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# Species integrity and ploidy stability despite extensive gene flow via introgressive hybridization: the case of *Betula* species in Iceland

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## Abstract

**Background** Introgressive hybridization is common in natural birch woodlands in Iceland, where two birch (*Betula*) species (diploid dwarf birch *B. nana* and tetraploid tree birch *B. pubescens*) coexist and hybridize readily. Our previous morphological, cytogenetic and palynological studies show that triploid hybrids are likely to have mediated gene flow between the two species. Our previous molecular study based on chloroplast haplotyping confirms the hybrid introgression and provides information about the genetic origin of *Betula* species in Iceland. The question remains, however, as to what extent nuclear gene flow is involved in this hybrid introgression process. The objective of the present study was therefore to use nuclear markers to probe birch introgressive hybridization.

**Results** AFLP (Amplified Fragment Length Polymorphism) analysis was performed on genomic DNA isolated from 169 individual *Betula* plants (67 diploid *B. nana*, 82 tetraploid *B. pubescens* and 20 triploid hybrids), from birch woodlands in Iceland in comparison to those from northern Scandinavia. The generated 115 polymorphic markers were subjected to analysis of molecular variance across ploidy groups, locations, and major chloroplast haplotypes. A new R package, Linarius, was developed for use with this mixed ploidy dataset. All markers were considered nuclear as no allele specific to any chloroplast haplotypes was detected. The results were to a certain extent congruent with those from our previous chloroplast study. No ploidy- or species-specific alleles were detected. Almost all alleles were shared among all three ploidy groups, indicating gene flow via hybridization. The difference, however, was that the nuclear markers clearly differentiated between diploid *B. nana* and tetraploid *B. pubescens*, whereas the chloroplast haplotype variation between species was non-significant. The triploid hybrid group was scattered within both ploidy clusters, in line with its role as a bridge to introgression. This nuclear separation between the two species is comparable to that from our previous analysis based on species- specific morphological characters, implying that the whole genomes may be selected for species adaptability in their different habitats. Furthermore, the present AFLP study depicted a clear east–west geographical separation among Icelandic *Betula* populations, based on both genetic distance analysis and anamorphosis modelling. This geographical separation is prominent in *B. nana* while *B. pubescens* is more genetically homogeneous.

**Conclusion** The present study shows that despite extensive gene flow, *Betula* species maintain their species integrity and ploidy stability. This in turn allows the long-term survival of the species in their local habitats.

**Keywords** AFLP, Birch, Gene flow, Forest, Introgression, Hybridization, Polyploidy, Woodland

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## Introduction

Introgressive hybridization (hybrid introgression) is a process by which hybridization leads to gene flow between species through backcrossing of the hybrid with its parental species [1]. This process allows the transfer of neutral or adaptive traits from one species to another and can increase genetic polymorphism in one or both species [2]. In the case of birch (*Betula* L.) in natural woodlands in Iceland, we have shown using various approaches that introgressive hybridization is indeed a common occurrence [3].

Two species of *Betula* coexist in Iceland: the diploid ($2n = 2x = 28$) dwarf birch (*B. nana* L.) and the tetraploid ($2n = 4x = 56$) downy tree birch (*B. pubescens* Ehrh.). Although closely related the two species are morphologically distinct from one another [4, 5]. The leaf of *B. nana* is orbicular in shape, with a rounded base, a crenate margin with single teeth, an obtuse leaf tip and a sessile petiole. On the contrary, the leaf of *B. pubescens* is ovate, with a cuneate base, a multi-toothed dentate margin, an acute leaf tip and a non-sessile petiole. While the dwarf birch *B. nana* is a circumpolar species, the tree birch *B. pubescens* has its main distribution in the temperate region of Europe and Eurasia [6, 7]. As these two species grow together sympatrically in natural birch woodlands, they hybridize, producing triploid ($2n = 3x = 42$) hybrids [8]. This triploid hybrid constituted 9.5% of 461 ploidy-identified (karyotyped) *Betula* plants from birch woodlands throughout Iceland [9].

This triploid hybrid is thought to have bridged gene flow between the two *Betula* species via backcrossing, resulting in a very large variation in morphology, especially that of the tetraploid *B. pubescens* [3, 8, 9]. We therefore evaluated viability of pollen and seed produced by these natural triploid birch individuals and found that the triploids are partially fertile [10], thus suitable as a bridge to gene flow between the two species. Only a few, partially fertile hybrids are required for introgression to occur across the species boundary [11]. For *Betula*, our botanical and cytogenetic studies [8, 9] reveal a significant overlapping of morphological variation across the ploidy boundaries, most probably resulting from gene-flow between the two species via hybridisation. Backcrossing of synthesized triploid hybrids with pollen from the tetraploid species *B. pubescens* produced triploid and tetraploid progenies having similar morphological variation as that exists in natural birch woodlands [12]. These crossing experiments indicated for the first time introgression via triploid bridge in Icelandic birch.

The occurrence of birch introgressive hybridization in Iceland using a molecular method of chloroplast cpDNA haplotyping was demonstrated in our previous study [13]. The three most common haplotypes (which together accounted for 83% of the Icelandic samples) are shared among all three ploidy groups, indicating bidirectional introgression. This introgressive hybridisation is supported by the statistical analysis of IG (introgression) indices. Furthermore, the overall introgression index for Iceland (all woodlands) is nearly twice as large as the value for northern Scandinavia, presumably indicating an active *Betula* hybridization zone in Iceland. The study [13] not only confirms the introgression, but also reveals a phylogeographical structure that points to multiple postglacial immigration events from northern Europe onto the island. This previous study is, however, based solely on organelle markers, which are maternally inherited. Therefore, in the present study we use bi-parentally inherited nuclear marker system to investigate introgression in the same sample set as with the cpDNA study. Some *Betula* studies show that although chloroplast and nuclear markers are only in part congruent, they provide different insights into the evolutionary relationship in each set of species [14, 15].

Here we aimed to analyse introgression and the genetic structure of *Betula* species in natural birch woodlands in Iceland using the multilocus nuclear marker AFLP (Amplified Fragment Length Polymorphism) method and compare it to the cpDNA variation generated previously from the same set of plant materials [13]. The AFLP technique [16] has been used to evaluate patterns of variation and genetic structure in several arctic and alpine plant species [17–20], as well as in *Betula* [21–23]. The present study is expected to produce results with good resolution, since *Betula* plants investigated are morphologically and ploidy-identified, such that the triploid hybrid group can be separated from the two species groups (the diploid *B. nana* and the tetraploid *B. pubescens*). Without ploidy identification it is difficult to distinguish hybrids from the parental species morphologically. In birch woodlands in Iceland, most of the hybrid-looking plants are introgressed (tetraploid) *B. pubescens* and half of the triploid plants have hybrid morphology, while other triploid plants resemble introgressed (diploid) *B. nana* [9].

## Materials and methods

### Plant material

Plant samples were collected during our field expeditions in 2000–2004 from 14 natural woodlands throughout Iceland as described in our botanical and cytogenetic paper [9] and later in woodlands in Norway and Sweden as described in our chloroplast haplotyping paper [13]. All sampling was conducted in locations freely accessible to the public. Voucher specimens and plant samples for the morphological study that were preserved in vacuum-sealed sheets, were deposited at Plant Genetics Laboratory, Institute of Life and Environmental

Sciences, University of Iceland. KAJ and ÆThTh identified the plant samples. Samples used in the present study are listed in the supplementary Table S1. The method of morphological evaluation, based on Flora Europaea [4], was described in our previous studies [8, 9]. Species-specific characters used for establishing the morphology index (Table S1) were mainly those of the leaf: leaf shape, leaf tip, leaf base, leaf margin, leaf teeth and the petiole. For each individual plant, 30 leaves were used in both qualitative (morphology index) and quantitative measurements [9]. Samples for the extraction of DNA (young leaves) and chromosomes (shoot tips) were collected in the field from the same plants that were morphologically identified as stated above.

The samples in the present study are a subset of those used in our chloroplast haplotype study [13]. AFLP analysis was performed on genomic DNA isolated from 169 individual *Betula* plants, from seven birch woodlands throughout Iceland (139 plants) and five from northern Norway and Sweden (30 plants). Their locations are mapped in Fig. 1 together with the data on chloroplast haplotypes identified in our previous study [13]. Information about the sampling locations and numbers of plants included in the study from each ploidy group in each location are listed in Table 1. These plants were ploidy identified by chromosome counting in our previous studies [9, 13], whereby metaphase chromosomes were extracted from shoot tips collected from *Betula* trees/shrubs in the field using enzymatic protoplast dropping method [24]. Of these 169 plants, 67 were diploid *B. nana*, 82 tetraploid *B. pubescens* and 20 triploid hybrids. One of the triploid plants, ID number B- 72 from the woodland Bifröst, is shown in Fig. 2.

### AFLP experiment

The AFLP experiments were conducted using the facilities at the Department of Biology, University of Copenhagen, Denmark. The technique was performed with fluorescent dye labelling and detection technology, with all AFLP reagents, adapters, primers, enzymes, size standards and fluorescent dyes obtained from Applied Biosystems®. Restriction and ligation reactions were carried out in accordance with the AFLP™ Plant Mapping Protocol (Applied Biosystems®) for average sized genomes. Fragment detection was performed on the ABI Prism 3730 Genetic Analyzer (Applied Biosystems®), an automated capillary electrophoresis device. The AFLP experiments were established and conducted exactly as described previously [20]. Selective primers were chosen after several tests to provide sufficient polymorphic marker without any risk of confusion between fragments of similar sizes (results not shown). The chosen primers are presented in Table 2.

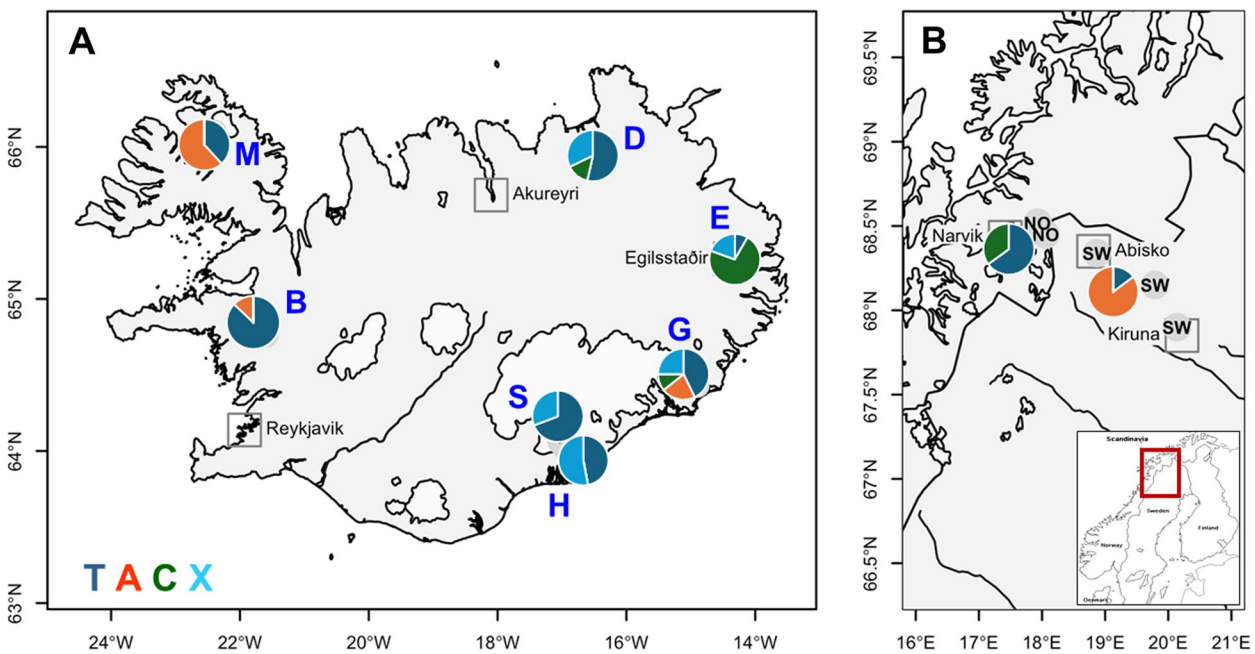


**Fig. 1** Sampling map of Iceland (**A**) and Scandinavia (**B**). The inset in map B shows location of the Scandinavian sampling sites in northern Europe. Pie-charts on both maps show proportion of *Betula* individuals carrying major chloroplast haplotypes (T, **A** and **C**) and X for all other (minor, unique) haplotypes together [13]. Numbers of individuals carrying each of the haplotypes, all ploidies combined, from each woodland are shown in the supplementary Table S2

**Table 1** Sampling locations, population labels, location coordinates and elevations of *Betula* trees under study, i.e. diploid *B. nana*, triploid hybrids and tetraploid *B. pubescens*. Chromosome number of individual plants was identified in previous studies [9, 14]

| Country | Collection site | Label | Location | | Number of samples analysed | | |
|---|---|---|---|---|---|---|---|
| | | | Lat./long | Masl | Diploid | Triploid | Tetraploid |
| IS—Iceland | Bifröst | B | 64.76/– 21.59 | 62 | 6 | 1 | 6 |
| | Ásbyrgi | D | 66.01/– 16.50 | 41 | 14 | 5 | 10 |
| | Eidar | E | 65.32/– 14.36 | 49 | 12 | 5 | 15 |
| | Jökulsá í Lóni | G | 64.43/– 14.90 | 36 | 3 | 1 | 9 |
| | Skaftafell | H | 64.03/– 16.98 | 267 | 10 | 3 | 12 |
| | Kaldalón | M | 66.10/– 22.39 | 36 | 8 | 3 | 7 |
| | Bæjarstadarskógur | S | 64.05/– 17.04 | 125 | 0 | 0 | 9 |
| NO—Norway | Jemvatna | | 68.52/17.93 | 321 | 3 | 0 | 4 |
| | Pettersenvatnet | | 68.45/18.06 | 520 | 4 | 2 | 3 |
| SW—Sweden | Abisko | | 68.34/18.87 | 437 | 3 | 0 | 4 |
| | Rensjon | | 68.15/19.79 | 465 | 1 | 0 | 1 |
| | Kiruna | | 67.90/20.14 | 479 | 3 | 0 | 2 |

## Data analysis

### *Statistical analyses*

These were performed with gnu-R (R Development Core Team) [25] using the packages ape, vegan, BoSSa and Poppr [26]. Distances provided by BoSSa were divided by 1000 to correct the unit that was incorrectly indicated as kilometres (km) rather than meters (m) in the manual. Furthermore, the 'Linarius' package was developed in the present study for use with this AFLP dataset, which contains mixed ploidy levels. It was considered an appropriate method fitting both ploidy and allele frequency data. It is an algebraic average of Hardy–Weinberg equilibrium (or binomial law) according to ploidy. The package may be downloaded for free at https://github.com/giby/Linarius. It provided us with the interpopulation indices Reynolds, Roger and Nei, while other packages offered mainly inter-individuals distances, such as the Jaccard index. In the present study Jaccard index was used for calculating distance between individual genotypes, but Reynolds for distance between populations based on allele frequencies between samples. Linarius also provided the means to perform clustering based on allele frequencies independent of ploidy levels. Lastly, Linarius allowed us to generate graphical presentations, such as a tri-polar heat map (unpublished) and 'anamorphosis' in the present study.

*AMOVA* A bootstrap method was first used to generate 10 000 random datasets fitting experimental allele frequencies and ploidies. AMOVA (package Poppr in ade4 framework) was then performed on these datasets and the distribution of these random values was used as a reference for determining the significance of AMOVA. P-values were calculated with the approximation of normal distribution and the results are presented in the table format. AMOVA was performed to test the variation by ploidy (Table 3), by location (Table 4) and by cpDNA haplotype (Table 5).

### *Anamorphosis design*

Once the distance calculation method was chosen for our dataset (see discussion), a method to position image points accordingly was needed, hence anamorphic analysis. Anamorphosis is a geometric function, a distorted projection that requires the viewer to occupy a specific vantage point and/or use special devices to view a recognizable image. Values for anamorphosis were computed according to functions present in the *Linarius* package. Geographic data were projected in the UTM zone 27W using the R package rgdal. Then, anamorphic analysis was performed with Darcy 2.1 [27].

The anamorphosis generates a single cartogram that combines geographical and molecular information. In the present study, the anamorphic cartogram is a map representing an adjustment of every distance according to the population-based genetic distance. In the area where the map is shrinking, the genetic distances there are closer to one another than average, and where it is extending the genetic distances are larger than average.

## Results

### AFLP variation

The AFLP analysis included 169 *Betula* individuals from 12 populations. Random replicates were used to remove

**Fig. 2** Triploid plant no. B- 72 in woodland Bifröst (population B), western Iceland. The morphology index of this plant is four (Table S1), which is considered a morphological intermediate within in the scale from zero (*B. nana*) to 10–13 (*B. pubescens*). This triploid plant has orbicular leaf shape with crenate margin from *B. nana*, multitoothed margin from *B. pubescens*, whereas intermediate characters are short petiole and subacute leaf tip. The red autumn colour of its leaves is typical of *B. nana*. This triploid plant produces plenty of female catkins. However, seed germination experiments revealed 0% seed germination [10]. The inset shows the somatic chromosome number 2n = 3x = 42

**Table 2** Primers used in the study (Applied Biosystems®). Samples from all locations were analysed with primer G18[a], whereas sample from Iceland locations were analysed with both

| Primer ID | | Primer sequence |
|---|---|---|
| BG7 | *Eco*RI—AAC | 5′-fam-GAC TGC GTA CCA ATT CAA C- 3′ |
| | *Mse*I—CTG | 5′-GAT GAG TCC TGA GTA ACT G- 3′ |
| G18[a] | *Eco*RI—AGG | 5′-joe- GAC TGC GTA CCA ATT CAG G- 3′ |
| | *Mse*I—CAC | 5′-GAT GAG TCC TGA GTA ACA C- 3′ |

nonreproducible markers and to ensure there were no shift between reads. Manual checking of all the reads were performed to remove artefactual, unreplicable and suspicious markers. Samples were removed when in a duplicate the marker was not present in both. After this, 115 polymorphic and reproducible markers were generated. Diploid plants produced 35 fragments, triploids produced 35 and tetraploids 36 fragments. In the final dataset, the number of markers was not significantly higher in tetraploids (average 16.6 fragments) than in diploids (average 15.2 fragments), except for the primer pair G18 when taken alone that the difference was significant with $P = 0.003$ according to Mann and Whitney test [28].

All alleles were shared by every ploidy level and location, except for a few very rare alleles. No allele specific to either diploids or tetraploids was observed, that is, no species-specific alleles were detected. Although some alleles were overrepresented in a ploidy level, either diploid or tetraploid (supplementary Table S3), there was no obvious specific pattern. Additionally, no allele that was specific to any chloroplast haplotype was observed and therefore we interpreted such that all the AFLP markers in the present study were nuclear markers.

There was no allele specific to any geographical locations, indicating that there was a common and widespread gene flow in our samples. Nevertheless, certain alleles were more frequent in some locations (supplementary Table S4), more so in western than in eastern Iceland. This appears to agree with the study of chloroplast haplotypes [13], whereby rare and unique haplotypes are prevalent in the western part of the country. Other location-prevalent alleles in the present study were scattered, that is, not region specific.

If we consider that an allele that is more frequent in one species originates from the other species, the presence of that allele is clearly due to introgression. We can then identify samples without such evidence of introgression. These were sample E- 11 for *B. nana* and samples E- 19, E- 32, G- 37 and M- 09 for *B. pubescens*. From these five samples without introgressive markers, four were from the eastern sites E (Eidar) and G (Jökulsá í Lóni), and only one was from the western site M (Kaldalón). No sample from population S (Bæjarstadarskógur) in the south contained introgressive markers from *B. nana*, however, this *B. pubescens* population is unique in the sense that there is no *B. nana* in the forest.

### Clustering analysis

The statistical results produced clear pattern of clustering of polymorphic AFLP markers according to ploidy levels (Fig. 3). The upper dendrogram (A) was constructed from the Icelandic dataset (seven woodlands), but the lower dendrogram (B) was based on data from all woodlands, including those five from northern Scandinavia.

**Table 3** Analysis of molecular variance (AMOVA) by **ploidy**, all locations. Ploidy groups (colour codes as in Fig. 3A-B): diploid (2x, red label) vs. tetraploid (4x, blue label)

| Source of variation | Df | Sum sq | Mean sq | Variation (σ) | Variation (%) | P |
|---|---|---|---|---|---|---|
| Between groups | 1 | 69.1222 | 69.1222 | 1.2504 | 9.0391 | 0.0344 |
| Within groups | 90 | 1132.4538 | 12.5828 | 12.5828 | 90.9609 | |
| Total | 91 | 1201.5761 | 13.2041 | 13.8332 | 100 | |

**Table 4** Analysis of molecular variance (AMOVA) by location, all ploidy levels (2x, 3x and 4x). Location groups in Iceland: East (D-Ásbyrgi; E-Eidar; G-Jökulsá; H-Skaftafell), blue label vs. West (B-Bifröst; M-Kaldalón), red label. The location S-Bæjarstadarskógur was excluded

| Source of variation | Df | Sum sq | Mean sq | Variation (σ) | Variation (%) | P |
|---|---|---|---|---|---|---|
| Between groups | 1 | 21.2201 | 21.2201 | 0.2554 | 1.9510 | < 0.001 |
| Within groups | 94 | 1206.7695 | 12.8380 | 12.8380 | 98.0490 | |
| Total | 95 | 1227.9896 | 12.9262 | 13.0934 | 100 | |

**Table 5** Analysis of molecular variance (AMOVA) by cpDNA haplotype, all locations (IS, NO and SW) and all ploidy levels (2x, 3x and 4x). Haplotype identity as in [14]. Three most common cpDNA haplotypes were statistically tested: A, C and T. All other haplotypes were not tested

| Source of variation | Df | Sum sq | Mean sq | Variation (σ) | Variation (%) | P |
|---|---|---|---|---|---|---|
| Between groups | 2 | 29.5318 | 14.7659 | 0.07713 | 0.5828 | NS |
| Within groups | 69 | 907.9682 | 13.1590 | 13.1590 | 99.4125 | |
| Total | 71 | 937.5000 | 13.2042 | 13.2361 | 100 | |

Samples from all locations were analysed with one primer pair (G18), whereas samples from Iceland locations were analysed with both primer pairs, BG7 and G18 (Table 2).

Figure 3A, B showed clustering according to ploidy levels. Diploids (red, *B. nana*) and tetraploids (blue, *B. pubescens*) were mostly in separate clades, whereas triploids (green) were scattered within both clades. The dataset from northern Scandinavia, when treated as a single population, supported the diploid-tetraploid separation. Of the two triploid samples from Norway, one was clustered with the diploid group and the other with the tetraploid group. Triploid samples, from all woodlands combined, did not form their own clade.

Cluster analysis of polymorphic AFLP markers was also performed according to geographical locations and major cpDNA haplotypes, but no specific pattern of clustering emerged (dendrograms not shown). Markers from the Icelandic woodlands were distributed all around the dendrogram. However, among the Icelandic populations, samples from the southern forest S (Bæjarstadarskógur) were not dispersed, but clustered mostly with tetraploids from the eastern group: north-eastern woodland D (Ásbyrgi) and the south-eastern woodlands G (Jökulsá í Lóni). The S population is comprised of tetraploid *B. pubescens* trees only. The Scandinavian populations (NO and SW) were present everywhere, except in the clade containing the S population. Overall, while a distinct pattern of clustering was not observed, there were several small clusters of samples according to regions in Iceland, even for samples with different ploidy levels. For example, individuals from all three ploidy levels from population D (Ásbyrgi) tended to cluster together, as did those from population E (Eidar), both in eastern Iceland. This appears to correspond relatively well with the pattern of chloroplast haplotype compositions, whereby the eastern populations are similar to one another, but different from those in the western part (Table S2, Fig. 1).

No clear pattern of clustering based on major cpDNA haplotypes emerged either. Three major cp-haplotypes [13] were included in this analysis: T (most common or 49% of all haplotypes in Iceland); C (19%, Scandinavian type); and A (15%, European type). Nevertheless, AFLP (nuclear) markers from the eastern woodlands (D, E and G) tended to be near those from forests in Scandinavia, which is in good agreement with the cpDNA results.

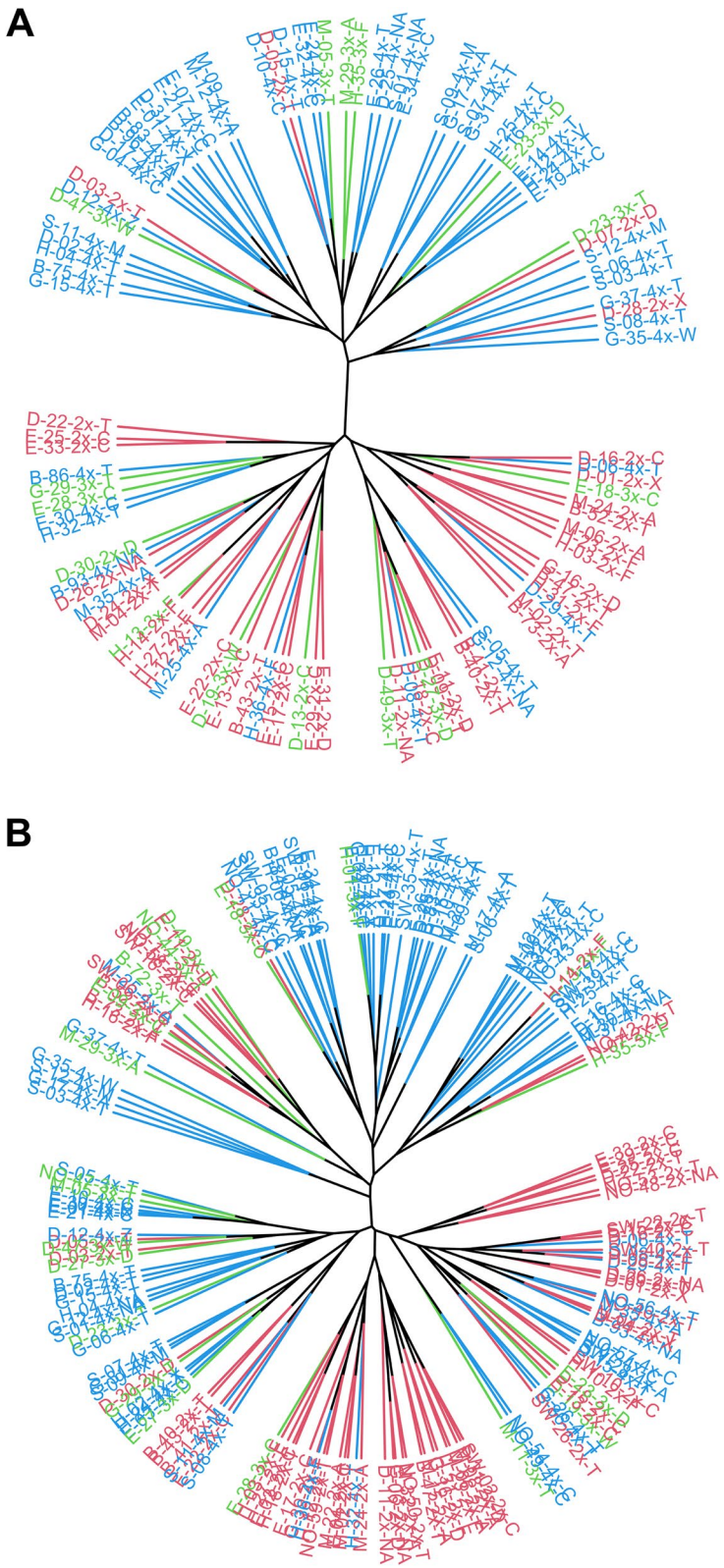


**Fig. 3** Dendrograms of the clustering analysis by ploidy of *Betula* samples from Iceland only (**A**) and all samples from Iceland and Scandinavia (**B**). Samples from Icelandic woodlands (**A**) were analysed using both AFLP primer pairs, BG7 and G18 (Table 2), whereas those from all locations (**B**) were analysed using only one primer pair, G18. Both dendrograms show good separation between diploid (*B. nana*) and tetraploid (*B. pusbescens*). Red; diploid (2x); green: triploid (3x); and blue: tetraploid 4x

### Analysis of Molecular Variance (AMOVA)

AMOVA was performed across groups: ploidy levels, locations, and chloroplast haplotypes. The results of AMOVA with ploidy levels are provided in Table 3. The variation was 9.039% for our dataset. The bootstrap of randomly generated data set produced values between 4.2% and 11.1% with a mean of 7.290% (SD = 0.963). The one-sided P-value was 0.03 and the two-sided was 0.07; both were statistically significant. The variation tested here was between the diploid (2*x*) and tetraploid (4*x*) groups, from all locations (see Fig. 3A-B), excluding the triploid group. This AMOVA result conflicts with that of the cpDNA variation in [13], which shows nonsignificant variation among ploidy groups, with or without the triploid group. Nuclear gene flow between the two *Betula* species is evidently less than gene flow via maternally inherited chloroplast genomes.

The results of AMOVA for locations in Iceland, from all ploidy levels (2*x*, 3× and 4*x*) are provided in Table 4. The variation between location groups, east vs. west, was 1.951% for our dataset. The bootstrapping gave values between −1.6% and 2.3%, with a mean of −0.086% (SD = 0.505). Both one-sided and two-sided P-values were smaller than 0.001, and therefore highly significant. The variation tested here was between the east (D-Ásbyrgi, E-Eidar, G-Jökulsá and H-Skaftafell) and the west (B-Bifröst, M-Kaldalón) locations. This supports the east–west separation of biogeography in Iceland with nuclear markers.

The results of AMOVA with cp-haplotypes, from all 12 locations (Iceland and Scandinavia) and from all three ploidy groups, are provided in Table 5. The observed variation was 0.583% for our dataset. The bootstrap treatment produced values between −1.9% and 2.5%. The mean was −0.046% (SD = 0.567). The P-value was > > 0.95, thus nonsignificant. The AMOVA results are in good agreement with the cp-haplotype clustering (Fig. 3E-F), in that there are no differences between groups. The AFLP nuclear markers in the present study are independent of the plastid markers.

### Geographic distance versus genetic distance

The Jaccard index was used to estimate relationship between the genetic distance among individual *Betula* samples and geographic distance for every possible pair of samples within our dataset. The overall results indicated a relatively low genetic distance (not shown here). Therefore, further statistical analysis was needed to interpret this data as the geographic distance did not fit a normal distribution. Kendall rank test of correlation [29] was then used to test association between genetic and geographical distances among individual samples. Table 6 shows high correlation when considering data from Iceland only; such a relationship was not observed when the Scandinavian samples were included. The correlation between these two distances originated mostly from diploid *B. nana*. Tetraploid *B. pubescens*, on its own, did not show any significant correlation. This statistical result is in good agreement with the analysis of cp-haplotypes from the same sample set [13], whereby genetic distance increases with geographical distance in *B. nana* but not in *B. pubescens*.

### Genetic distance between populations

The Reynolds distance matrix between populations is provided in Table 7. Based on these values, an anamorphosis cartogram (Fig. 4A) and a dendrogram of the population differentiation were constructed (Fig. 4B).

The anamorphic map (Fig. 4A), a visualization of genetic distances in a biogeographical background, shows a separation between eastern and western populations in Iceland. The southeastern population G (Jökulsá í Lóni) appears drifting away from all other locations, meaning that the genetic distance between G and any other populations is considerably more than the average of all distances in the study, presumably due to high variation in the nuclear allele frequencies in G compared with other populations. Interestingly, the cpDNA haplotypes in G seem to be most diverse (Fig. 1). The anamorphic map also shows increasing genetic distance in the other eastern populations, i.e., E (Eidar, north of G) and D (Ásbyrgi, northwest of E), in the same direction as G, that is away from the west. These two eastern populations are similar to one another in their cpDNA haplotype composition (Fig. 1). The western populations behave in the anamorphic model differently. While the southwestern population B (Bifröst) drifts eastwards, the northwestern population M (Kaldalón) remains static,

**Table 6** Correlation (by Kendall rank test) between genetical and geographical distances

| Ploidy groups | All locations | | | Iceland only | | |
|---|---|---|---|---|---|---|
| | τ | pv | P | τ | pv | P |
| Diploid (2x) | − 0.0120 | 0.4139 | | 0.0740 | 0.0034 | |
| Tetraploid (4x) | 0.0025 | 0.8321 | | 0.0294 | 0.2060 | |
| Total | − 0.0047 | 0.4167 | | 0.0646 | < 0.001 | |

**Table 7** Reynolds distances between populations in Iceland

| | B | D | E | G | H | M |
|---|---|---|---|---|---|---|
| B | 0.0000000 | | | | | |
| D | 0.1140946 | 0.0000000 | | | | |
| E | 0.1304104 | 0.1006316 | 0.0000000 | | | |
| G | 0.1669105 | 0.1570456 | 0.1378863 | 0.0000000 | | |
| H | 0.1282267 | 0.1121184 | 0.1035107 | 0.1389077 | 0.0000000 | |
| M | 0.1237846 | 0.1207251 | 0.1299984 | 0.1618284 | 0.1117115 | 0.0000000 |

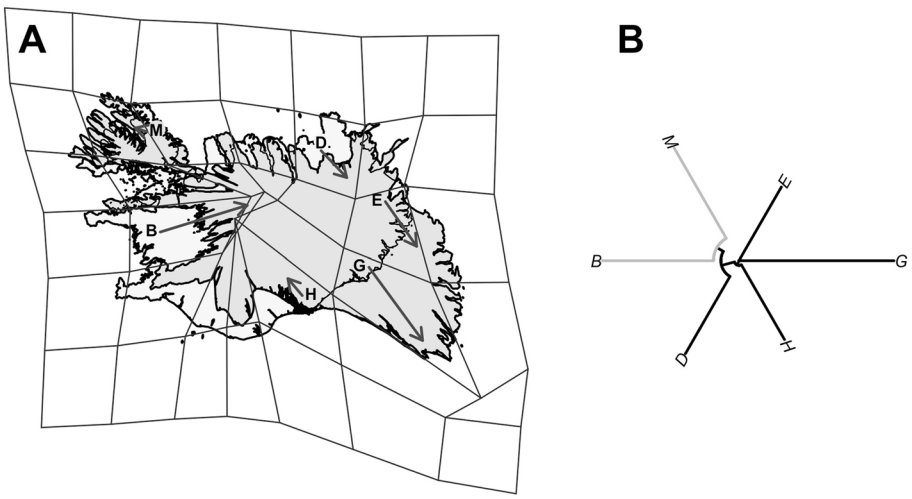


**Fig. 4** Anamorphic cartogram of genetic distances among *Betula* populations in Iceland (**A**) and its supporting dendrogram (**B**)

and the southwestern population H (Skaftafelll) shows little movement. Reynolds genetic distance, when visualized in the form of anamorphosis, is longest between G (SE) and M (NW). This is also reflected in the dendrogram (Fig. 4B), where overall eastern and western populations are mostly differentiated from each other. This is supported by the AMOVA analysis of AFLP variations (Table 4) and by the Kendall rank test of correlation between genetic and geographic distances (Table 6), whereby the correlation originated mostly from diploid *B. nana*. This geographical separation is similar to that found in our study of chloroplast haplotype variation in Iceland (Fig. 1, Table S2), which was interpreted as *Betula* having different origins, especially that of *B. nana* [13].

## Discussion

The present study analysed AFLP variation within and among birch woodlands in Iceland, where diploid dwarf birch (*Betula nana*), tetraploid downy birch (*B. pubescens*) and their triploid hybrids coexist sympatrically. Various statistical analyses were applied to this nuclear dataset, and in some cases chloroplast haplotypes generated in our previous study [13] were included. The results confirm gene flow across ploidy boundaries and phylogeographical differentiation among populations. In contrast to the chloroplast study, the AFLP results revealed statistically significant separation between diploid and tetraploid groups (*B. nana* versus *B. pubescens*). The triploid individuals were randomly scattered within the diploid and tetraploid clusters: they did not form their own cluster like that generated with morphological and chloroplast markers. The AFLP analysis of all samples including those from northern Scandinavia showed no major deviation from the results described above.

### Birch introgressive hybridization

All but a few rare AFLP alleles were shared by all ploidy groups, indicating introgression via hybrids, also known as hybrid introgression or introgressive hybridization. Without the triploid hybrid group, the shared genomic variation between the two co-existing *Betula* species could have been the result of incomplete lineage sorting rather than due to reticulate evolution by introgressive hybridisation. Triploid individuals carry the same alleles as diploid and tetraploid plants, meaning that such alleles have been transferred via backcrossing of the

hybrids with their parental species, presumably in both directions. As over 97% of our samples indicated signs of introgression, we cannot identify a preferred direction. One of the ways to infer the directionality of introgression with genomic data such as that in the present study is to include unadmixed outgroups in the sampling design for comparison. Therefore, the possibility for a study later would be to collect samples from populations of relatively pure *B. nana* and *B. pubescens* based on morphology according to floras. For *B. nana*, we have in our collection samples from the highland populations, such as "Blöndulón" (location 65.36 N/19.80 W, altitude 452 m), which is not included in the present study, but this population includes 97% diploid *B. nana* and 3% triploids [9]. There is no pure *B. pubescens* in Icelandic woodlands, that is, no individuals with the morphology indices of 11–13 based on Flora Europaea (see also Table S1). Therefore, we plan to include samples of *B. pubescens* from the Carpathians through collaborations.

As stated in the introduction, our studies of morphological variation and chloroplast DNA haplotyping indicated bidirectional introgression [9, 13], and our crossing experiments confirmed at least the diploid-to-tetraploid direction [12]. Bidirectional introgression, symmetrical or not, is likely to be enhanced especially in the northern latitudes where the growing season is short but light-intensive (that is, with long summer days). Periods of the flowering and pollination among related plant species tend to overlap in this region, leading to hybridization. The two *Betula* species in Iceland (*B. nana* and *B. pubescens*), and elsewhere in the arctic, overlap in both their distribution and phenology [6, 7, 30]. Triploid hybrids can be formed readily by an open-pollinated *B. nana* plant [31], indicating the abundance of *B. pubescens* pollen at the time the female flowers of *B. nana* are receptive. Also, as pollen from tree/shrub *B. pubescens* can travel further in the air, compared with pollen from low-lying dwarf *B. nana*, triploid hybrids can be pollinated easily by *B. pubescens* (backcross), thus an introgressive gene flow occurs more readily from *B. nana* to *B. pubescens*. This could be the reason why bidirectional introgression between these two birch species in the arctic region can be such asymmetrical [22], the study that further indicates that AFLP introgression from *B. nana* to *B. pubescens* increased at more northerly latitudes. It is therefore likely that introgression between *Betula* species in more southern latitudes may tend to be unidirectional. Diploid-to-tetraploid birch introgression in Britain is a good example, whereby both diploid *B. nana* and diploid *B. pendula* have evidently introgressed into the tetraploid *B. pubescens* [32, 33]. It is, however, not clear at this stage whether this pattern holds true for other regions of natural *Betula* distribution. As of North America, a recent molecular study of *Betula* shrubs [34] depicted gene exchange and unidirectional introgression from diploid *B. glandulosa* Michx. into tetraploid *B. pumila* L.

The diploid-to-tetraploid gene flow may also occur in the absence of triploid bridge, that is, via hybridisation between unreduced (2*n*) gametes in the diploid species and normally reduced (2*n*) gametes of the tetraploid species and backcrossing there after [35]. In the case of *Betula* in the present study, gene flow from *B. nana* to *B. pubescens* could happen directly via unreduced gametes at the same time as gene flow via triploid bridges. But gene flow in the opposite direction could only happen via the triploid bridge. Our meiotic study of triploid hybrids [36] showed that although normal triporate pollen occurred in a very low frequency, the pollen grains were present in two sizes, presumably 1*n* and 2*n* euploid sizes. Morphometric measurements of triporate pollen grains from 22 triploid *Betula* plants from ten woodlands around Iceland (20 of these triploid plants were analysed in the present AFLP study) revealed two pollen sizes, but the small (1*n*) *B. nana* size was far more prevalent than the larger (2*n*) *B. pubescens* size [37]. Pollen from these triploid plants was found to be variably viable – while most triploid plants had a pollen germination of less than 2%, some showed an exceptionally high pollen viability of 11–79% [10]. If the 1*n* viable pollen of the triploid hybrid backcrosses with *B. nana*, gene flow can occur in the tetraploid-to-diploid direction, that is via triploid bridge.

In the present AFLP study we also found that all alleles are shared in all but one location/woodland in Iceland, meaning that the introgression is widespread. This is largely congruent with our previous chloroplast haplotype variation analysis [13]. The cpDNA study found the most common T-haplotype to be distributed in all 12 woodlands around Iceland as well as in the same five woodlands in Norway and Sweden as those analysed in the present study (T forms 49% of total haplotype diversity in Iceland and 43% in northern Scandinavia, but 0% in Scotland). Gene flow occurs via both plastid and nuclear genomes, and independently, as the present study finds no pattern of association between the two marker types. The other half of chloroplast haplotype diversity is divided geographically, forming an east–west pattern in Iceland. Our AFLP results support this statistically, both by AMOVA (highly significant) and anamorphosis. The AFLP results provide additional insights. The smaller Reynolds distance observed among western populations, compared with eastern populations, may be indicative of a more recent origin, or older populations having been through high genetic drift.

It is also possible that the (genetic) east–west separation is due to long distances and physical barriers to gene flow, such as mountains and glaciers. The anamorphic

analysis in the present study shows genetic distance to be the longest between the southeastern (G- Jökulsá, near Vatnajökull Glacier) and northwestern (M- Kaldalón, near Drangarjökull Glacier) locations. Indeed, both plastid and nuclear markers detected statistically significant correlation between genetic and geographical distances, especially in the dwarf birch *B. nana*. The tree/shrub birch *B. pubescens* appears to be more homogeneous genetically, that is, with more gene flow and less differentiation between populations. This pattern is compatible to that found in the AFLP analysis of *B. nana* and *B. pubescens* throughout their range of distribution [22], whereby *B. nana* in Iceland is genetically differentiated, due to the difference in its postglacial origins, while *B. pubescens* is relatively homogeneous.

#### Species integrity and ploidy stability despite gene flow

The present study revealed clear separation between diploid (2*x*) dwarf birch *B. nana* and tetraploid (4*x*) downy birch *B. pubescens*, as shown by the clustering of allele frequencies using Linarius and the AMOVA by ploidy. This result is opposite to that obtained from the chloroplast haplotype variation analysis of the same sample set [13], where the variation among ploidy groups is not statistically significant. Other studies on *Betula* have also observed nuclear separation despite extensive introgression in the chloroplast genome, for example, in North American birch using microsatellites [38] and among Eurasian species [39]. Furthermore, a clear difference based on AFLPs was also shown between *B. nana* and *B. pubescens* in the Arctic [22] and between diploid *B. humilis* and *B. pubescens* in Poland [40].

The species separation obtained from the AFLP dataset in the present study, however, is comparable to that of a morphological analysis based on species-specific botanical characters [9], whereby the diploid and the tetraploid groups can be differentiated more than 95% of the time. Their morphology is distinct, their ploidy different, but their genetic markers are almost identical. This could mean that birch introgressive hybridization favours nuclear alleles that keep species-specific phenotypic features intact, which are, logically, those adapted to their environment. The number of these genes does not need to be large if they affect many traits and have broad expression across plant tissues and organs. For example, the analysis of genomic data from thousands of individuals from 25 plant species [41] identified core genes enriched for signatures of repeated local adaptation to climate, including many genes with well-known functions in the abiotic stress response. In the case of *Betula*, there could be genes that drive adaptation for the species to thrive in its own habitats, and these genes may be introgression resistant. The two coexisting species in Iceland occupy different habitats, although they are mostly sympatric. Tetraploid downy birch (*B. pubescens*) occupies lower elevations and drier habitats, whereas diploid dwarf birch (*B. nana*) is more prevalent in the interior highlands and at colder sites [30]. Even in the same woodland, such as at Bifröst (population B in this study), *B. nana* grows on wet ground around Lake Hredarvatn, but *B. pubescens* grows as large shrubs on the hill slope, on dry land away from water (our own observation). Triploid birch individuals in this woodland, for example B72 (Fig. 2), grow in an open area in between the two habitats. This example of habitat preference supports the notion that the two *Betula* species are not in competition for resources above or below ground. It also means that they are preferentially adapted to their own environmental niches.

Although triploid birch plants thrive well in these transition zones, there are no data on fitness or adaptability of the triploids in their own niche or in habitats occupied by the two species. Nevertheless, the triploid hybrid is not likely to invade into the occupied habitats, as it does not have sufficient sexual fertility to expand its population size, due to its meiotic constraints [36]. Triploid hybrids appear only to play the role of bridging introgression (gene flow) between the two parental species via backcrossing, because they are sufficiently fertile [10]. No AFLP markers were found to be specific to the triploid group. The AFLP clustering placed triploid individuals within either the diploid or the tetraploid groups. However, we discovered one 179 bp AFLP marker that was overrepresented among triploids, compared with the diploid and the tetraploid groups. This could be a marker of biological interest, for example, for hybrid survival.

This *Betula* introgression is not likely to be random, but which probably provides the two species with broader genetic diversity serving as resources for adaptability, for example, in a changing environment. Indeed, the so-called 'adaptive introgression' has been discovered in many species, plants and animals [42–44]. Numerous adaptive traits in plants include tolerance to environmental stresses and disease resistance [45]. In *Betula*, a functional analysis of *B. pubescens* loci containing alleles introgressed from *B. nana* identified multiple genes involved in climate adaptation [46]. The genome-wide molecular study of introgression in poplar (*Populus* L.) in the Rocky Mountain region of the United States and Canada [47] showed that adaptive introgression from one species into the genomic background of another may constitute a mechanism facilitating adaptation at range limits. *Populus* species at these range limits are faced with gradients of photoperiod and temperature. The analysis of genetic and morphological variability of several rowan species (*Sorbus* L.) from the Tetra Mountains,

the highest mountain range in the Carpathians with typical alpine soil and climate conditions, also indicated adaptive introgression [48].

Species integrity despite geneflow is shown here to be the case with *Betula* in Iceland. Nuclear markers have revealed this pattern in several *Betula* species throughout their respective distribution range [22, 32], and in other tree species, for examples, *Picea* (Pinaceae) in North America [49] and *Brownea* (Fabaceae) from the Amazon [50]. Such maintenance of species integrity is thought to be through environmental selection. In the case of *Betula* in Iceland, the two species appear to be adapted to different habitats within their shared location, as described above. But the environments change. Numerous studies of *Betula* microfossil pollen in Iceland since the deglaciation, the beginning of Holocene epoch, revealed alternating periods of birch woodland expansion and decline depending mostly on the climate [51, 52]. Expansion of the woodland dominated by shrub birch *B. pubescens* followed climate warming in northern Europe and the decline appeared to occur after climate cooling, due to, for example, major volcanic eruptions and glacial floods. Further loss of woodland vegetation during the Anthropocene is also well documented [53]. Our work on fossil birch pollen showed period of intense hybridisation between *B. nana* and *B. pubescens* following birch woodland expansion during the warming periods, at the onset of the deglaciation around 9.5–7 cal. ka BP and again around 5–3.5 cal. ka BP within the mid-Holocene Northern Hemisphere warming [54]. With ongoing global warming, a new wave of birch hybridisation appears to have started in the last few decades in Iceland. Birch woodlands are likely to become more widespread. Introgressive hybridization in birch is expected to increase, providing more natural genetic variation, a valuable resource for adaptation in the changing environment.

#### Methodological aspects of the data analysis

A unique feature of the present study is that the analysis of genetic distance was complemented by the designing of an anamorphosis cartogram. Several distance indices have been described. The most common is Nei's distance [55]. An issue arises when calculating Nei's distance: should we consider an absence of a band an allele? Several polymorphisms may lead to absence of a band, including polymorphisms at either ends of, or mutations within, the fragment, forming a new restriction site. Therefore, only the presence of a band may be considered an allele. With such an assumption, Nei's index appears very noise-sensitive and potentially irrelevant when considering dominant markers. Cavalli-Sforza chord distance [56] cannot be computed as it requires knowledge of all possible alleles. If not, it results in the square root of a negative value. Computing such an index would be complex for dominant markers. Euclidian-based distances seem more natural for dominant markers. True Euclidian distance is not bounded in [0,1], thus may not be suitable for some calculations. Reynolds [57] and Rogers [58] distances were also considered. For anamorphosis purposes, the use of Rogers index resulted in an identical image to the one obtained when using Euclidian distance. Reynolds distance was therefore chosen because one of its assumptions, that of the nonmutation model, was consistent with the observed pattern of gene flow.

In the present study we used Reynolds distance calculation method to generate an anamorphic map of *Betula* populations in Iceland. To our knowledge, anamorphosis has never been used in the field of molecular biology. Its general usage to weight a phenomenon according to area is documented in several fields, such as health sciences, demography and economics [59]. It has been used in epidemiology, which seems to be its only use in a field related to biology [60]. The representation of distance by anamorphosis is still at an experimental stage – currently, a common use is in the coordination of transportation networks [61]. In the present study, the methodology we designed consisted of three mains steps. The first step was to consider a position as fixed, then the distance was adjusted between this point and every other according to the genetic distance. This provided only partial information, as locations genetically closer to the focal point tended to collapse with it, while others were spread out. The second step was to apply this method to every point. The result was a cartogram that represented an adjustment of every distance according to the population genetic distance. The cartogram is visually more readily understandable, but still has a drawback: the result depended on the order in which we considered each point as the focal point. As with our dataset, bootstrapping and averaging resulted in a stable cartogram. The last step was a dilation using centre of gravity as centre and calculate the ratio of its distance to the original (source) points over its distance to the image points, to get a more harmonic scale between source and image. This improved the readability of the anamorphic cartogram. This methodology can still be improved, but in its current implementation, it already allows a view of the results that is more readily interpretable than a dendrogram, especially for readers who are not familiar with the sampling areas.

## Conclusion

While confirming introgressive hybridization between the diploid *Betula nana* and the tetraploid *B. pubescens* via triploid hybrids, the present study shows that the introgression involves the whole nuclear genomes of both species. There is no genome- or species-specific marker, and thus, a given allele can be present in any homoeologous or ohnologous loci. Nevertheless, *Betula* maintains its species integrity and ploidy stability. We postulate that birch introgressive hybridization could favour nuclear alleles that keep species-specific phenotypic features intact. These features are likely those that have enabled adaptation to the environment and habitat. Based on our previous studies, the ploidy stability itself is most likely an (advantageous) outcome of a stringent prezygotic reproduction barrier within triploid hybrids, preventing the formation of aneuploidy. The AFLP study also indicates that *B. nana* populations in the western part of Iceland are distinct from those in the east, while *B. pubescens* populations are more homogenous, yet the diploid *B. nana* and the tetraploid *B. pubescens* have coexisted almost everywhere in Iceland. The coexistence naturally promotes introgressive hybridization. Such introgression is likely to have genetic advantages that allow both *Betula* species to survive in their woodland habitats over time and in a constantly changing environment. The triploid bridge enables gene flow between the two species and at the same time serves as a reproduction barrier preventing homogenization across species boundaries.

## Supplementary Information

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Supplementary Material 1.

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**Authors' contributions**
BL conducted the AFLP experiments and statistical analyses, created the Linarius package and drafted the first version of the manuscript. KAJ and ÆTT conceived the idea of this study, designed the experiments, conducted field sampling both in Iceland and Scandinavia, and karyotyped all samples. KAJ secured the funding, supervised the project and led the manuscript writing. All authors contributed to the article and approved the submitted version.

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**Data availability**
All data and materials can be available upon request.

## Declarations

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
All authors give consent for publication.

**Competing interests**
The authors declare no competing interests.

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## Publisher's Note