónsson & Tómasson, 1990) and from open-pollinated *B. nana* plants (Anamthawat-Jónsson & Tómasson, 1999), the proportion of triploid hybrids among the progeny is much higher and the triploid plants are not sterile. Such interspecific hybrids can mediate gene flow between the parental species via back-crossing of the hybrids. This process, so-called

introgressive hybridisation or introgression, has been shown to occur in birch woodlands in Iceland (see review in Anamthawat-Jónsson, 2003a). The multivariate data analysis of species-specific morphological characters (Thórsson et al., 2007) has confirmed the occurrence of bidirectional introgression and indicated that the triploid group may have played an important role in maintaining genetic variation of the woodland birch species.

In northern Europe, birch has played a key role in vegetation succession as glaciers retreated at the beginning of the Holocene, and for the northernmost parts, such as Iceland and Fennoscandia, birch woodlands remain important features of the vegetation. As different birch species represent different ecosystems, several attempts have been made to differentiate sub-fossil pollen of *Betula*, especially between *B. pubescens* and *B. nana* (see Caseldine, 2001). Pollen of *B. pubescens* is on average larger than pollen of *B. nana*, has more protruding apertures and a larger vestibulum (Birks, 1968). However, size frequency curves overlap significantly and interpretation of sub-fossil data has to rely on mean sizes rather than individual grains.

Pollen grains of *Betula* are triporate with a distinct vestibulum and a grain diameter of usually 17 – 27 µm (e.g. Mäkelä, 1996; Caseldine, 2001). No clear characteristics of pollen separate species within the genus *Betula*, but differences in mean size, relative size of vestibulum and minor structural features of pores make identification on species level just about possible (Blackmore et al., 2003). In our recent study (Karlsdóttir et al., 2007), pollen size was measured and compared between samples of *B. nana* and *B. pubescens* that were classified based on chromosome number. Due to the introgressive hybridisation described above, morphological variation in Icelandic *Betula* is so great that species identification based on morphology is rendered difficult. But the chromosome number of Icelandic birch plants is stable: only three groups of plants have been identified – diploid, triploid and tetraploid plants (Thórsson et al., 2001, 2007). Pollen of diploid *B. nana* was therefore compared with that of tetraploid *B. pubescens*; the results showed significant difference in the mean size of pollen grains of these two species, regardless of the large overlap in plant morphology (Karlsdóttir et al., 2007).

As triploid birch hybrids are quite common in natural woodlands in Iceland, it is important to know to what extent pollen from the triploid hybrids has contributed to the pollen pool and how it has affected the species differentiation of birch pollen in general. This should be useful when interpreting data from sub-fossil pollen with an aim to establish the vegetation history. Sub-fossil *Betula* pollen is

found in Icelandic sediments and peat throughout the Holocene and even in late-glacial sediments (Hallsdóttir, 1990; Rundgren, 1995, 1998; Caseldine, 2001; Caseldine et al., 2003). If we could identify specific characteristics of pollen from triploid birch, it would be possible to study the history of hybridisation and introgression in Iceland using sub-fossil pollen. The aim of this study was to compare pollen of triploid birch plants with pollen of diploid *B. nana* and tetraploid *B. pubescens* from the same woodlands in Iceland.

Material and methods

Pollen samples were collected from 92 individual trees/shrubs in ten different sites in Iceland: Brekkuskógur, Bifröst, Ásbyrgi, Eidar, Jökulsá í Lóni, Skaftafell, Reykjanes, Kjálkafjörður, Kaldalón and Mývatn (for description of the sites and map, see Thórsson et al., 2007). All of these plants had been identified beforehand by chromosome number, which was obtained from direct counting of metaphase chromosomes from leaf bud cells as in Anamthawat-Jónsson (2003b). The counting yielded three groups of plants (Table I): diploid *Betula nana* ($2n=28$), tetraploid *B. pubescens* ($2n=56$) and triploid hybrids ($2n=42$). In early spring, male catkins were either collected at the place of origin or allowed to mature on cut branches indoors. All samples were prepared under the same laboratory conditions. The analysis of pollen from the diploid *B. nana* and the tetraploid *B. pubescens* was reported in Karlsdóttir et al. (2007), and as a continuation of that work the analysis of pollen from the triploid plants is presented here in comparison with pollen of the two parental species.

The pollen samples were prepared and mounted in silicon oil as in Karlsdóttir et al. (2007). Images of 120 – 130 pollen grains from each individual tree/shrub were taken using a Nikon digital camera DXM1200F on a Nikon Eclipse E800 microscope with $400\times$ microscopic magnification. Grain diameter (D) and pore depth (P) were defined and measured for each pollen grain as in Karlsdóttir et al. (2007). Only triporate pollen grains (normal grains with three pores) lying in polar view were measured. The grain diameter (representing pollen size) was measured digitally with an accuracy of approximately 0.3 µm. The measurement of pore-depth was less accurate, owing to varying visibility of the inner wall and a relatively greater effect of the visual angle.

Abnormal pollen grains from the same preparations as used for the size measurement were examined. Approximately 1000 grains per individual plant were counted and all types of abnormalities (anomalies or deviations from normal appearance)

Table I. Mean pollen size by ploidy groups.

Species and chromosome number		No. of trees	Diameter (μm)			Pore depth (μm)			Diameter/Pore depth ratio		
			n	mean	SD*	SE*	mean	SD*	SE*	mean	SD*
<i>B. nana</i>	28	31	20.4	1.6	0.3	2.2	0.2	0.04	9.5	0.9	0.16
Hybrid	42	22	20.1	1.3	0.3	2.8	0.4	0.08	7.5	1.0	0.22
<i>B. pubescens</i>	56	39	24.2	1.5	0.2	2.8	0.3	0.05	8.8	0.9	0.14

*among individual trees

were noted. In eleven cases, pollen from an individual tree/shrub was sampled in two consecutive years and the average outcome was used in the results. These were five *B. nana*, four triploid hybrids and two *B. pubescens* plants.

ANOVA and Tukey's HSD were performed to test pollen size differences among the ploidy (chromosome number) classes, one-sample Kolmogorov-Smirnov goodness-of-fit test was used for comparison to normal distribution and Pearson's correlation coefficient was also performed, all using SPSS software version 10.0.5 (© SPSS Inc. 1989–1999).

Results

Triplicate pollen

In this study and in Karlsdóttir et al. (2007), all measurements were made on pollen grains having three pores (triplicate), which is considered to be the normal type in *Betula*. Mean pollen diameter (D), mean pollen pore depth (P) and the D/P ratio differed significantly (ANOVA: $p < 0.001$) among ploidy groups, i.e. diploid *B. nana*, tetraploid *B. pubescens* and triploid hybrid between the two species.

Triplicate pollen grains from the triploid plants were often similar in size to *B. nana* pollen, but smaller than pollen of *B. pubescens* (Figure 1, Figure 2A). The mean grain diameters of the diploid, triploid and tetraploid groups were 20.5,

20.1 and 24.2 μm, respectively (Table I). The Tukey HSD test showed a significant difference ($p < 0.001$) in pollen size between the triploid group and *B. pubescens*, but not between the triploid group and *B. nana*. Based on these mean grain diameters, pollen grains of *B. nana* and the hybrid were similar in size but smaller than *B. pubescens* pollen. However, when looking at size distribution within plants, pollen grains from the triploid hybrids were not uniformly small compared to size distribution within individuals of *B. nana*. Pollen sizes were normally distributed within each individual of *B. pubescens* and *B. nana* (as shown by the K-S goodness-of-fit test). Of the 22 triploid hybrids, four showed significant diversion from normal size distribution and in one case a distinct bimodal distribution. Of the 18 triploid hybrids that showed the normal size distribution, ten individuals resembled *B. nana* type (Figure 3A); the most common sizes clustered around the mean value for the diploid species while the largest diameters were seldom above 24 μm. Eight triploid plants showed similar size distribution to Figure 3A, but the majority of the grains were larger than the *B. nana* average and some (5 – 10%) were even bigger than the average *B. pubescens* size (above 24 μm). Of the four plants that showed significant deviation from the normal distribution curve, one triploid tree produced mainly large pollen grains with the same mean diameter as *B. pubescens* (Figure 3B), whereas another tree showed two peaks of size distribution: small and large pollen grains (Figure 3C). None of

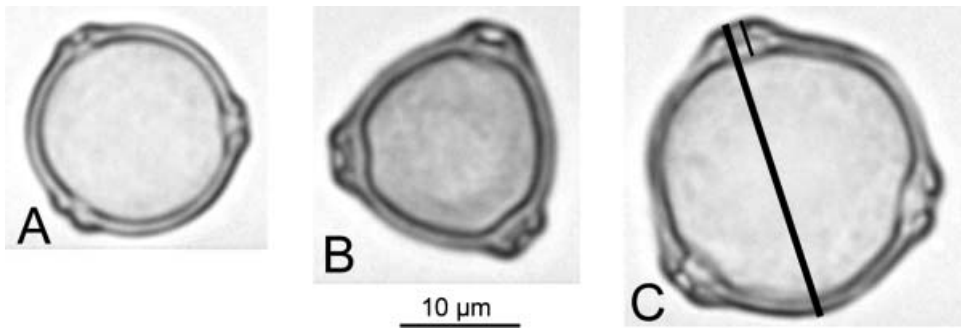


Figure 1. Pollen from diploid *Betula nana* (A), triploid hybrid (B) and tetraploid *B. pubescens* (C) showing grain diameter (27.4 μm) and pore depth (2.9 μm). The 10 μm scale bar applies to all pollen grains in this figure.

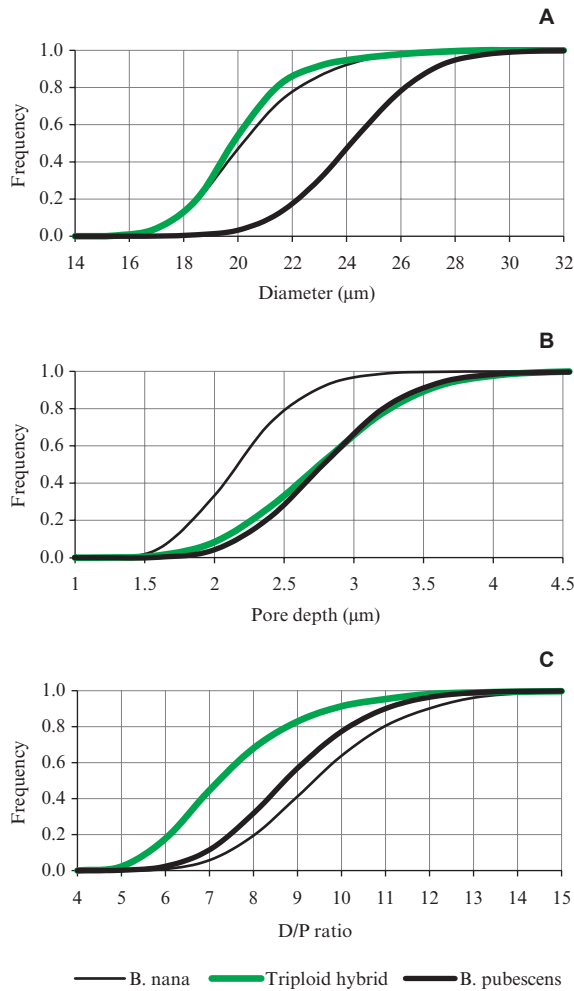


Figure 2. Cumulative distribution of pollen parameters by ploidy groups [diploid *B. nana*, triploid hybrid and tetraploid *B. pubescens*] - pollen grain diameter (A), pollen pore depth (B) and diameter to pore depth ratio (C).

the triploid plants showed a clear case of hybrid peak, i.e. containing pollen grains mainly of intermediate size.

The mean depth of pollen pore (P) also differed significantly among the ploidy groups, but regarding this parameter pollen of the triploid group differed significantly from *B. nana*, yet not from *B. pubescens* (Figure 1, 2B). The mean pore depths in the diploid, triploid and tetraploid groups were 2.2, 2.8 and 2.8 μm , respectively (Table I). As the triploid hybrids produced mostly small pollen like *B. nana* and deep pores like *B. pubescens*, their D/P ratio became the lowest compared to the ratios obtained from the two species (Table I, Figure 2C). The D/P ratio in hybrids often results in a characteristic triangular shape of pollen grains. Differences in D/P ratio were statistically significant among all three groups (Tukey's HSD: $p < 0.01$ for *B. nana* vs. triploid hybrid; $p < 0.01$ for *B. pubescens* vs. hybrid; and $p < 0.05$ for *B. nana* vs. *B. pubescens*).

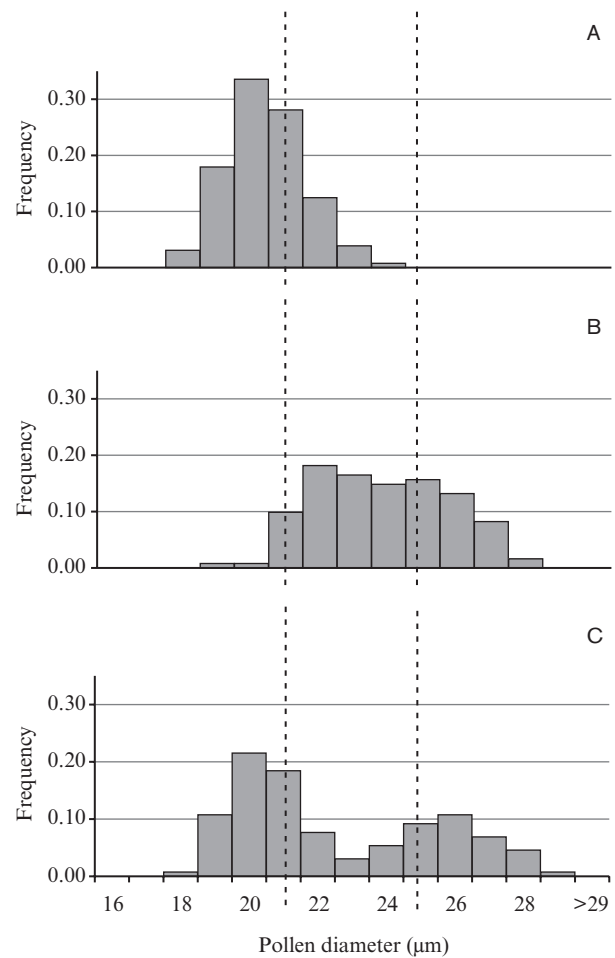


Figure 3. Examples of distribution of pollen grain diameters within individual triploid hybrid: tree numbers J11 (A), A13 (B) and J23 (C), based on 128, 121 and 130 triplicate grains measured, respectively. Two vertical dotted lines represent mean grain diameters of *Betula nana* (20.4 μm) and *B. pubescens* (24.2 μm).

Abnormal pollen

Eleven classes of structural abnormalities were recorded (Figure 4): three classes were presumably caused by damage to normal grains (shrunken, flattened or broken grains, presumably caused by environmental factors or during preparation of samples) and eight classes included deformed grains (0, 1, 2, 4, 5, 6 or more pores, fused grains, and grains with uneven pollen wall or other abnormalities). Both groups, damaged and deformed, were significantly more frequent among pollen samples from the triploid hybrids than those from *B. pubescens* or *B. nana* (ANOVA: $p < 0.001$; Tukey's HSD: $p < 0.01$) (Figure 5). The difference between *B. pubescens* and *B. nana* pollen was not statistically significant. Within ploidy groups, the proportion of abnormal pollen varied considerably among individual plants, as reflected in the large standard deviation and standard error (Figure 5). In some triploid hybrids, half of the pollen grains were

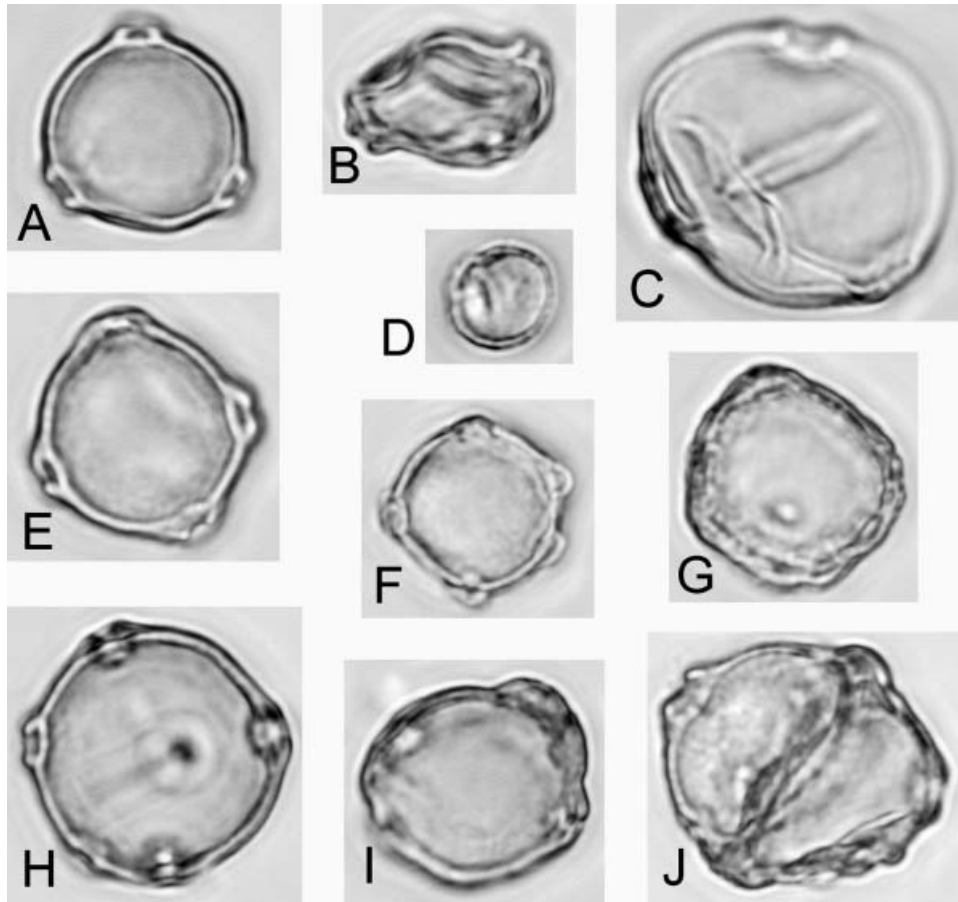


Figure 4. Examples of deformed and damaged pollen grains found frequently among pollen samples from triploid hybrids, compared to the normal triplicate type as in A, B, C. Damaged grains: (B) shrunk, (C) flattened. D–J. Deformed grains: (D) dwarf size with one pore, (E) four pores, (F) five pores, (G) abnormal and uneven exine, (H) multiple pores, (I) uneven exine, (J) fused grains.

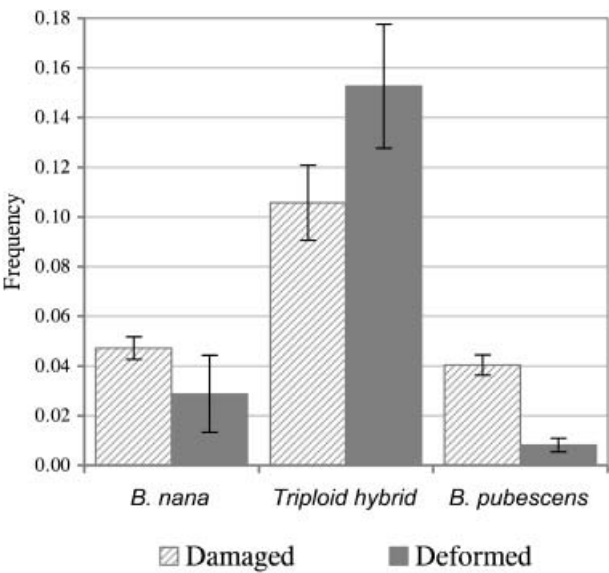


Figure 5. Frequencies of abnormal pollen by ploidy groups [diploid *Betula nana*, triploid hybrid and tetraploid *B. pubescens*]. Vertical bars represent standard errors.

deformed. Eleven of 22 hybrid individuals had more than 10% deformed pollen grains. This was rarely found among samples from the two species, occurring in only two of 31 *B. nana* individuals and one of the 39 *B. pubescens* trees.

The most frequent type of deformity in pollen morphology was pollen with four pores instead of the normal three. Pollen with four pores was found in 2.3% of *B. nana* pollen examined, 0.7% of *B. pubescens* pollen, but 10.8% in the hybrid pollen. Other deviations from three pores were rare. Non-triporate pollen grains combined were found in 2.4% and 0.7% of *B. nana* and *B. pubescens*, respectively, but 12.2% in the triploid hybrids.

In the cases where pollen was sampled from the same individuals in two consecutive years, no significant difference in the frequency of abnormal pollen grains was found between years. Furthermore, the proportion of deformed pollen was significantly correlated between years ($r=0.85$; $p<0.001$), as well as the proportion of non-triporate

pollen ($r=0.84$; $p<0.001$). The correlation in damaged pollen was not significant.

Discussion

Pollen of diploid *B. nana* and tetraploid *B. pubescens* from Iceland differs in average size and shape, although frequency curves overlap on grain diameters (D), pore depths (P) and D/P ratios (Karlsdóttir et al., 2007). Pollen of the triploid hybrids between *B. nana* and *B. pubescens*, as shown in the present study, differs from pollen of the parental species and several anomalies in its morphology (shape and structural features) are common. Hybrid pollen is not intermediate in size and shape, but possesses characters from both or either parental species. The majority of pollen grains from the triploid hybrids have the small size of *B. nana* pollen whereas the deep pollen pore is more like *B. pubescens*, which gives some of the hybrid pollen a characteristic triangular appearance. This finding may provide a means to detect the presence of hybrid pollen in a mixed sample, by its unique morphology, and to calculate proportions of hybrid pollen according to distribution curves, especially those with low D/P ratios. Birks (1968) emphasized the usefulness of D/P ratio in distinguishing pollen of the tree birch *B. pubescens* from that of the dwarf birch *B. nana* (lower D/P ratio in *B. pubescens*), but the problem seemed to be with the pollen of mountain birch (*B. pubescens* ssp. *tortuosa*). As defined in the introduction, we do not separate *B. pubescens* in Iceland into subspecies, which is based on morphology. Repeated investigations (recently published in Þórsson et al., 2007) have shown that the hybrid-like morphology (*tortuosa*-type) is found in all ploidy groups of Icelandic woodland birch samples, and most interestingly only half of the triploid birch has hybrid morphology. In the study reported here, we examined pollen from triploid birch plants, which was formed under meiotic restrictions and only a very small fraction of microspores survived to be normal and viable pollen grains (unpublished results). The normal looking triporate pollen from these triploid hybrids differs statistically in its morphology from pollen of the parental species. By this means, it should be possible to identify interspecific hybridisation in sub-fossil pollen samples.

Furthermore, anomalies from normal pollen morphology are very frequent among pollen from triploid hybrids. As comparable anomalies are rare in the diploid and tetraploid species, this might prove to be a true indicator of hybridisation if abnormal pollen grains are frequent in a mixed sample. The proportion of non-triporate pollen grains is of special

interest in this context, as they can easily be recognized in sub-fossil pollen. Repeated sampling shows that the rate of non-triporate pollen formation does not fluctuate much between growing seasons.

High frequencies of structural abnormalities in pollen of the triploid hybrids are not unexpected, due largely to meiotic irregularities occurring in PMCs (pollen mother cells) of interspecific hybrids. This has been observed in a number of *Betula* natural hybrids (e.g. Woodworth, 1929; Johnsson, 1949; Brown & William, 1984). As a consequence of pairing problems during the first meiotic division, especially between the homoeologous genomes, a huge number of variants among the microspores (and pollen) are often formed. Woodworth (1929) reported that pollen grains from a triploid hybrid between *B. pendula* and *B. pubescens* from northern Sweden were variable in size, and the sizes among pollen grains of a triploid hybrid between *B. lenta* and *B. pumila* varied from being microcytes (micronuclei) to more or less normal-sized grains, to large double-sized grains. Pollen of a presumed triploid birch hybrid from Poland (Szwabowicz, 1976) contained empty grains (devoid of cellular content), deformed grains with a thicker wall and a greater number of pores (4, 5 up to 8 pores), and viable grains which varied greatly in size. The present study shows a similar range of abnormality among hybrid pollen.

These deformed or malformed pollen grains are not likely to be viable, as selection would act against meiotic configurations that generate unbalanced gametes. Previous studies involving triploid F1 hybrids from *B. nana* $\times$ *B. pubescens* crosses and backcrossed progeny of the triploid hybrids, essentially with *B. pubescens* (Anamthawat-Jónsson & Tómasson, 1990), indicated that the triploid hybrids produced viable euploid gametes containing a full genome complement, i.e. having 14 ($1 \times$) or 28 ($2 \times$) chromosomes. All other numbers must have been eliminated, either via gametic or sporophytic selection, as only backcrossed progeny having diploid, triploid and tetraploid chromosome numbers were recovered. No aneuploid was found among these backcrossed progeny, nor has any aneuploid ever been found in Icelandic woodlands (Anamthawat-Jónsson & Þórsson, 2003; Þórsson et al., 2007). Unreduced gametes ($3 \times$) might have been formed among the hybrid pollen, but no $5 \times$ or $6 \times$ birch plants have ever been discovered. However, unusually large, presumably unreduced, pollen grains have often been observed in birch hybrids (Woodworth, 1929). In the present study, unusually large grains were found among those having more than three pores, but non-triporate pollen grains were not size-measured. Unreduced gametes may have a critical role in polyploid

formation, especially tetraploid fixation in diploid or mixoploid populations of horticultural plants (Ramsey & Schemske, 1998, 2002; Husband, 2004).

Within a given taxonomic or phylogenetic framework, ploidy level (chromosome number) appears to correlate positively with genome size and cell size (Beaulieu et al., 2007; Gregory et al., 2007). In *Betula*, 1C DNA value (genome size) tends to be directly related to ploidy and is considered to be close to the theoretical expected value for the 42, 56 and 70-euploid numbers (Taper & Grant, 1973). Pollen of diploid dwarf birch species (*B. nana* and *B. glandulosa*) is considerably smaller than that of polyploid tree birch species examined, such as *B. pubescens* and *B. papyrifera* (Caseldine, 2001; Clegg et al., 2005; Karlsdóttir et al., 2007). However, in relation to the diploid and tetraploid species in the present study, the mean size of pollen from the triploid individuals is not different from that of the diploid species *B. nana*.

If chromosome number was a dominating factor in determining pollen size, one would expect pollen produced by the triploid *Betula* hybrids to be larger on average than *B. nana* pollen. Clearly there has been selection in favour of the small size of *B. nana* (presumably having 14 chromosomes) among triporate pollen grains from the triploid hybrids. Eighteen (of 22) triploid hybrids produced small pollen grains similar in size to the diploid species *B. nana*. However, a small proportion of hybrid pollen consists of large pollen grains similar in size to those of *B. pubescens*, and in some samples the large size is prominent. No intermediate size of triporate pollen grains is clearly observed. This supports the hypothesis proposed initially in the crossing study of Anamthawat-Jónsson and Tómasson (1990), i.e. that triploid hybrids produce viable euploid gametes, hence gametic selection. Szwabowicz (1976) observed that in meiosis of the (triploid) hybrid birch there was a tendency towards reduction of the number of chromosomes to $n=14$ (by chromosome elimination) in both the first anaphase and telophase stages and also in the second meiotic division. Preferential chromosome elimination is known to occur in synthesized hybrids from interspecific crosses, for example in wheat and barley (Laurie & Bennett, 1988; Anamthawat-Jónsson et al., 1993). Preferential transmission of chromosomes via (triploid) hybrids has also been shown to be genome- or species specific, for example in *Brassica* whereby the effect of the chromosome number on fertilization fitness is more pronounced in some hybrids, as they show a stronger tendency in keeping their gametes toward euploid numbers (Lu & Kato, 2001; Leflon et al., 2006). Aneuploidy, in both plants and animals, may disturb gene expression and, as a consequence, the phenotypic characteristics

(Birchler et al., 2001). It has been suggested that the aneuploid effects result from altered stoichiometry of transcriptional regulators and that there is a tendency for genes to exhibit a “per cell” expression that is directly correlated with ploidy. In a study with maize aneuploids (Guo & Birchler, 1994), monosomics and trisomics are often less vigorous than euploid normals. Natural plants that thrive in extreme environments, like sub-arctic *Betula* in this study, can not afford aneuploidy.

In northern Europe, birch has a key role in the early Holocene vegetation history. When the history is reconstructed using sub-fossil pollen from sediments as a main source, emphasis is usually made on differentiating the tree-birch pollen of *B. pubescens* from the pollen of dwarf-shrub birch *B. nana* (e.g. Birks, 1968; Mäkelä, 1999; Caseldine, 2001). The results of the present study suggest that the hybridisation of the two species, which has presumably led to the establishment of mountain birch (*B. pubescens* ssp. *tortuosa*) near the tree-line, could be traced back by observing sub-fossil birch pollen, both in terms of the structural changes and the occurrence of deformed pollen grains.

Conclusions

Triploid *Betula* hybrids produced pollen with distinct characteristics which may be used to detect interspecific hybridisation in sub-fossil samples. Large and protruding pores tended to distinguish hybrid from *B. nana* pollen, which was of equal size but smaller than pollen of *B. pubescens*. Several anomalies in pollen morphology, including unusual numbers of pores, were frequent in pollen samples from the triploid hybrids but were very rare among pollen grains from the parental species.

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