Morphometric discrimination among *Betula nana, Betula pubescens* and their hybrids using the shapeR package

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10 eininga ritgerð sem er hluti af
Baccalaureus Scientiarum gráðu í Líffræði

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Líf- og umhverfisvísindadeild
Verkfræði- og náttúruvísindasvið
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Útdráttur

Ilmbjörk (*Betula pubescens*) og fjalldrapi (*Betula nana*) eru náttúrulegar skóglenda tegundir á Íslandi og mikilvægar í íslenskum landvistkerfum. Þær eru báðar af sömu ættkvísl og geta myndað blendinga sín á milli. Þar sem ilmbjörk er fjórlitna og fjalldrapi tvílitna verða blendingar þeirra þrílitna. Blendingar ilmbjarkar og fjalldrapa geta síðan æxlast við ilmbjörk eða fjalldrapa og þannig flutt erfðaefni milli tegundanna tveggja. Þetta hefur orðið til þess að íslenskir stofnar ilmbjarkar og fjalldrapa deila ýmsum útlitseiginleikum og er því stundum erfitt að greina á milli þeirra og þá sérstaklega milli blendinganna og tegundanna tveggja. Þessi rannsókn gerir tilraun til þess að auðvelda aðgreiningu tegundanna og blendinga þeirra með því að nota ShapeR pakkann í R, sem gerir notandanum kleift að draga útlínur og stika af myndum, lýsa breytileika ásamt því að framkvæma tölfræðileg próf. Með því að nota þenna pakka ásamt tveim öðrum pökkum fyrir tölfræðigreiningu gagna var unnt að greina á milli ilmbjarkar, fjalldrapa og blendinga út frá lögun laufblaða þeirra með talsverðri nákvæmni. Þær breytur sem voru gagnlegastar til að aðgreina tegundirnar og blendinga þeirra voru lengd og breidd laufs, ásamt hlutfalli ummáls þeirra og flatarmáls. Aðferðin gæti nýst við rannsóknir á stofnum ólíkra tegunda birkis og blendingum þeirra.

Abstract

Downy birch (*Betula pubescens*) and dwarf birch (*Betula nana*) are natural woody species in Iceland and play important roles in the terrestrial ecosystems. They both belong to the same genus and can hybridize. Since downy birch is tetraploid and dwarf birch is diploid, their hybrids become triploid. These hybrids can then backcross with either one of their parental species and thus transfer genes between the two species. This has resulted in a considerable amount of similar morphological characteristics between the two species, and consequently the hybrids are not clearly distinguishable from their parent species. This study attempts to facilitate discrimination between the two species and their hybrids using the ShapeR package in R, which enables the user to extract outlines and several traditional morphometric parameters from image samples and to conduct statistical testing. Using the ShapeR package in conjunction with other R packages used for statistical analysis, it was possible to discriminate between both species and their hybrids with unprecedented accuracy. Surprisingly, the most effective discriminants were among the simple traditional morphometric parameters that the ShapeR package extracts. These were leaf width, length and ratio of leaf perimeter and leaf area. This approach can be used efficiently and reliably in the species-hybrid differentiation in population-based studies of *Betula* species.

Þakkir

Ég vil þakka leiðbeinendunum mínum, Kesöru Anamthawat-Jónsson og Snæbirni Pálssyni fyrir mikilvæga aðstoð og leiðsögn. Einnig vil ég þakka Lísu Anne Libungan fyrir kennslu á shapeR pakkann, Hildi Magnúsdóttur fyrir að lána mér myndavél og kenna mér á hana og Ægi Þór Þórssyni fyrir að safna sýnum sem ég notaði við verkefnið.

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Introduction

Downy birch (*Betula pubescens*) is the only plant species in Iceland that forms continuous natural woodland and thus is of considerable importance to local ecosystems. *B. pubescens* originates in mainland Europe, and is represented by two subspecies; *pubescens* and *tortuosa*. The nominal subspecies *pubescens* can grow up to 25 meters tall, either monocormic or polycormic (with single or many stems), whereas the latter subspecies tortuosa has a much lower stature forming shrubs or small trees and has a distribution in mountainous regions in northern Europe and is thus called mountain birch (Walters 1964; Atkinson 1992). This phenotype is believed to be a result of introgressive hybridisation with the dwarf birch *Betula nana* L. (Elkington 1968; Anamthawat-Jónsson 2012), which is a prostrate shrub growing only up to 1m in height, represented by subspecies *nana* in Europe and western Asia and subspecies *exilis* in eastern Asia and North America (Atkinson 1992; de Groot *et al.* 1997).

The *B. pubescens* population in Iceland shares many characteristics with the Fennoscandian *tortuosa* subspecies, but is not treated here as a subspecies itself due to extensive morphological variation within and between populations in Iceland. These variations can probably be explained by extensive hybridization and consequent introgression between dwarf and downy birch in Iceland, facilitated by significant overlap in distribution and flowering times between the two species and possibly because of climatic factors having a negative effect on the efficiency of their incompatibility systems (Thórsson *et al*. 2007).

Historically, *B. pubescens* is believed to have covered most of Iceland's lowland before settlement around the ninth century but receded into isolated pockets covering only around 1% of the total land area today due to extensive deforestation over the last centuries (Jónsson 2004). However, the recent survey by Snorrason *et al*. (2016) has shown that birch woodland has indeed expanded to being about 1.5% land area during the past twenty years, due mainly to new growth (young plants), and this appears to correlate with an increase in mean summer temperature in certain regions of the country.

Hybridisation between dwarf and downy birches in Iceland is not only a current event but seems to have taken place at several periods in the Holocene, mainly due to downy birch expansion facilitated by a warming climate (Karlsdóttir *et al*. 2016). Although both monocormic and polycormic plants are found in Iceland, the latter dominates natural woodlands. There is also considerable variation in height among plants, some mature trees growing up to 10-12m in inland areas, but the majority of natural woodland being dominated by plants of less than 2m height (Jónsson 2004). These variations may be explained by environmental factors, but as discussed above, genetic factors might have a significant explanatory power as well (see review by Anamthawat-Jónnson 2012).

Since downy birch (*B. pubescens*) and dwarf birch (*B. nana*) have different ploidy levels, $2n=4x=56$ and $2n=2x=28$, respectively, their hybrids have an intermediate ploidy level of $2n=3x=42$. This can be used to distinguish with great accuracy between the two species and their hybrids. Counting chromosomes is, however, labourious and time consuming so alternative methods of classification would be of great value for further research on the subject.

An alternative method of discrimination between *B. pubescens* and *B. nana* has been tested in previous studies on dwarf and downy birch in Iceland, involving traditional morphometric analysis of birch leaves (Thórsson *et al.* 2007). The morphology of leaves is especially useful in this context since they are relatively independent of environmental factors. This method revolved around a score system, so-called morphology index, taking several discriminants into account, and giving each plant a rating based on the prominence or existence of different morphological features. Quantitative discriminants such as leaf length and area were also measured and incorporated into the score system. In this method, 30 leaf samples were taken from each individual plant and given scores based on their discriminants. Plant-wide discriminants such as procumbence habit and plant height were also taken into consideration. The mean of scores for each plant was then used to identify its species. The results of this study were verified by analysing ploidy levels of each plant. The morphology proved to be a useful method for differentiation between dwarf and downy birch but the hybrids were more difficult to classify, since they could both display the morphological score of either parent species or exhibit an intermediate morphology. Leaf length (including petiole) proved to be the single most effective discriminant in this study (Thórsson *er al.* 2007), identifying up to 40% of the triploid hybrids correctly, whereas many other discriminants contributed very little to the classification and one variable even had a negative effect on differentiation between groups.

The quantitative discriminants used in the study by Thórsson *et al.* (2007) were extracted automatically using an image scanner and the WinFolia software (Regent Instruments, Quebec, Canada). The qualitative discriminants, however, were evaluated ‘manually’ by the researchers themselves (Thórsson *et al*. 2007). This kind of evaluation is dependent on skilled evaluators and is somewhat vulnerable to human error and inconsistency. If leaves can be differentiated effectively without the use of these qualitative discriminants it could be of considerable utility to further studies in the field. This present study aims therefore to establish dependable quantitative discriminants that can differentiate between *B. pubescens, B. nana* and their triploid hybrids with sufficient accuracy.

ShapeR (Libungan & Pálsson 2015) is an R package (R core team 2017) which attempts to facilitate computerized classification of any two dimensional object. Although originally developed to analyse fish otolith shape to discriminate between fish stocks it is suitable to analyse variation in any two dimensional object. Several morphometric methods are utilised in shapeR, mainly outline analysis based on independent wavelet or Fourier coefficients, but the program also extracts traditional morphometric parameters such as maximum length and width, perimeter and area. The package then facilitates further statistical analysis, including a linear discrimination-model in order to distinguish between specimens and predict their affinities to certain samples, and also to establish which parameters best describe the variance between groups (Libungan & Pálsson 2015).

ShapeR is a promising tool for analysis of leaves, since parameters are extracted automatically by the program and the analysis is largely automated. However, the package does require some degree of sentience in converting samples to a format recognizable by the package. This includes capturing images of leaf samples and using image manipulation programs to maximise contrast between an object and its background for shape extraction to work properly.

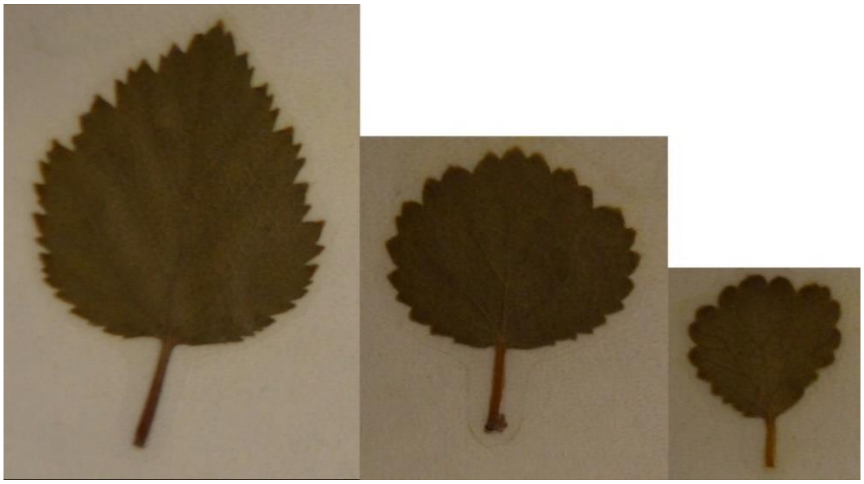

Figure 1: Examples of leaves from *Betula* pubescens (4n), hybrid (3n) and *Betula* nana (2n).

In this study, morphological variation in birch leaves from two birch species (*B. pubescens*, *B. nana*) and their hybrids (Fig. 1) is evaluated and used to distinguish between the three ploidy groups, by using traditional morphometry along with outline analysis, applying the shapeR package (Libungan and Pálsson 2015) to this end.

Methods

Sampling

Leaf samples preserved from the previous study by Thorsson *et al.* (2007) were used. The samples were collected from three sites (Bifröst, Vagla-Hálsskógur and Ásbyrgi) in Iceland (Fig. 2, Table. 1).

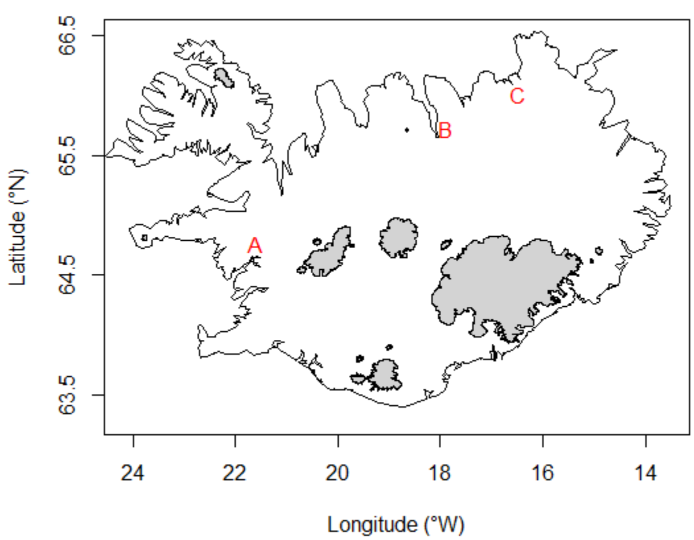

Figure 2: Map of Iceland showing sampling locations. A: Bifröst, B: Vagla-Hálsskógur, C: Ásbyrgi. Further information is given in table 1.

The samples used were gathered when leaves had matured, mostly in July. Thirty leaves were originally gathered from each plant (with a total of 461 plants), and from as many branches as possible. Most of the leaves collected were from positions 3-5, counted from the shoot tip (Thórsson *et al*. 2007). A subset of these leaves, including one leaf per plant were used in this study, representing the three different ploidy groups of *B. nana*, *B. pubescens* and their hybrids (Table 1).

Table 1: Information about the samples used, including location and ploidy levels. Labels refer to sites in Fig.1.

Location	Label	N	Diploid	Triploid	Tetraploid
Bifröst	A	28	19	2	7
Vagla-Hálsskógur	B	11	1	0	10
Ásbyrgi	C	0	0	7	2

Images and data files

Images were captured of a single leaf from each plant and stored on a computer file system in separate sub-folders according to their ploidy group, which had been established in the previous study using ploidy level analysis by Thorsson *et al.* (2007).

Another folder was created for images after they had been manipulated to maximise contrast between leaf and background (Fig.3). The manipulated images were then stored in the same manners as the original images within this new folder. Both of these folders were then stored in a super-folder. The setup of the folders followed recommendation from the shapeR package (Libungan and Pálsson 2015).

A data file in csv format (*.csv), containing information about the samples (such as image name, ploidy level and ploidy group), was also included in the super-folder.

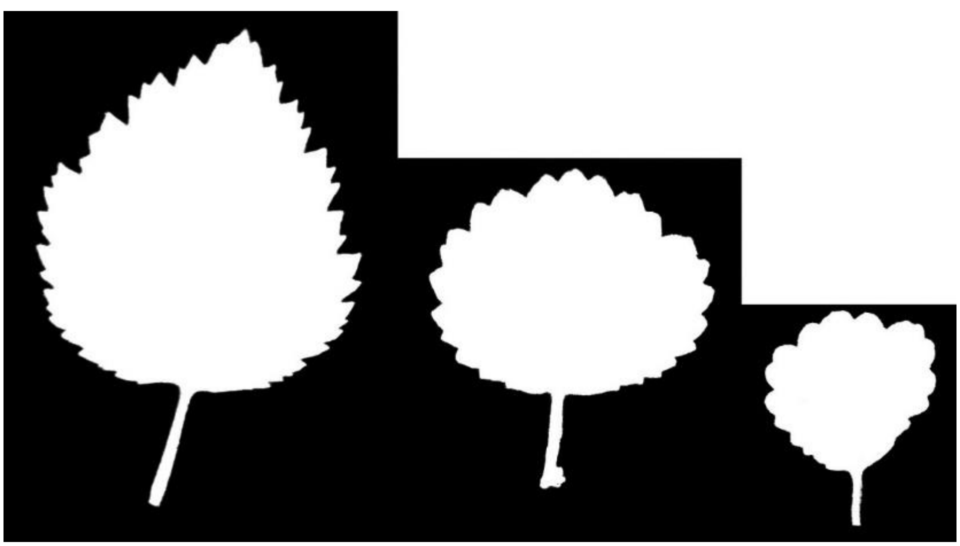

Figure 3: An example of how the images look when the contrast between the leaves and their background has been adjusted. Each leaf is analysed individually. From the left; *B.pubescens*, hybrid, *B. nana*.

Morphometric analysis

Outlines were extracted from each sample along with four traditional morphometric discriminants; maximum leaf length and width, leaf area and perimeter length using the **detect.outline** function from 'shapeR' package. It should be noted that the longest axis of the leaf, or any other object measured using shapeR is labeled as 'otolith.width' and the orthogonal axis as 'otolith.length'. In this paper the axis from the leaf tip to the end of the petiole (leaf stalk) is called 'length'. Shape coefficients were also extracted from the leaf outlines in an automated manner with Wavelet and Fourier analysis using the generateShapeCoefficients function from the 'shapeR' package.

The variation of the shape coefficients, along with the leaf outline in polar coordinates were analysed using standard deviations partitioned among groups by calculating the intraclass correlation coefficient (Sokal & Rohlf. 2012), using **plotWaveletShape** and **plotFourierShape** functions from the 'shapeR' package. This gave an idea of which areas of the leaves showed the greatest variation between the ploidy groups (i.e. species and their hybrids) or how it was distributed along the leaf outlines.

All discriminants (including the traditional morphometric discriminants) were tested with anova-like permutation tests using the **anova.cca** function from the'vegan' package (Oksanen *et al.* 2013), and then visualized with cluster plots and box plots in order to estimate which of them showed the greatest divergence between ploidy groups. The traditional discriminants were also combined in order to remove the size variable, providing a better picture of their morphometric relations within ploidy groups.

Finally, the classificatory power of the discriminants was estimated using linear discriminant analysis. This was done using the **errorest** function from the 'ipred' package (Peters *et al.* 2011) which utilises cross-validation and bootstrapping (see Libungan and Pálsson 2015).

Results

Reconstruction plots of the leaf shape using the Wavelet and Fourier shape coefficients showed some amount of variation between ploidy groups, with *B. pubescens* showing the most distinctive double-tooth outline (Fig.3). It seems that Wavelet coefficients probably give a better approximation of the actual leaf shapes, but the Fourier coefficients seem to generate more rounded outlines. This is probably due to the Wavelet coefficients taking more discrete variations into account, such as the dentate leaf margins of *B. pubescens* leaves. The Fourier coefficients seem to show a much greater variation between *B. pubescens* and the other groups with respect to the width axis (or the y-axis in the figures).

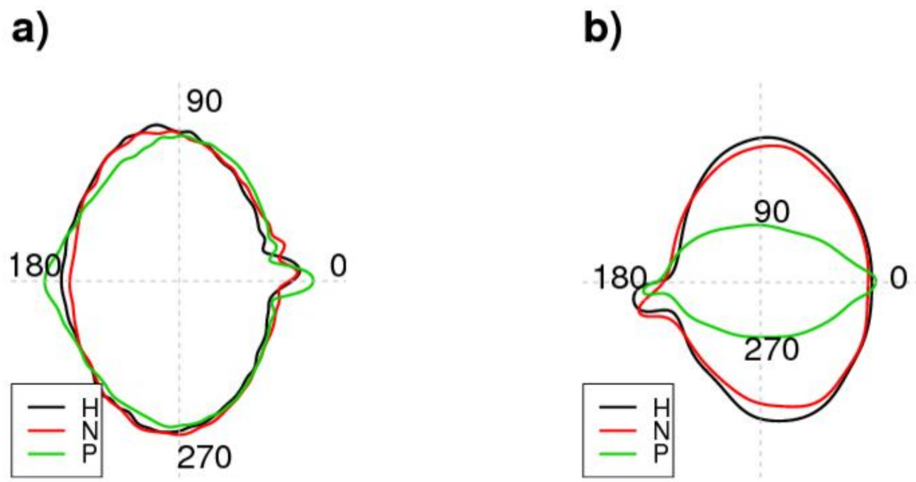

Figure 4: Mean shape using Wavelet and Fourier coefficients, respectively. The numbers represent angle in degrees.

The quality of the reconstruction plots was estimated by comparing proportional deviation from outline with the number of Wavelet levels or Fourier harmonics used for the reconstruction (Fig.5). This gave an idea of how many Wavelet levels or Fourier harmonics were necessary to reconstruct outlines accurately. Much fewer Wavelet levels were needed to accurately describe the outlines than Fourier harmonics. Since the Wavelet coefficients seemed to be more effective at describing the real leaf outlines they were used instead of the Fourier coefficients.

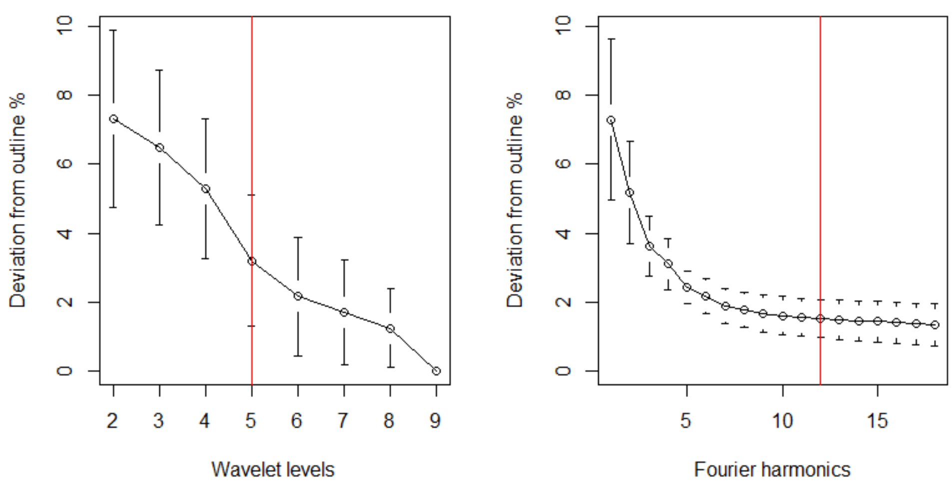

Figure 5: Outline reconstruction plots showing quality of reconstruction based on Wavelet levels and Fourier Harmonics. The red vertical lines show how many Wavelet levels or Fourier harmonics are needed for 98.5% accuracy of the reconstruction.

Variation between samples and among ploidy groups along the outline was also explored, showing that the greatest variation within ploidy groups was found around the 180° angle, which is the angle at which the leaf tip was generally located. This seems consistent with previous studies, but *B. pubescens* generally have a much longer and pointier tip than *B. nana* (Thórsson *et al.* 2007). The greatest variation between samples was found at the the 0°and 360°angles, which represent the petiole (Fig.6).

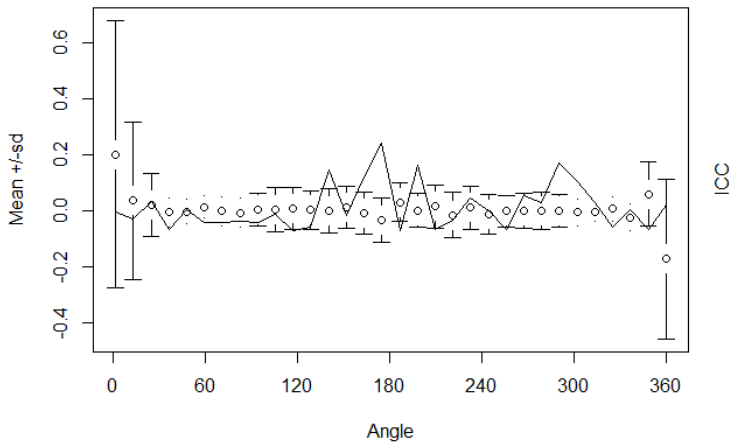

Figure 6: Mean and standard deviation of Wavelet coefficients combined from all ploidy groups. The intraclass correlation (ICC) is represented by a line to show proportional variance among the ploidy groups. The horisontal axis represents the angle along the leaf surface, starting from the leaf stalk.

Canonical Analysis of Principal Coordinates (CAP) was used on the Wavelet and Fourier coefficients to analyse the variation in shape among the ploidy groups. Using an ANOVA-like permutation test (anova.cca from the vegan package in R), by comparison of the partition of distances among groups versus distances within groups (Table 2). The test's results show clear differentiation among the three ploidy groups based on the Wavelet coefficients but not for the analysis based on the Fourier coefficients.

Table 2. Results of ANOVA-like permutation tests on Canonical Analysis of Principal coordinates with Wavelet and Fourier coefficients.

Method	df	Var	F	Pr (=>F)
Wavelet				
Modal	2	0.518	5.051	0.001
Residual	45	2.308		
Fourier				
Modal	2	1.642	1.738	0.192
Residual	45	21.255		

Next, ANOVA-like permutation tests were performed on the Wavelet CAP values of two ploidy groups at a time (Table 3). The results of these tests showed that CAP values of *B. pubescens* compared with both hybrids and *B. nana* were statistically significant, but when *B. nana* and hybrids were compared they were not statistically significant. This suggests that the *B. nana* and hybrid ploidy groups will prove the hardest to differentiate using these discriminants.

Table 3. Results of ANOVA-like permutation tests on Canonical Analysis of Principal coordinates with Wavelet and Fourier coefficients with two ploidy groups tested at a time.

Ploidy groups	df	Var	F	Pr(=>F)
***B.pub* & hybrids**				
Modal	1	0.376	4.385	0.001
Residual	27	2.314		
B.pub* & *B. nana				
Modal	1	0.503	8.349	0.001
Residual	37	2.228		
***B. nana* & hybrids**				
Modal	1	0.157	1.631	0.095
Residual	26	2.499		

A cluster plot of the CAP axes indicated some degree of unique distribution within ploidy groups but not without overlap. The hybrids seemed to be distributed differently than the parent species but still had considerable overlap with them, occupying a fairly distinct area between the parent species but generally being positioned closer to *B. nana* than *B. pubescens* (Fig.7).

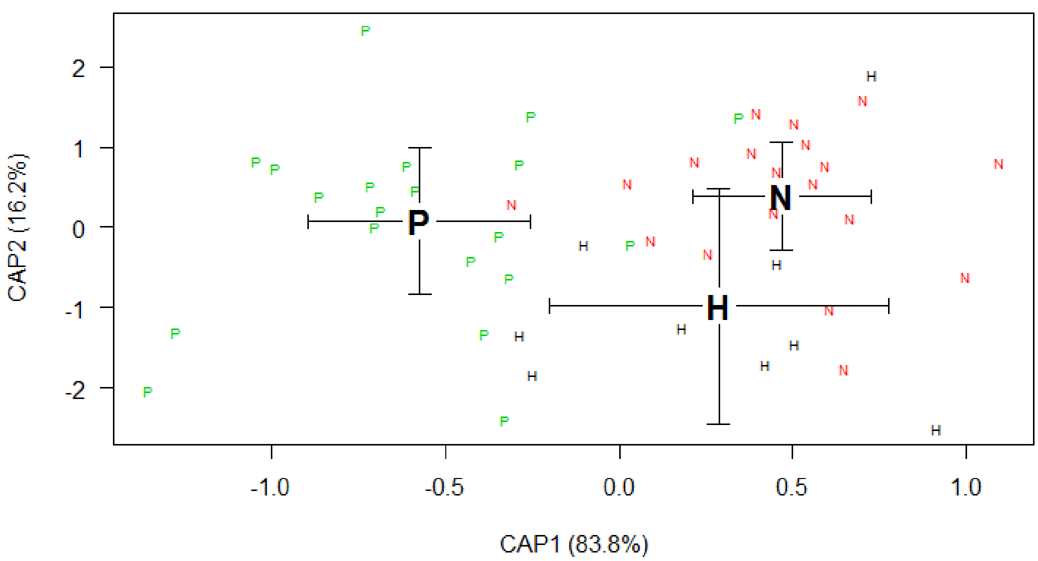

Figure 7. Canonical scores of constrained discriminating axes CAP1 and CAP2 based on Wavelet coefficients showing the proportion of variation among ploidy groups explained by either axis. 'H' represents hybrids, 'N' represents *B. nana* and 'P' represents *B. pubescens*. Large black letters represent the means of ploidy groups, and the intervals surrounding them represent one standard error. The smaller letters represent individual leaves.

Wavelet coefficient distribution of ploidy groups were compared and showed considerable overlap between ploidy groups and a high variation within ploidy groups (Fig.8), indicating that they might have limited effectiveness as discriminants.

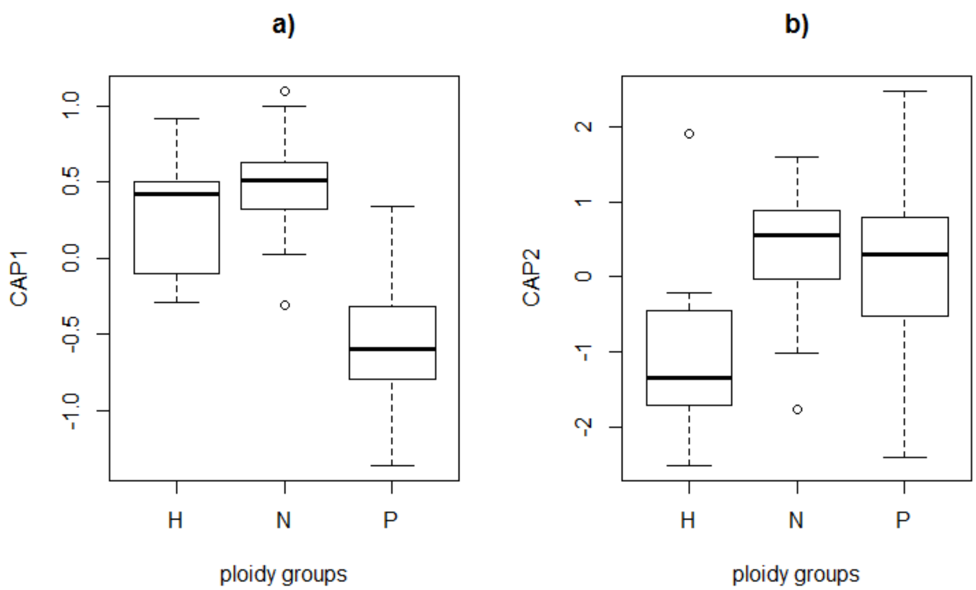

Figure 8: Distributions of canonical scores of constrained axes CAP1 (a) and CAP2 (b) based on Wavelet coefficients within ploidy groups. 'H' represents hybrids, 'N' represents *B. nana* and 'P' represents *B. pubescens*.

The traditional morphometric discriminants observed (length, width, area and perimeter) all showed fairly unique and consistent distributions between ploidy groups (Fig.9 and 10), perhaps due to differences in leaf size between *B. pubescens* and *B. nana* and the somewhat intermediate size often displayed by hybrids. This indicated that they were possibly useful for classification.

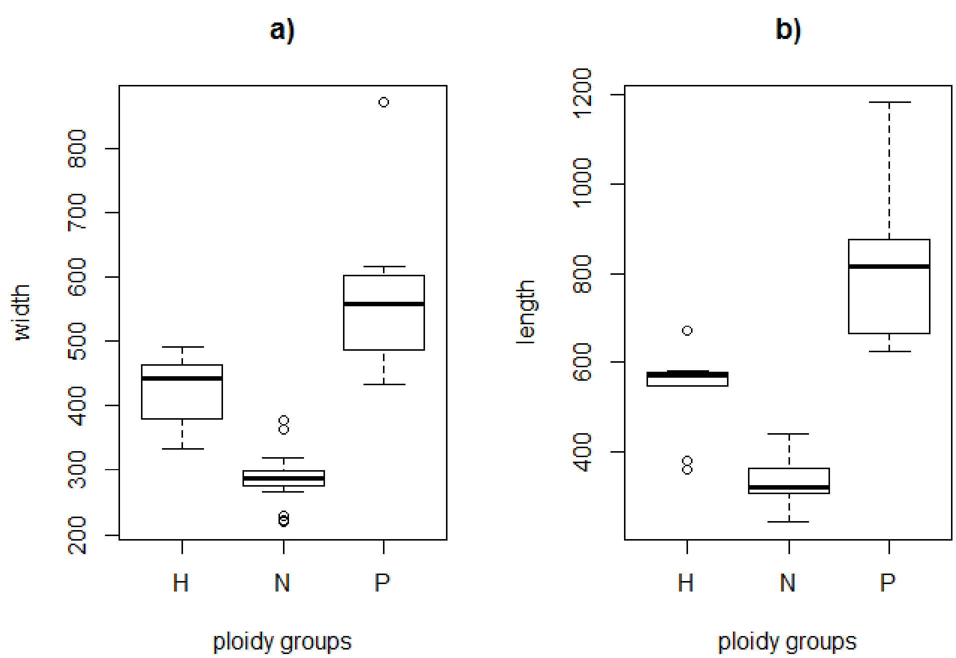

Figure 9: Distributions of maximum widths (a) and lengths (b) within ploidy groups. 'H' represents hybrids, 'N' represents *B. nana* and 'P' represents *B. pubescens*. On this scale, 100 equals ~0.44cm.

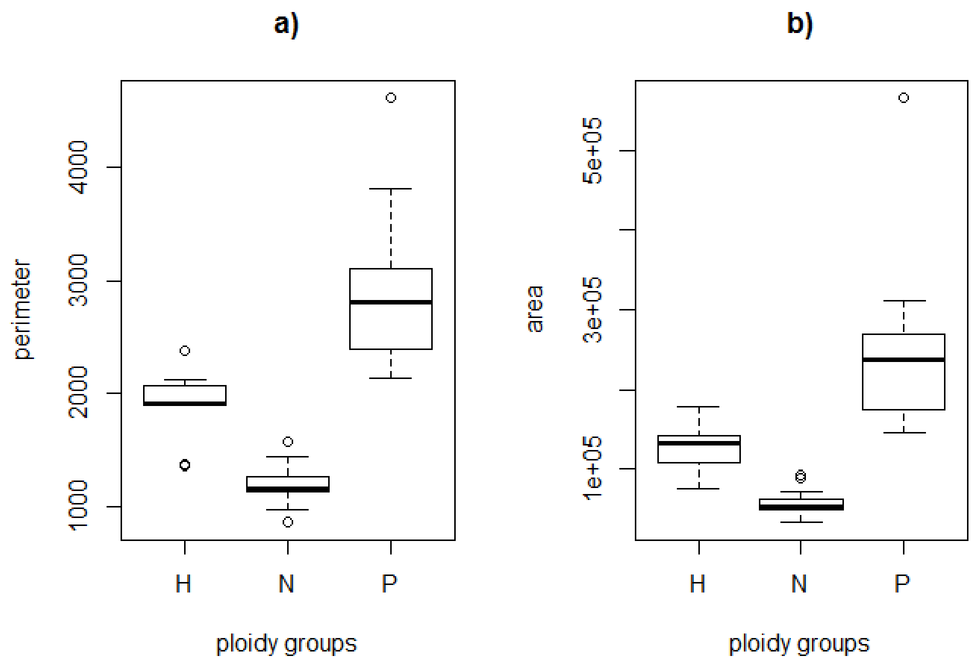

Figure 10: Distributions of area (a) and perimeter length (b) within ploidy groups. ‘H’ represents hybrids, ‘N’ represents *B. nana* and ‘P’ represents *B. pubescens*. On this scale, 1000 equals ~4.4cm.

Combining traditional morphometric discriminants eliminated the size variable and gave a better idea of how they related to each other within ploidy groups. The relationship between perimeter and area showed the most distinctive distribution, whereas combining width and length showed severely overlapping distributions, especially between *B. nana* and hybrids (Fig.11).

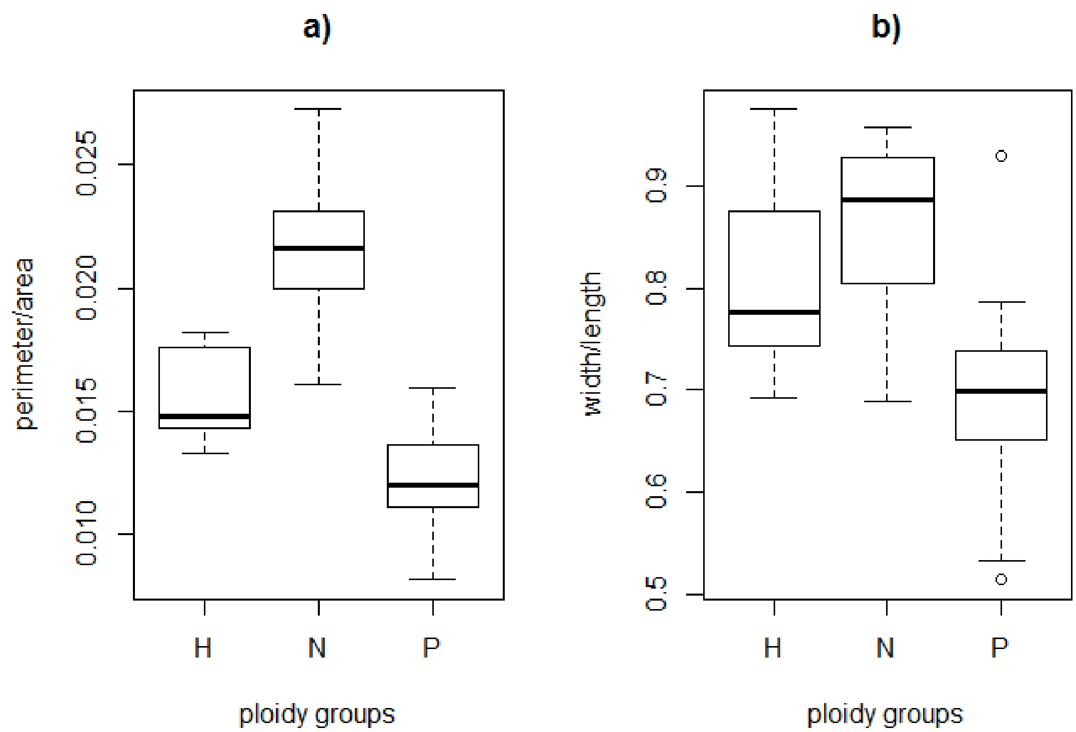

Figure 11: Distributions of perimeter/area (a) and width/length (b) within ploidy groups. 'H' represents hybrids, 'N' represents *B. nana* and 'P' represents *B. pubescens*.

Cluster plots using the traditional morphometric discriminants against the combined discriminants showed a fairly good divergence between ploidy groups, with hybrids generally occupying intermediate areas between the parent groups. Cluster plots showing perimeter or area against the combined discriminant of width/length showed a very narrow distribution of hybrids mostly occupying an intermediate area (although with some overlap) with the parent species having well defined areas distributions on either side of the hybrids, indicating that these parameters showed promise as potential classifiers (Fig.12). Cluster plots where width or length was plotted against the combined discriminant of perimeter/area showed a rather linear distribution, with a seemingly higher degree of overlap between the hybrids and parent species (Fig.13), but still showing similar trends as the former cluster plots. Cluster plots with the combined parameters against each other showed similar results as the former cluster plots, although with a wider distribution (Fig.14).

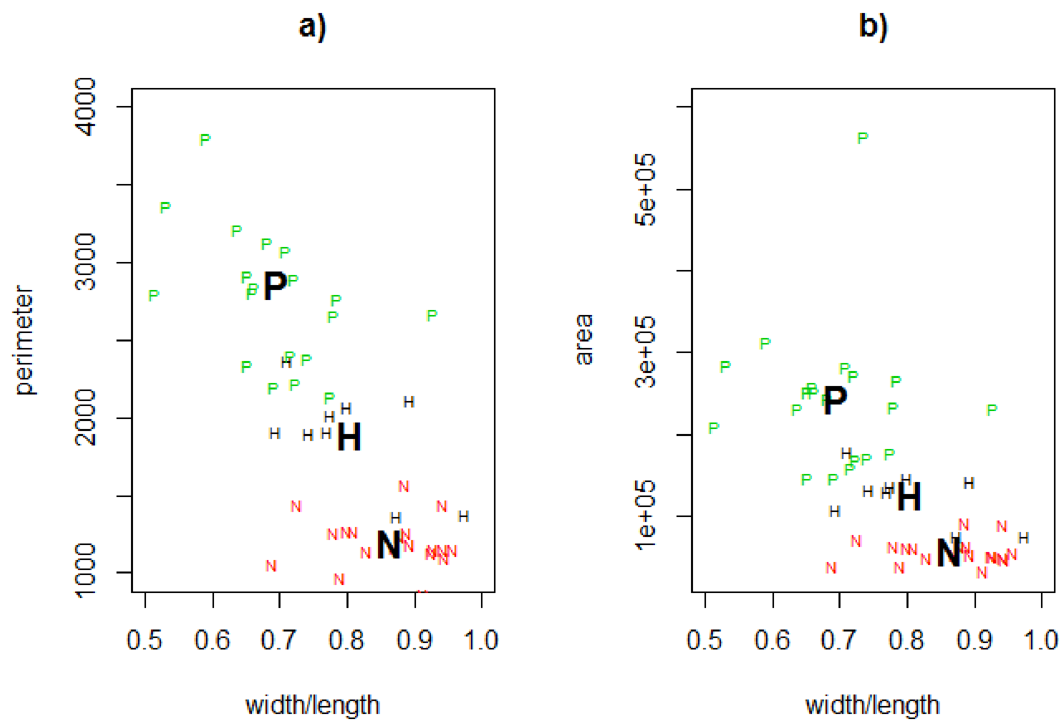

Figure 12: Perimeter (a) and area (b) against width/length. 'H' represents hybrids, 'N' represents *B. nana* and 'P' represents *B. pubescens*. Large black letter represents the means of ploidy groups and the smaller letters represent individual leaves. On the scale displayed on the y-axis, 1000 equals ~4.4cm.

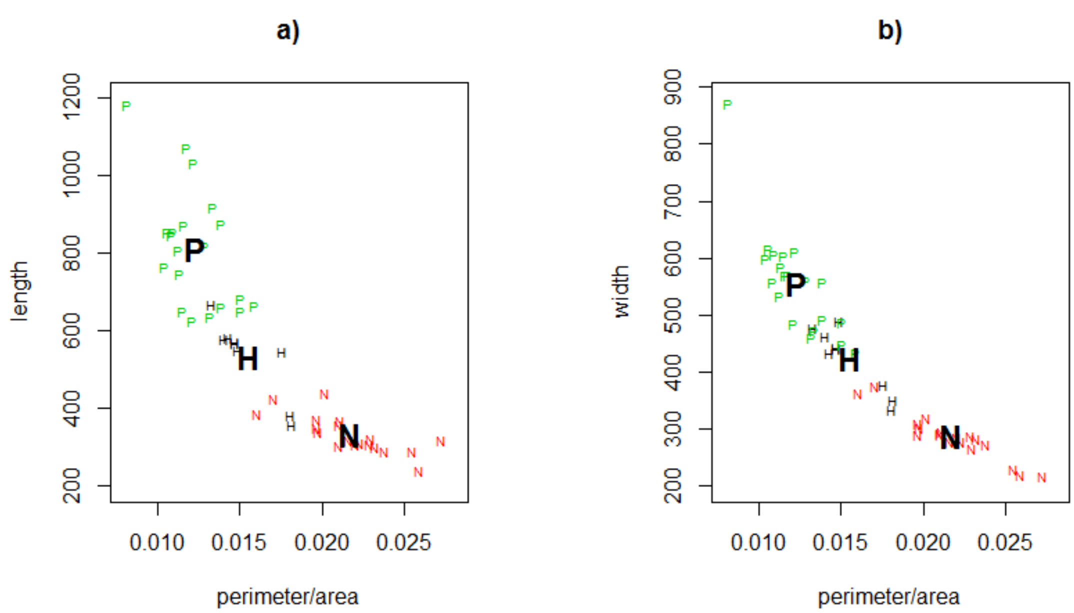

Figure 13: length (a) and width (b) against perimeter/area. 'H' represents hybrids, 'N' represents *B. nana* and 'P' represents *B. pubescens*. Large black letter represents the means of ploidy groups and the smaller letters represent individual leaves. On the scale displayed on the y-axis, 100 equals ~0.44cm.

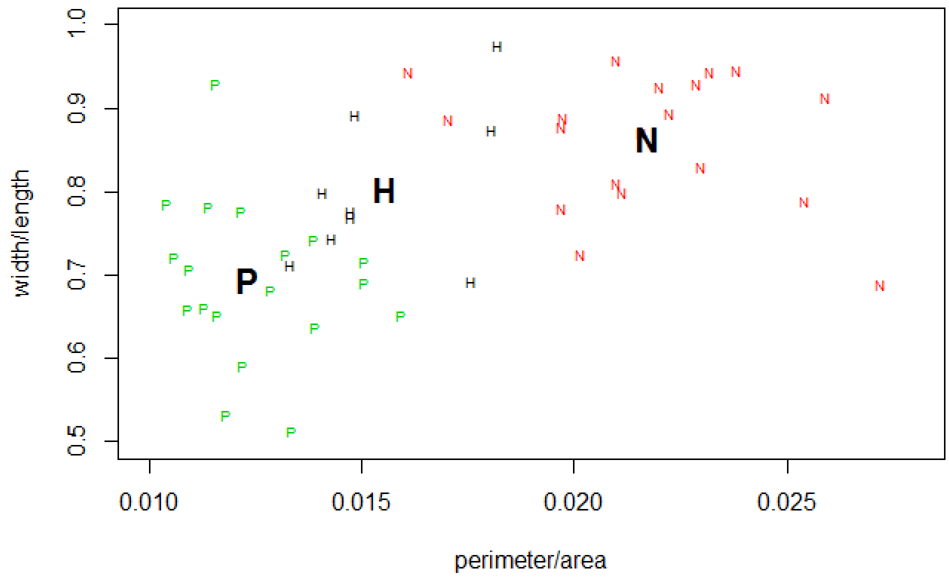

Figure 14: Cluster plot showing relatonships between both combined discriminants. 'H' represents hybrids, 'N' represents *B. nana* and 'P' represents *B. pubescens*. Large black letter represents the means of ploidy groups and the smaller letters represent individual leaves.

The most promising combinations of discriminants as well as the Wavelet coefficients and CAP axes were then tested with cross-validation and bootstrapping to evaluate their effectiveness (Table 4). The Wavelet coefficients proved to be ineffective. The CAP values obtained from these coefficients were fairly effective at classifying between species but less so when hybrids were also included. Some of the traditional morphometric discriminants were very effective, especially when used along with combined discriminants. The most effective discriminants overall were length or width in conjunction with the combined discriminant perimeter/area, as well as width in conjunction with length/perimeter. It is important to note that cross-validation splits the variables into two sets; a test set and a validation set, and the outcome of the error estimation depends on which variables end up in which set. This makes the outcome vary slightly so they are not 100% accurate, but can still indicate discriminant effectiveness.

Table 4: Error estimates (proportion of incorrectly assigned leaves to ploidy groups) of cross-validation tests with 1000 bootstraps using the discriminants examined.

Discriminants	*B.pub.* / *B. nana*	*B.pub.* / hybrid	*B. nana* / hybrid	All ploidy groups
Wavelet coefficients	0.384	0.276	0.679	0.583
Width/length, perimeter	0	0.138	0.071	0.146
Width/length, area	0.026	0.241	0.107	0.188
Perimeter/area, length	0	0.103	0.107	0.125
Perimeter/area, width	0	0.069	0.107	0.125
Perimeter/area, width/length	0	0.207	0.143	0.208
Cap1, Cap2	0.077	0.172	0.143	0.208

Discussion

It was possible to discriminate effectively between different ploidy groups of birch, along with their hybrids using the R package ‘shapeR’ in conjunction with statistical functions from the ‘vegan’ and ‘ipred’ packages. The more complex discriminants (CAP axes and Wavelet coefficients) proved to be ineffective in comparison to the simpler ones (leaf length, width, perimeter, area and combinations thereof), among which leaf length and width and the ratio between leaf perimeter and area were the most effective, enabling discrimination between all ploidy groups with up to 87.5% accuracy and with even better accuracy when only two ploidy groups were discriminated (Table 4). This is a considerable improvement over the previous method used in Thórsson *et al.* (2007), in which a linear discriminant analysis was only able to identify the hybrids 49% correctly of the time. The method used in this study allows for classification between Icelandic birch hybrids and either *B.nana* or *B.pubescens* with 89.3% and 99.31% accuracy, respectively. This increase in accuracy was achieved with the addition of the ratio between perimeter and area as a discriminant, but the method used in Thórsson *et al.* (2007) did not make use of leaf perimeter in their analysis.

The more complex discriminants (CAP axes and Wavelet coefficients) proved to be ineffective in comparison to the simpler ones (width, length, perimeter, area and combinations thereof). However, these parameters have previously been useful in analysing shape variation in Atlantic herring otoliths (Libungan *et al.* 2015). This indicates that discriminants should be chosen on a case for case basis. Removing the petiole could possibly increase the effectiveness of these discriminants, since the angle of the petiole has been shown to have a negative effect on differentiation between ploidy groups (Thórsson *et al.* 2007). This is possibly because of the petiole’s seemingly random angle that could increase individual variance regardless of ploidy group.

Using shapeR for morphometric analysis of leaf shapes could be very useful in studies involving plant systematics since it expedites classification greatly and bypasses the need for skilled taxonomic experts to classify each specimen. However, the method outlined in this study does require some amount of preparation of samples, including capturing images of each sample in a conform manner and modifying the images in such a way that ShapeR can extract the necessary parameters.

It should be noted that relatively few samples were used in this study, and this affected the validity of the cross-validation tests somewhat. Further studies might elaborate on this, and verify the results using larger sample sizes. It would also be of great interest to establish if the results presented here are representative of the general birch population in Iceland, both by using larger sample sizes and samples from different areas.

Variance within the different sample areas (or populations) in this study was not analysed particularly, and the sample areas used do not represent the full geographical distribution of birch in Iceland. Different populations could have been affected differently by different hybridization events (different segments of genetic material crossing between the two species) and different environmental factors. The shapeR program could be used to establish how the leaves of

B.pubescens, *B.nana* and their hybrids vary between different populations and thus give insights into how hybridisation and environment can affect morphology in varying ways. Since some of the most effective parameters used in this study are dependent on scale, they would be vulnerable to changes in leaf size due to environmental factors, especially if the size of *B.pubescens* and *B.nana* leaves change differently in response to environment.

Individual variance might signify genetic divergence or different environmental factors that affect leaf morphology and should be analysed further. Variance within single plants should also be analysed in order to establish if there is significant variation between leaves present within each plant. Analysis of morphological variance of leaves within plants could be used to further illuminate the extent of influence on morphology posed by genetic and environmental factors. Leaf size is known to be dependent on environment, especially of light quality and irradiance, whereas leaf morphology is generally dependent on both environment and genetics (deCasas *et al.* 2011).

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Supplementary Material

Finally, a small number of birch samples from Greenland and Scotland were tested along with the icelandic samples in order to give an idea of how well the parameters that best described the icelandic ploidy groups could describe other unconnected groups. These samples included *B. pubescens* and *B.* glandulosa samples from Greenland and *B.pendula* samples from Scotland. Some of the groups were easy to discriminate between, and classification with all of the groups present was possible with 68.4% accuracy. Due to the few samples used from the groups from Greenland and Scotland, the results were not very reliable, but nevertheless gave an idea (if somewhat vague) of further possibilities for classification of birch species using the most efficient discriminats described in this paper.

Table S1: Error estimates (proportion of incorrectly assigned leaves to groups) of cross-validation tests with 1000 bootstraps using the discriminants examined for the supplementary samples.

Discriminants	*B.nana*/*B.glandulosa*	hybrids/*B.pub.* (Greenland)	*B.pub.*/*B.pub.* (Greenland)	*B.pub.*/*B.pendula*	All groups
Perimeter/area, length	0.250	0.091	0.136	0.136	0.351
Perimeter/area, width	0.208	0	0.136	0.091	0.316

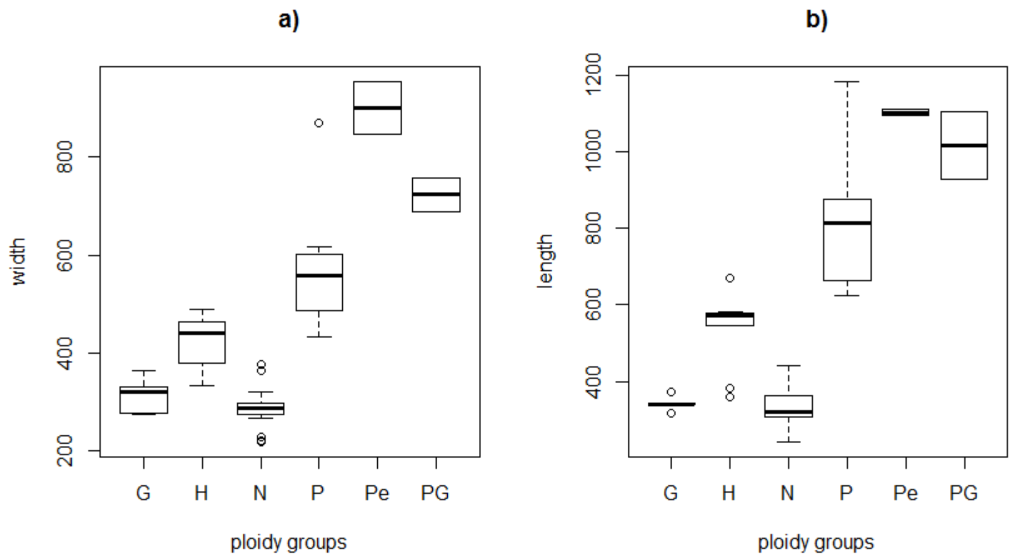

Figure S1: Distributions of maximum widths (a) and lengths (b) within groups. 'G' represents *B. glandulosa*, 'H' represents hybrids, 'N' represents *B. nana* and 'P' represents *B. pubescens*, 'Pe' represents *B.pendula* and 'PG' represents *B. pubescens* samples from Greenland. On this scale, 100 equals ~0.44cm.

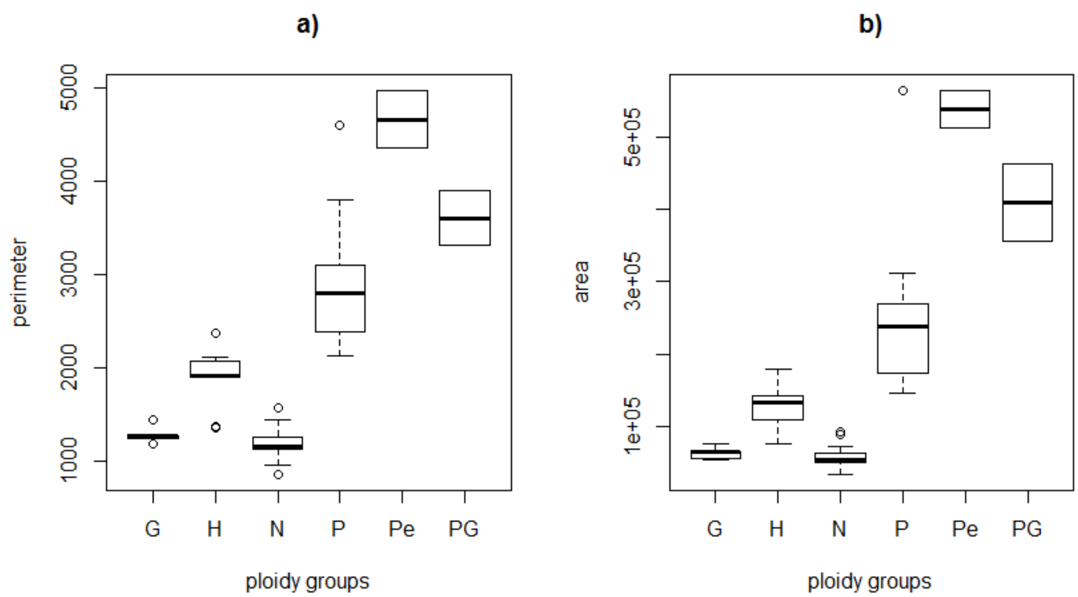

Figure S2: Distributions of area (a) and perimeter length (b) within groups. 'G' represents *B. glandulosa*, 'H' represents hybrids, 'N' represents *B. nana* and 'P' represents *B. pubescens*, 'Pe' represents *B.pendula* and 'PG' represents *B. pubescens* samples from Greenland. On this scale, 100 equals ~0.44cm.

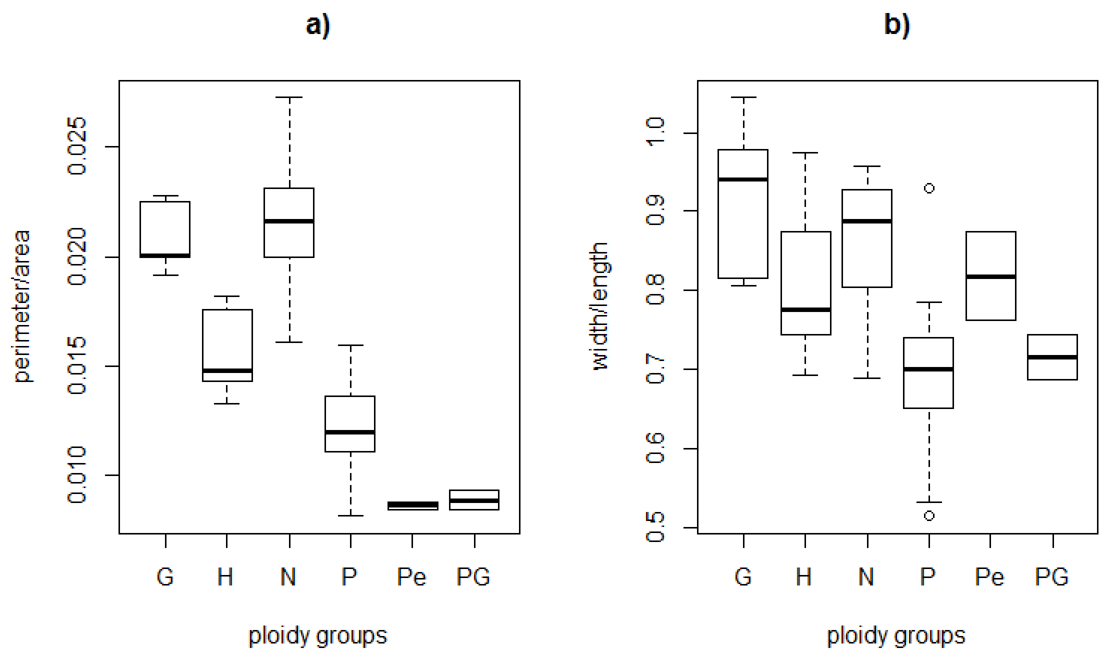

Figure S3: Distributions of perimeter/area (a) and width/length (b) within groups. ‘G’ represents *B. glandulosa*, ‘H’ represents hybrids, ‘N’ represents *B. nana* and ‘P’ represents *B. pubescens*, ‘Pe’ represents *B.pendula* and ‘PG’ represents *B. pubescens* samples from Greenland. On this scale, 100 equals ~0.44cm.

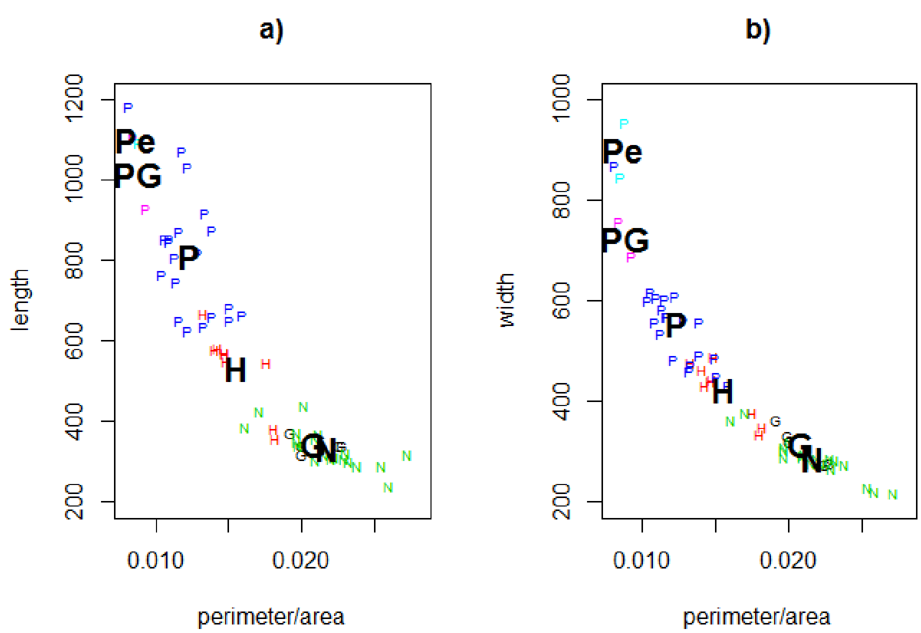

Figure S4: length (a) and width (b) against perimeter/area. ‘G’ represents *B. glandulosa*, ‘H’ represents hybrids, ‘N’ represents *B. nana* and ‘P’ represents *B. pubescens*, ‘Pe’ represents *B.pendula* and ‘PG’ represents *B. pubescens* samples from Greenland. Large black letter represents the means of ploidy groups and the smaller letters represent individual leaves. On the scale displayed on the y-axis, 1000 equals ~4.4cm.