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Historical Cultivars of *Buxus sempervirens* L. Revealed in a Preserved 17th Century Garden by Biometry and Amplified Fragment Length Polymorphism (AFLP)

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Summary

Buxus sempervirens L. extant in a preserved renaissance parterre dating from ca. 1680 at Milde (Bergen, Norway) has been provisionally classified as the morphs 'Pendula', 'Suffruticosa', and "Yellow". These were thought a priori to represent distinct taxa, viz. cultivars. Shoot length, leaf hairiness, leaf width, leaf curvature, and lamina width-to-length ratio measured on 30 leaves in each individual allowed separation of the morphs in discriminant analysis. In AFLP analyses 7 primer combinations yielded informative data, producing 168 (57 %) polymorphic fragments. Based on the AFLP data, the three morphs in neighbour-joining bootstrap analyses formed well supported clades (100 % BP) and in a principal coordinate analysis formed distinctly separated and close clusters, indicating that the morphs are genetically distinct and the individuals of each morph are closely related. Biometric analysis and finger printing techniques (AFLP) consistently show that the three morphs are both morphologically and genetically distinct. The three morphs are assumed to represent genetically distinct old cultivars introduced during the 17th or 18th century.

Key words. boxwood parterre – *Buxus sempervirens* cultivars – conservation – garden history – Norway – renaissance garden

Introduction

Buxus sempervirens L. (Common Boxwood) today is native in Southern and Western Europe northwards to Central France and Southern England (Tutin et al. 1968), and occurs as a naturalised species further north (Decocq et al. 2004). It has been cultivated for edging and topiary at least since Roman Times (Ciarallo 2004), and was well known in medieval gardens of Europe (Harvey 1981, 1987; Dickson 1994). The introduction of the species to the Nordic countries probably only followed the breakthrough of the renaissance garden style that originated in Italy in the 15th century and reached the north during the 16th and 17th centuries (Lorentzon 1998; Christensen 1999; Dietze 2000; Moe et al. 2006). Old specimens of *Buxus* thought to date from these early introductions are still found extant in a few historic gardens and parks in Scandinavia (Moe 1991; Lorentzon 1998; Moe et al. 2006).

The Milde Estate Garden

The Milde Estate Garden is situated in Bergen, Norway, close to the North Atlantic coast (60 ° 15 ' N. lat., 5 ° 16 ' E. long., altitude 35 m a.s.l.) with a climate characterized by mild winters (mean January temperature range: +4 to 0 °C) and moderate summer temperatures (mean July temperature range: 12 to 16 °C) (Aune 1993), and an annual precipitation of 1000 to 2500 mm depending on topography (Førland 1993). The garden is surrounded by large stone walls and probably dates from before 1530 (Salvesen and Moe 2005; Moe et al. 2006). In written sources it is, however, only known since the early 18th century, and then "flower quarters" and "pyramid trees" are mentioned (Schübeler 1888). The maintenance of the garden apparently has been sporadic since ca. 1815, and little seems to have been added to its contents by the owners since then (Schnitler 1915; Moe et al. 2006). The original vegetation is therefore partly still in existence, including some 40 species and cultivars of extant garden plants, and a rectangular parterre (sized 25 m by 15 m) overgrown with a 5–7 m high entangled shrubbery of *Buxus sempervirens* preserved in front of the manor house. The *Buxus* material has been classified into three morphs, provisionally named 'Pendula', 'Suffruticosa', and "Yellow" (Salvesen and Moe 2005; Moe et al. 2006). The former two morphs compare well with known cultivars, whereas the latter does not. It has a notable yellowish green leaf colour, and was therefore tentatively named "Yellow", the double quotes signalling its uncertain affinity. Six 'Pendula' specimens, presumably remnants of the "pyramid trees" mentioned by Schübeler (1888), are still standing as one-stemmed, bowed trees. The largest, featuring a stem diameter of 32.6 cm, has been dated by annual ring counts at 320 ± 47 years of age. The "Yellow" morph comprises strongly growing shrubs apparently originally trimmed into spherical shapes, each with sev-

eral stems protruding from the base. The age of the largest stem (diam. 19.7 cm) is estimated at ca. 200 years. In the smaller 'Suffruticosa', making branching shrubs partly subdued by shade, an age of ca. 205 years has been estimated for the largest stem (diameter 10.5 cm) (Moe 1991; Salvesen and Moe 2005). The age of 'Suffruticosa' and "Yellow" may reflect the time when the maintenance and renewal of the trimmed hedges of the parterre halted. The ages of the 'Pendula' morphs presumably give an age estimate for the parterre itself, as pollen analysis from the site indicate the presence of *Buxus sempervirens* since it was established (Moe et al. 2006).

The aim of the present study is to examine if the provisional classification of *Buxus* morphs is supported by a more detailed study using biometrical and finger printing techniques. And if so, to substantiate if these morphs represent ancient cultivars, and therefore to establish a basis for the further study of *Buxus* found in old gardens.

Materials and Methods

Plant material

Leaves were collected from *Buxus sempervirens* in the Milde Estate Garden for biometric and AFLP analyses (26 samples), from all the 6 specimens of 'Pendula' preserved (P1–6), and from ten specimens each of the 'Suffruticosa' (S1–10) and "Yellow" (G1–10) morphs. The samples for biometric and AFLP analyses were all taken from the same stand, but may not include exactly the same specimens in the two data sets. The effect this would have on the resulting comparison is expected to be low, as the total number of *Buxus* specimens in the garden hardly exceeds 30.

Biometry

From each of the 26 specimens (P1–6, G1–10, S1–10), three branchlets were collected, and from each branchlet, five young (last year's) shoots were picked where possible. In each shoot, two well developed leaves near the middle of the shoot were measured. Thus, in general a total of 30 leaves were measured from each of 26 specimens. In a few instances additional leaves were measured where variation between measurements was judged to be exceedingly large, and occasionally some cases were discarded due to missing data (total number of measurements, n=817). The characters scored from each leaf were: total leaf length, lamina length, and lamina width (Fig. 1), lamina curvature (arbitrary scale; −2 = strongly concave, −1 = concave, 0 = flat, 1 = convex, 2 = strongly convex), and hairiness of the petiole and leaf nerve (arbitrary scale; 0 = no hairs, 1 = scattered hairs, 2 = hirsute, 3 = densely hirsute; the mean hairiness of petiole and nerve was entered in the statistical analysis). In addition the length of the selected shoot was measured.

AFLP-analysis

Fresh leaf material from each of 26 specimens was sampled (P1–6, G1–10, S1–10; Table 1). To test the reproducibility of our results, nine additional samples were taken

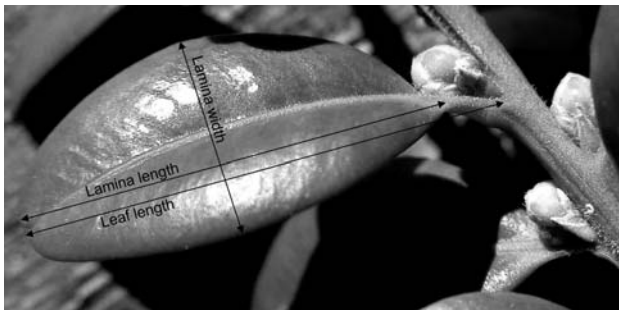


Fig. 1. Position where leaf characters total leaf length (mm), lamina length (mm), and lamina width (mm) were measured in *Buxus sempervirens*.

Table 1. *Buxus sempervirens* material utilized in the AFLP study.

Taxon	Specismens	Reference / collector (herbarium voucher)	Origin
'Pendula'	P1–10	Salvesen PHS06.01 (BG)	Norway, Milde Estate, Bergen
	P6 1–9	Salvesen PHS06.15 (BG)	Norway, Milde Estate, Bergen
	P10 1–9	Salvesen PHS06.15 (BG)	Norway, Milde Estate, Bergen
"Yellow"	G1–6	Salvesen PHS06.01 (BG)	Norway, Milde Estate, Bergen
	G1 1–9	Salvesen PHS06.15 (BG)	Norway, Milde Estate, Bergen
	G6 1–9	Salvesen PHS06.15 (BG)	Norway, Milde Estate, Bergen
'Suffruticosa'	S1–10	Salvesen PHS06.01 (BG)	Norway, Milde Estate, Bergen
	S2 1–9	Salvesen PHS06.15 (BG)	Norway, Milde Estate, Bergen
	S3 1–9	Salvesen PHS06.15 (BG)	Norway, Milde Estate, Bergen
	suf1	Kanz (BG)	Germany, Atrops tree nursery, Rheurdt
not specified	Bar		Norway, Rosendal Barony, Kvinnherad

BG: The Bergen herbarium

from two individuals per morph (P1 1–9, P6 1–9, G6 1–9, G10 1–9, S2 1–9, S3 1–9). In addition to the 26 samples collected at Milde Estate Garden, one sample (Bar) supplied from a shrub of uncertain morph affiliation in the garden at the Rosendal Barony (Kvinnherad, Hordaland, Norway), and one sample (suf1) of the 'Suffruticosa'-morph obtained from the Atrops tree nursery in Germany were included. Samples were stored in silica gel immediately after collection. Voucher specimens are deposited in the Bergen herbarium (BG).

DNA extraction

Total genomic DNA was isolated from one leaf of each of the 82 samples using the DNeasy® Plant Mini Kit (Qiagen) following the manufacturer's instructions with minor variations in order to recover a sufficient amount of genomic DNA. Cell lysis was performed over night. Final DNA concentrations were determined electrophoretically, using a 100 Bp-DNA ladder (Roth) with known amounts of DNA as standards.

AFLP procedure

AFLP analyses were performed according to the Beckman Coulter application information A-2015A (Hayashi et al. 2005) with the modifications indicated in the following. An amount of 50 ng total genomic DNA was used for the restriction and ligation reactions with unlabelled *Mse*I (1 µM) and fluorescence-labelled *Eco*RI (0,1 µM; both Fermentas) restriction enzymes and T4 DNA Ligase (1U; Fermentas) in a final volume of 10 µl during 2 h at 37 °C in a DNA Engine Dyad® cycler (MJ Resaerch™). Preselective amplifications were performed using primer pairs with a single selective nucleotide, *Mse*I-C and *Eco*RI-A and AFLP Core Mix (Applied Biosystems). Selective amplifications were performed using primer pairs with two (*Mse*I) or three (*Eco*RI) selective nucleotides, respectively, and AFLP Core Mix (Applied Biosystems). PCR reaction parameters were chosen as described in Meister et al. (2006). Out of 28 primer combinations, seven primer pairs were found to be informative with clearly detectable peaks and high degrees of polymorphism (Table 2).

After DNA precipitation, DNA pellets were vacuum dried and dissolved with a mixture of Sample Loading Solution (SLS) (Beckman Coulter) and CEQ Size Standard 600 (Beckman Coulter). The fluorescence-labelled selective amplification products were separated by capillary gel electrophoresis on an automated sequencer (CEQ 8000, Beckman Coulter). Raw data were then exported to GeneMarker (version 1.5, SoftGenetics, LLC) for scoring of fragments. The automatically constructed presence/absence (1/0) matrices were edited by visual inspection of the electrophorograms. Weak and indistinct peaks were generally ignored. Reliability and reproducibility were tested with three primer pairs (*Mse*I-AT/ *Eco*RI-ACC, *Mse*I-AA/ *Eco*RI-AAG, *Mse*I-AA/ *Eco*RI-ACT) on nine samples of each morph (54 samples), respectively, at each step in the AFLP procedure from DNA extraction to AFLP analysis.

Since not all samples were tested with all seven primers, three 1/0 matrices were constructed for further study: (1) From 27 individuals using seven primer pairs (G1–10, P1–6, S1–10, Bar), (2) from 28 individuals using five primer pairs (G1–10, P1–6, S1–10, Bar, suf1) and (3) from 70 individuals using three primer pairs. Matrices (1) and (2) were converted to distance matrices based on the Nei and Li (1979) (= Dice) index of similarity.

Data analysis

Initially the morphological characters separating best between the three morphs were selected by evaluating the MORPH effect in separate one-way ANOVAs according to the model: Morph$ + Specimen$ + Branchl_no + Shoot_no + Constant = 0. Data for these characters were then included in three separate discriminant analyses using the GLM routine in the SYSTAT package v. 7.0 (Wilkinson 1997). In the first analysis the data for each pair of leaves were averaged within shoot (n = 395), in the second analysis data were averaged for each of the branchlets within specimen (n = 83), and finally, in the third analysis, data were averaged for each specimen (n = 26). A classification and a canonical scores plot including 95 % confidence ellipsoids for each morph were produced for each analysis.

Phylogenetic analyses of the AFLP data were carried out on the three 1/0 matrices using PAUP* 4.0b10 (Swofford 2002). Neighbour-joining bootstrap analyses based on 1000 bootstrap replicates were performed in order to display the relationships among individuals of the three morphs and to calculate branch support values. Results are displayed as 50 % majority rule consensus trees. The sample from Rosendal Barony was used as an out-group to root the trees. Principal coordinate analyses (PCoA) were conducted using NTSYSpc 2.10p (Rohlf 2000).

Results

Biometry

Significant differences between the morph means were found in one-way ANOVAs for shoot length, hairiness, leaf width, leaf curvature, and the lamina width-to-length ratio. These characters were used in the three discriminant analyses.

The first analysis with shoot means (Fig. 2a) yielded a classification matrix (Table 3) where the 'Pendula' morph was reasonably well separated from both the two other morphs (95 % correct identification), while only 80 %

Table 2. Primer combinations used for selective amplification and number of scorable fragments.

Primer combination	Number of scorable fragments	Number of polymorphic fragments
*Mse*I-AT/ *Eco*RI-AAC	58	29 (= 50 %)
*Mse*I-AT/ *Eco*RI-ACC	46	25 (= 54 %)
*Mse*I-AA/ *Eco*RI-AAG	39	18 (= 46 %)
*Mse*I-AC/ *Eco*RI-AAG	41	18 (= 44 %)
*Mse*I-AC/ *Eco*RI-ACG	29	22 (= 76 %)
*Mse*I-AA/ *Eco*RI-ACT	43	28 (= 65 %)
*Mse*I-AC/ *Eco*RI-ACA	40	28 (= 70 %)
Total	296	168 (= 57 %)

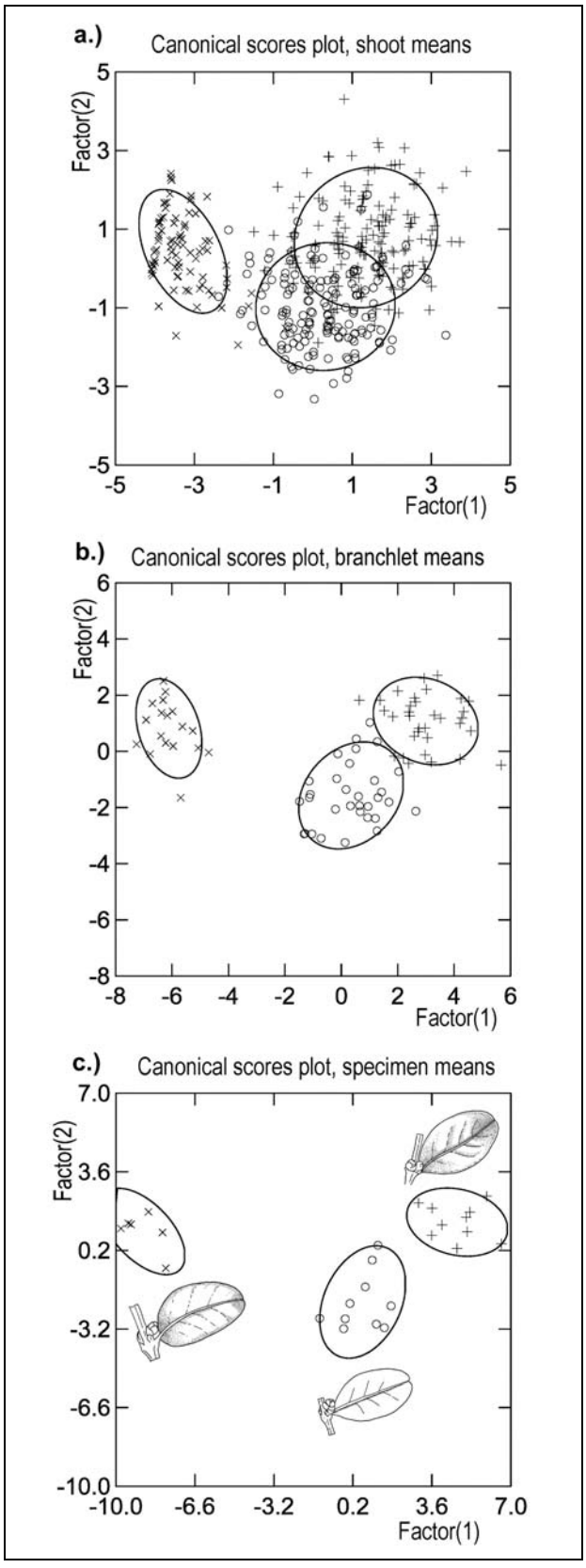


Fig. 2. Discriminant analyses of leaf character data for three morphs of *Buxus sempervirens*, 6 specimens of the morph 'Pendula' (x), 10 of "Yellow" (+), and 10 of 'Suffruticosa' (o). a.) shoot means (n = 395); b.) branchlet means (n = 83); c.) specimen means (n = 26).

Table 3. Discriminant analysis classifications of leaf character data for three morphs of *Buxus sempervirens*. (P = 'Pendula', G = "Yellow", S = 'Suffruticosa', for further details see Fig. 2 and text.

Group membership estimates based on	Cultivar	P	G	S	% correct
Shoot means	'Pendula'	75	0	4	95
	"Yellow"	1	124	30	80
	'Suffruticosa'	6	28	127	79
	Total	82	152	161	83
Branchlet means	'Pendula'	18	0	0	100
	"Yellow"	0	33	0	100
	'Suffruticosa'	0	2	30	94
	Total	18	35	30	98
Specimen means	'Pendula'	6	0	0	100
	"Yellow"	0	10	0	100
	'Suffruticosa'	0	0	10	100
	Total	6	10	10	100

and 79 % of the specimens of "Yellow" and 'Suffruticosa', respectively, were correctly classified. In the plot the separation between these two morphs is obscured by a large overlap. The second analysis based on branchlet means (Fig. 2b), gave a classification (Table 3) where the 'Pendula' and "Yellow" morphs were correctly identified (100%), while two shoots of 'Suffruticosa' were reclassified as "Yellow". The plot shows a good separation of 'Pendula' from the two others, while the 95 % confidence ellipses for "Yellow" and 'Suffruticosa' are barely separated. In the last analysis with specimen means (Fig. 2c) all specimens were allocated to the initially determined morphs (Table 3). The plot shows a clear separation of the three groups with a 95 % confidence level of significance.

Taken as a whole, the discriminant analyses of leaf morphology indicate that, on average, based on a large number of leaves it is possible to identify a plant correctly to one of the initially assumed morphs present in the old garden at Milde Estate. In smaller samples taken at random, this will not always be possible. In particular, the distinction between the morphs 'Suffruticosa' and "Yellow" may prove problematic.

AFLP data

Scored fragments ranged from 63 to 531 nucleotides in length (Fig. 3). The AFLP analyses yielded a total of 324 fragments of which 168 (57 %) were polymorphic and included in further statistical analyses. The number of polymorphic fragments per primer ranged from 18 to 29 (average 24). Blind samples as a negative control, showed no amplification. The test of reproducibility of peak patterns revealed an error rate of 1.5 % (75 differences out of 4940 comparisons). It appeared that the main reason for non-reproducible patterns was slight differences between the DNA extraction protocols used on

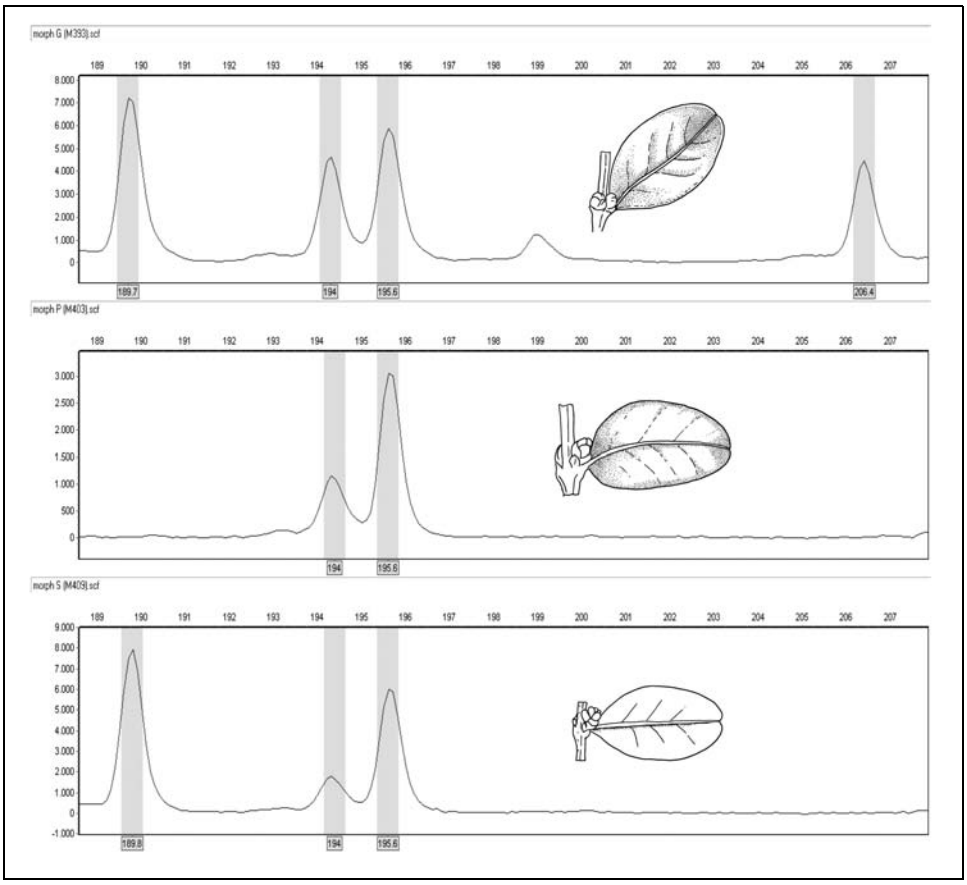


Fig. 3. Partial AFLP with primer pair *Mse*I-AC/*EcoR*I-ACA, examplary for the capability of primers to separate morphs from each other. Morph P: *B. sempervirens* 'Pendula', morph G: *B. sempervirens* "Yellow", morph S: *B. sempervirens* 'Suffruticosa'.

the different sets of samples to gain sufficient amounts of DNA.

Fig. 4 displays the neighbour-joining dendrogram from the analyses of 27 individuals using seven primer pairs (Table 2), with bootstrap values above 50 %. Individuals from all three morphs form well supported clades (100 % BP). The sample from Rosendal Barony (Bar) is isolated from the rest, indicating that the *Buxus* material at Milde Estate Garden is genetically different from this individual. The suf1 sample of the 'Suffruticosa'-morph was correctly assigned to the 'Suffruticosa'-clade based on the combination of five primer pairs (see arrow in Fig. 4). The rest of the branches on the tree are only supported by comparatively lower bootstrap values (less than 91.5 %). Based on the data set available, it is thus considered uncertain that there are any genetic differences between specimens within the morphs.

The three morphs formed distinctly separated clusters in the PCoA-analysis (Fig. 5). The first two axes accounted for 87.6 % of the total genetic variation in the sample. The single individual from Rosendal Barony occupied an intermediate position between the three morphs from Milde Estate Garden. The spread of data points within clusters is small, reflecting the low genetic variation between specimens within a morph.

Discussion

Gardeners in earlier days travelled across Europe taking employment at houses of wealthy nobility, clergy, or merchants (Hobhouse 1992). In particular, gardeners leading the introduction of the renaissance garden style to the north, like Hans Raszmussøn Block (Block 1647) and Christian Gartner (Gartner 1694) were trained and skilled in the art after years of experience in the finest gardens of their time (viz. Royal Gardens in Denmark and France). The *Buxus sempervirens* plants required were apparently brought from one garden to the next as rooted suckers propagated by vegetative means during maintenance of box parterres (Christensen 1999; Batdorf 2004), and thus constituting clones or groups of very closely related clones when planted. Different selections also were brought for different purposes, thus facilitating the spread of specific clones from within the originally variable species. The affinity of the morph here named "Yellow" is still obscure, and may not be similar to any of the cultivars with yellowish coloured leaves mentioned by early writers in England and France, such as Parkinson (1629) or Tournefort (1700). Forms with "golden" leaves became, however, extremely popular when they first emerged during the 17th century (Christensen 1999). The morph here named 'Pendula' is clearly different from the present-day cultivar 'Pendula' of Batdorf (2004), but there seems to exist more than one form with pendulous branches treated under this name in Scandinavia (Lorentzon 1998). The 'Pendula' plants at Milde Estate Garden may represent a very early cultivar. The plants here called 'Suffruticosa' seem to correspond well with today's trade material of this cultivar, as is also indicated by the plant included from the Atrops tree nursery. They presumably represent the low-growing, sterile edging box planted in gardens, mentioned already in the 13th century by Albertus Magnus (Jessen 1887; Moe et al.

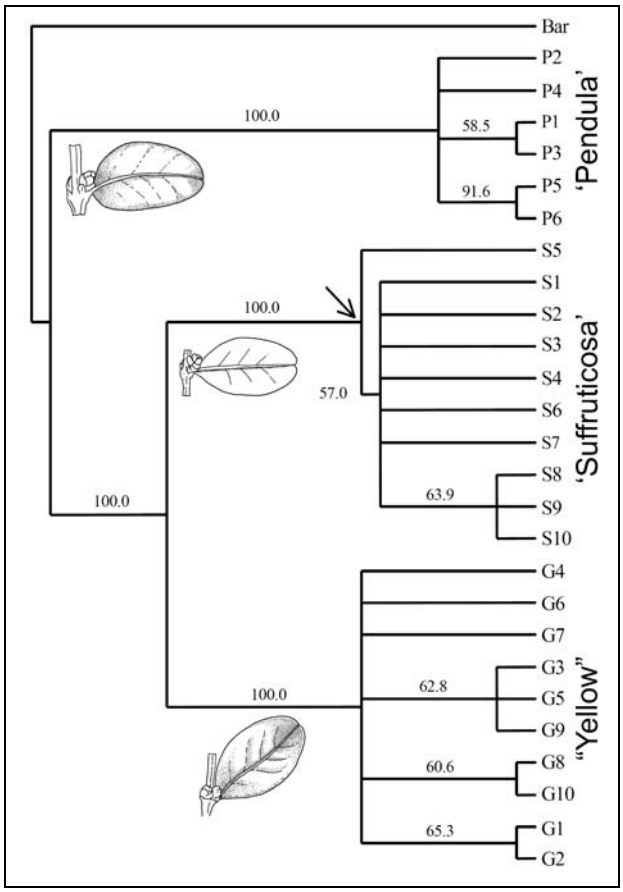


Fig. 4. NJ dendrogram from the analyses of seven primer pairs and 27 individuals combined with bootstrap values above 50 %. The arrow indicates the position of the 'Suffruticosa' –individual from the Atrops tree nursery in a data set with only five primer pairs.

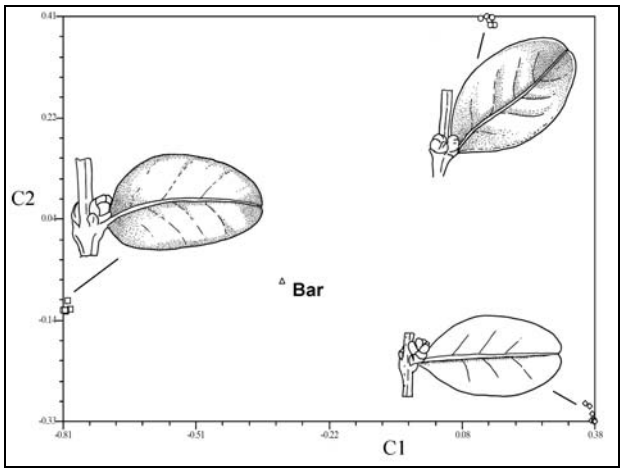


Fig. 5. Axes C1 and C2 from the principal coordinates analysis of AFLP data from *Buxus sempervirens*, based on the same data set as the NJ tree (Fig. 2). The axes are scaled in artificial units, 'Pendula' (❑), "Yellow" (o), 'Suffruticosa' (✧), and the sample from the Rosendal Barony (△).

2006) and corresponding to the *Humibuxus* and *Buxus humilis* of Dodoens (1554, 1557, 1616) and PARKINSON (1629). With this in mind it may be speculated that boxwood in the old garden at Milde Estate were brought there by highly skilled gardeners to construct a formal boxwood parterre in keeping with the current fashion in the 17th century aristocracy.

The vegetative morphology of plants is modified by environmental factors like temperature, moisture, soil fertility, cutting, and light. To eliminate such effects in biometrical studies, a large number of samples must usually be measured to produce the resolution necessary for separating between morphs. Since plants sampled generally – and certainly in the present case – have been growing under quite different environmental conditions, one would have to set up trials in an experimental garden under uniform conditions to be able to separate the genetic from the phenetic effects with reasonable certainty. As an alternative approach the molecular techniques have proved most useful (SZCEPANIAK et al. 2002; WINFIELD and HUGHES 2002; CONSAUL et al. 2008). In *Buxus*, ZHANG et al. (2003) found the combined use of morphological and molecular data to be a powerful tool for discriminating between cultivars.

Based on the small sample treated in the present study, it is evident that individuals classified to any of the three morphs proposed on the basis of leaf characters alone, are genetically distinct and separable from each other by the AFLP markers used. The AFLP method has proved superior to morphological characters in distinguishing genetically distinct groups of closely related individuals. The data do not, however, allow the assertion of any genetic differences between plants within the morphs or if the morphs correspond to monotypic clones. The three groups of specimens may, however, fairly certainly be taken to represent narrowly selected cultivars used to lay out the renaissance parterre at Milde Estate Garden, presumably all at once during the 17th century.

Work is in progress to compare old *Buxus* specimens from gardens in Northern Europe with a reference collection of named taxa in the Arboretum and Botanical Garden, Milde, Norway. A survey of *Buxus* found in old gardens of South Norway (Salvesen unpubl.) indicates that substantial morphological variation is present. The AFLP method will be used to elucidate the variation found, as a tool for separating between genetic traits and phenotypic plasticity. A detailed and accurate assessment of the genetic variation in plants extant in old gardens may prove crucial to the conservation of living relics of the gardens of the past, and also for selecting plant material for reconstruction of historic gardens where the original plants are no longer present. The long-term aim of the project is to establish unique markers for different cultivars and clones, and to trace their distributions across Europe.

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