Trees (2014) 28:389–398
DOI 10.1007/s00468-013-0957-y

ORIGINAL PAPER

The use of digital morphometrics and spring phenology for clone recognition in trembling aspen (*populus tremuloides* michx.) and its comparison to microsatellite markers

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Received: 25 April 2013 / Revised: 22 October 2013 / Accepted: 29 October 2013 / Published online: 15 November 2013

Abstract Aspen clones were traditionally identified based on similarities in several phenotypic traits including leaf shape. This required several visits of the stands, laborious measurements and subjective visual assessments. In this study, we investigated a novel approach to clone identification using digital morphometrics of leaf shape complemented with bark characteristics and spring phenology. Aspen clones were delineated based on molecular (microsatellite loci), morphological (leaf shape, bark colour and type) and phenological (when first fully expanded leaves appeared) characteristics. Leaves were scanned and images were analyzed using normalized elliptic Fourier descriptors and principal component analysis. Using microsatellite loci, 18 clones were identified among 60 aspen trees in three sites investigated in this study. When employing digital morphometrics, foliar types in two out of the three sites matched the clones defined by microsatellite markers. Many ramets from the third site were clustered erroneously into incorrect clones. The reclassification test indicated that leaf shape contains features according to which very similar clones can be differentiated with low error rates. However, because it was not possible to set a threshold for maximum distances within clones, application of digital morphometrics of complex leaf shape for clone identification in natural aspen stands with a high number of multi-ramet clones and many singletons is unfeasible. We judged spring phenology as the least reliable trait for clone recognition and suggested possible causes of heterogeneous leaf flushing among ramets of the same genotype.

Keywords *Populus tremuloides* · Clone identification · Leaf flush · Environmental effect · Morphometrics · Ramet · Foliar diversity · Spring phenology · Microsatellite markers · Clonal integration

Communicated by J. Carlson.

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Introduction

Natural populations of aspen exhibit a clonal growth as an admixture of clones of several ramets and singletons. Identification of clones was traditionally based on the morphological characteristics such as floral, foliar, stem, and bark traits, growth form, and susceptibility to diseases or injuries (Barnes 1966, 1969; Barnes and Han 1993). A putative large aspen clone consisting of 47,000 ramets was identified in this way (DeWoody et al. 2008). Owing to its size, this clone was referred to as "Pando". However, when molecular markers were used, many distinct genotypes were found in and around this clone (Mock et al. 2008). Morphological characterization for clonal identification was quickly abandoned mainly for two reasons: First, evaluation of some phenotypic traits such as leaf shape appeared too subjective to be reliable. Secondly, many phenotypic traits are likely to be influenced by environmental factors (Persson and Gustavsson 2001; Rumpunen and Bartish 2002; Lopez-de-Heredia et al. 2004). Hence, after the discovery of molecular markers, morphological identification of intermingling clones in aspen stands was quickly replaced by an almost exclusive use of a set of

microsatellites (Rajora et al. 2001; Wyman et al. 2003; Namroud et al. 2005; Suvanto and Latva-Karjanmaa 2005; Mock et al. 2008; De Woody et al. 2009; Jelínková et al. 2009; Liesebach et al. 2010).

Nonetheless, recent advances in morphometrics, in particular automated image processing, brought new possibilities for genotype recognition that could help overcome the above mentioned disadvantages of morphological traits assessment. For instance, there is an array of new methods for analyses of biological shapes (McLellan and Endler 1998; Jensen et al. 2002a; Neto et al. 2006; Viscosi and Fortini 2011; Cope et al. 2012). They have been primarily developed for the needs of taxonomists to give an objective, quantitative shape evaluation. Among a number of descriptor suits that have been proposed, elliptic Fourier descriptors (EFDs) have proved especially useful in a variety of contexts in several plant species (Mancuso 1999; Iwata et al. 2002a; Rumpunen and Bartish 2002; Yoshioka et al. 2004; Neto et al. 2006; Menesatti et al. 2008; Torres et al. 2008; Viscosi and Fortini, 2011).

Elliptic Fourier descriptors can delineate any type of shape with a closed two-dimensional contour and are sensitive to both subtle and complex changes in a specimen's outline (Kuhl and Giardina 1982; McLellan and Endler 1998).They have been shown to be more efficient in assigning plant material to correct clones than other descriptor suits (Persson and Gustavsson 2001; Rumpunen and Bartish 2002; Cope et al. 2012). Availability of software packages such as LAMINA or SHAPE makes the leaf shape assessment easy, fast, and inexpensive (Iwata and Ukai 2002; Bylesjo et al. 2008). Hence, unlike molecular markers, leaf shape could be used as an inexpensive marker for early clone identification in situ during a single field visit.

Besides the need for an objective evaluation, clone identification also requires a selection of phenotypic characteristics that exhibit low sensitivity to environmental effects. Development of leaf shape and size is a highly complex process under the control of many genes which is further modulated by hormonal and environmental factors (Wu et al. 1997; Wu 2000). Although leaf size can be conditioned by environmental factors (such as light exposure or water availability), leaf shape is usually less affected; Iwata et al. (2002b) tested genotype × environment interactions in a field trial of citrus and showed that the genotype was the main source of variation in leaf shape, but not in size. A study of European aspen (*Populus tremula*) also indicated that the genotype influence was smaller for size than for shape related traits (Lopez-de-Heredia et al. 2004). Moreover, leaf shape can be described by symmetrical and asymmetrical features. Asymmetrical features result from irregular growth when for instance the blade's apex is bended towards one side or when one half of the blade's base is more flat than the other one. It has been shown that symmetrical features are highly heritable, while the asymmetrical ones are consequences of environmental effects. The two types can be divided and analyzed separately (Iwata et al. 2002a; Iwata and Ukai 2002).

Studies employing both molecular and morphological markers are rare in forest tree species (Cannon and Manos 2001; Jensen et al. 2002b; Penaloza-Ramirez et al. 2010) and in genus *Populus* in particular (Lopez-de-Heredia et al. 2004; Suvanto and Latva-Karjanmaa 2005; Lexer et al. 2009). In the present study, we investigated a method for clone recognition that could be used in a variety of contexts of ecological research or in studies of genotype–phenotype correlation. The tested method employed image analysis of leaf shape complemented with bark characteristics and spring phenology. Aspen clones investigated in this study were at the same time identified with a commonly used set of microsatellite markers. To our knowledge, there is neither previous study applying digital morphometrics of complex leaf shape to aspen nor study comparing morphological and microsatellite markers. Unlike traditional morphometrics, the proposed method uses the whole leaf shape without selection of specific leaf measurements.

Materials and methods

Study sites

Aspen clones were delineated based on molecular (microsatellite loci), morphological (leaf shape, bark color and type) and phenological (when first fully expanded leaf appeared) characteristics in three natural stands in the southern boreal forest in northwest Quebec, Canada. The three sites (Mont Kanasuta, McWatters, Parc National d'Aiguebelle) consisted of pure even-aged trembling aspen forest that regenerated after clear cuts. The sites were not perfectly even regarding their age. At the time of this study, sites McWatters and Aiguebelle were 38 and 42 years old, while site Kanasuta was only 23 years old. The area stretches from 48°11′N to 48°30′N of latitudes and from 78°45′W to 79°23′W of longitudes. A plot of 30 m^2 encompassing 27, 16, and 17 aspen trees, respectively, was established at each site (Table 1).

Table 1 Number of trees and leaves sampled in the study sites

Sites	No. of trees	No. of leaves
McWatter	16	128
Aiguebelle	17	136
Kanasuta	27	216
Total	60	480

Microsatellite characteristics

Bark sample was taken from every stem within the plots for DNA extraction. DNA was extracted from cambial tissue with the help of the GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich Canada Ltd, Oakville, Canada) according to the manufacturer's protocol. Amplification of seven microsatellite loci PTR1, PTR2, PTR3, PTR4, PTR5, PTR6, and PTR8 (Dayanandan et al. 1998; Rahman et al. 2000) was done using dye-labeled oligonucleotide primers and *Taq* polymerase (Gibco, Invitrogen™ Life Technologies, Burlington, Canada). For more details on the protocol see Jelínková et al. (2009). The set of aspen trees characterized at the microsatellite loci was used for leaf shape description.

Leaf shape description

Leaves were collected from the mid-position in the crowns and from the same position on the branch (the oldest fully expanded leaf). By selecting leaves from the same position in the crown and of the same developmental stage, we expected to reduce within tree variation of leaf shape. Moreover, leaves from long shoots were avoided as they are known to be heteroblastic (Lexer et al. 2009). Eight healthy leaves with perfectly preserved contours were taken from every tree (Table 1). Petioles were removed and the blades were pressed and scanned with HP Laser Jet M1120 (resolution 300 dpi). The image analysis was conducted with the software package SHAPE v1.2 (Iwata and Ukai 2002) using elliptic Fourier coefficients (Kuhl and Giardina 1982). Precision of contour description increases with increasing number of harmonics (trigonometric functions describing the shape). As clonal differences in leaf shape can be minor, in particular when attempting to differentiate clones growing in close proximity, a series of elliptic Fourier transformations employing 20, 40, and 80 harmonics were used in this study. The elliptic Fourier descriptors (EFDs) were manually normalized to be invariant in size, thus the size component of the variation was excluded from the analysis (Kuhl and Giardina 1982). Moreover, only components describing the symmetrical features of the leaf shape were analyzed. These features not only describe the whole leaf shape but also selected characteristics such as length or width of leaf blades.

Leaf shape analysis

To summarize the information contained in normalized EFDs (nEFDs) and to reduce the number of variables describing every leaf, principal component analysis (PCA) was performed based on a variance–covariance matrix of nEFDs in SHAPE v1.2 (Iwata and Ukai 2002). The variation in leaf shape accounted for by every principal component score (PC) was visualized by letting the score be equal to the mean plus and minus two times the standard deviation and the remaining components be zero. A following inverse Fourier transformation allowed reconstruction of the mean shape and its variation described by every PC (FURUTA et al. 1995). Separate PCAs and subsequent inverse Fourier transformations also allowed for reconstruction of mean leaf shapes of clones defined by microsatellite markers.

Principal components were used as input variables for a cluster analysis using the unweighted pair-group method of averages (UPGMA) to generate dendrograms. Averages of the principal component scores were calculated from eight leaves of every tree prior to the clustering; clustering was, hence, based on an average leaf shape of every ramet.

Canonical variates analyses (CVA) were used to partition morphometric diversity into within- and between-clone and tree components. CVA provides a measure, Wilks' lambda (λ), which gives a proportion of the total diversity that is due to within-group variation. Within-group replicates were 432 leaves from multi-ramet clones and the groups were successively defined as 12 multi-ramet clones and 54 ramets obtaining λclone and λramet, respectively. 1–λclones expresses between clone components and λclones–λramet between ramet components. Analyses were done separately for PCs calculated from nEFDs based on 20, 40, and 80 harmonics.

A classification test was done to test the discriminatory power of the EFDs. This test requires prior knowledge of the groups. In our case, the groups were clones identified by microsatellite markers. The test reassigns samples (ramets) described by EFDs into groups to see if they can be classified correctly into groups defined by microsatellite markers. As eight leaves were available for every tree, four leaves were used as reference samples to create the group definitions and four as test samples to test the discriminatory power. Clones represented by only one ramet were excluded from this analysis as grouping of single trees is not feasible. Clustering, tree reconstruction, CVA, and reclassification tests were done with commands CLUSTER, TREE, CANDISC, and DISCRIM in SAS v9.1 (SAS Institute, Cary, NC, USA).

Phenological and bark characteristics

The plots were visited once every 5 days during the period of leaf flushing and dates were taken when the first fully expanded leaves appeared. As it is not easy to assess phenological characteristics in high trees, we decided to follow the dates when leaves were fully expanded and well visible rather than assessing a "bud burst" stage. Every stem was assessed visually for the color of the bark. Bark texture was evaluated as either smooth or rough.

Results

Microsatellite characteristics

The seven microsatellite markers distinguished 18 clones among 60 aspen trees in the three sites (Table 2). There were 12 multi-ramet clones and six clones were represented by only one ramet. Average clonal size was 3.8 ramets and the largest clone comprised 13 ramets. All clones identified in the three study sites differed by six or more alleles (data not shown). The frequency distribution of genetic distances did not indicate any somatic mutations or scoring errors. The resolution power of the microsatellites used was high (for more details see Jelínková et al. 2009).

Leaf shape characteristics

PCA of nEFDs identified several independent features of leaf shape variation (Fig. 1). An increased number of harmonics resulted in a higher number of PCs (Table 3). Nevertheless, the cumulative contribution of the first two components accounted for over 93 % of the total variance. Gaining additional PCs when employing 80 harmonics led to an increase of the total variance contained within the PCs by only 4.1 %. Corresponding PCs (calculated from 20, 40, and 80 harmonics) had similar contributions to the total explained variance and seemed to reflect similar characteristics of leaf shape variation when the reconstructed contours were evaluated visually (Fig. 1 shows contour reconstruction of the first 5 PC scores for 80 harmonics only). PCA left out 2.7–6.8 % of the diversity conveyed by nEFDs.

Leaf shapes reconstructed by inverse Fourier transformations indicated that the first PC was a good measure of the length to width ratio (Fig. 1). It accounted for over 80 % of the total variation of the original coefficients (Table 3). The second component was associated with the shape of apex that varied from pointed to flat and accounted for over 6 % of the total variation. It also expressed the shape of blade base that diverged between flat and domed. The remaining components comprising <3 % of variation each were ascribed to other types of variation which were more difficult to explain.

Means and standard deviations of PCs based on nEFDs for 80 harmonics were calculated for the 12 multi-ramet aspen clones identified by microsatellite markers in three sites. The mean leaf shape of every clone was then drawn using the inverse Fourier transformations of these values (Fig. 2).

Table 2 Numbers of clones defined by microsatellite markers

Sites	McWatters	Aiguebelle	Kanasuta	All
Stand age	38	42	23	
No. of ramets	16	17	27	60
No. of clones	5	2	11	18
No. of single-ramet clones	1	0	5	6
Mean No. of ramets per clone	3.4	9	3	3.78
Max. No. of ramets	9	13	11	13

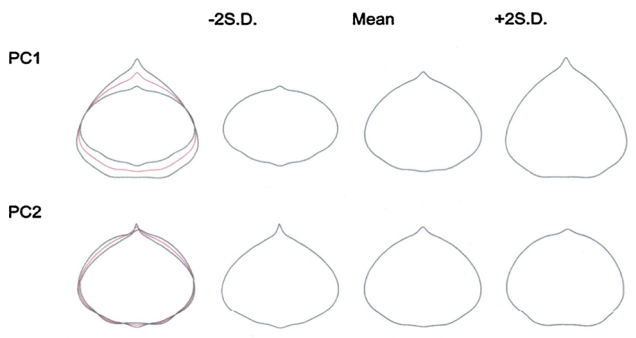

Fig. 1 Contour reconstruction by inverse Fourier transformation showing the effect of the first (PC1) and second principal component (PC2) scores calculated from 80 harmonics. Second, third, and fourth column depict the cases when the scores take −2 standard deviations, mean, and +2 standard deviations. The three contours are overlaid in the first column

Table 3 Eigenvalues and contributions of principal components calculated from normalized elliptic Fourier descriptors based on 20, 40, and 80 harmonics

PC	Eigenvalue [10^4]			Proportion [%]			Cumulative [%]		
	No. of harmonics			No. of harmonics			No. of harmonics		
	20	40	80	20	40	80	20	40	80
1	72.0	67.0	68.0	86.7	84.8	84.9	86.7	84.8	84.9
2	5.4	5.6	5.8	6.6	7.1	7.3	93.2	92.0	92.3
3		2.1	1.8		2.7	2.2		94.7	94.5
4		1.1	1.1		1.4	1.4		96.02	95.9
5			0.58			0.7			96.6
6			0.53			0.7			97.3

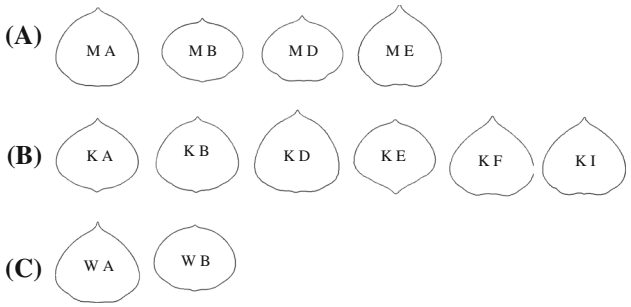

Fig. 2 Mean leaf shapes of the 12 multi-ramet aspen clones identified by the microsatellite markers. Clones are marked by the letters starting with M for site McWatters (**a**), K for site Kanasuta (**b**), and W for site Aiguebelle I (**c**)

Scatter plots of the within-ramet means of the first two PCs indicate variation both within and between clones and suggest possible grouping of individuals into groups (Fig. 3). Information contained within all PC scores is summarized by cluster analysis dendrograms in Fig. 4.

The morphological variation described by nEFDs for 20, 40, and 80 harmonics was further subjected to the canonical variates analyses (CVAs). All Wilks' lambdas (λ) were significant at $P = 0.05$ (Table 4). Using a higher number of harmonics and, hence, improving the precision of extracted leaf contours, a growing part of the total morphological variation was attributed to the between clone component, while the foliar diversity between ramets within clones and among leaves of individual ramets had a tendency to diminish. For 80 harmonics, <1 % of variation was found within individual trees (Table 4).

In classification tests, 87, 93, and 96 % of all aspen ramets from multi-ramet clones (Table 5) were correctly reassigned into microsatellite-defined clones. Tests were conducted separately for nEFDs using 20, 40, and 80 harmonics, respectively. Percentages of correctly reassigned ramets were high for most genes with the exception of KF and KI that could not be discriminated according to nEFDs of leaf shape.

Phenological and bark characteristics

Based on bark color and texture, three, two, and one phenotypes were distinguished at sites McWatters, Aiguebelle, and Kanasuta, respectively (Table 6). Two morphologically similar clones at site McWatters appeared genetically different when using a set of microsatellites. At site Aiguebelle, morphotypes matched the microsatellite-delineated clones. Ramets of all clones in site Kanasuta were uniform in bark color and type.

According to the timing of spring leaf flush, trees in sites McWatters, Aiguebelle, and Kanasuta were grouped into four, three, and three phenotypes, respectively (Table 6; Fig. 5). If bark and phenological characteristics were used together for clone identification, six, five, and three putative clones would have been distinguished. Groups defined by phenological features were not concordant with clones identified by microsatellite markers. When comparing genetic identity and spring phenology, not all the ramets from microsatellite-defined clones produced leaves at the same time (Fig. 5). Seven out of 12 multi-ramet clones identified by molecular markers were heterogeneous in the timing of leaf flushing. Almost one-fourth of ramets from these clones flushed at different times. Figure 5 shows maps of sites Aiguebelle and Kanasuta depicting leaf flush times and clones as defined by microsatellite markers. The picture also includes root maps (Jelínková et al. 2009) showing that some

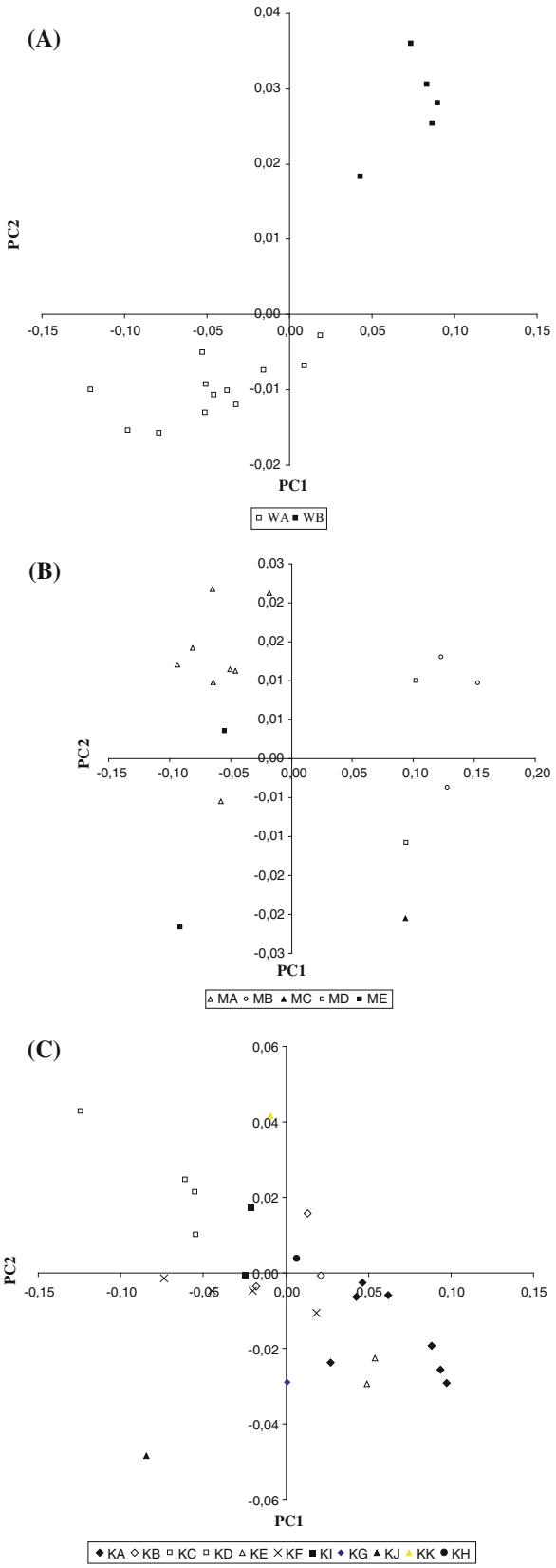

Fig. 3 Scatter-plots of within-ramet means of the first (PC1) and second (PC2) principal component based on nEFDs for 80 harmonics, **a** Aiguebelle **b** McWatters and **c** Kanasuta sits

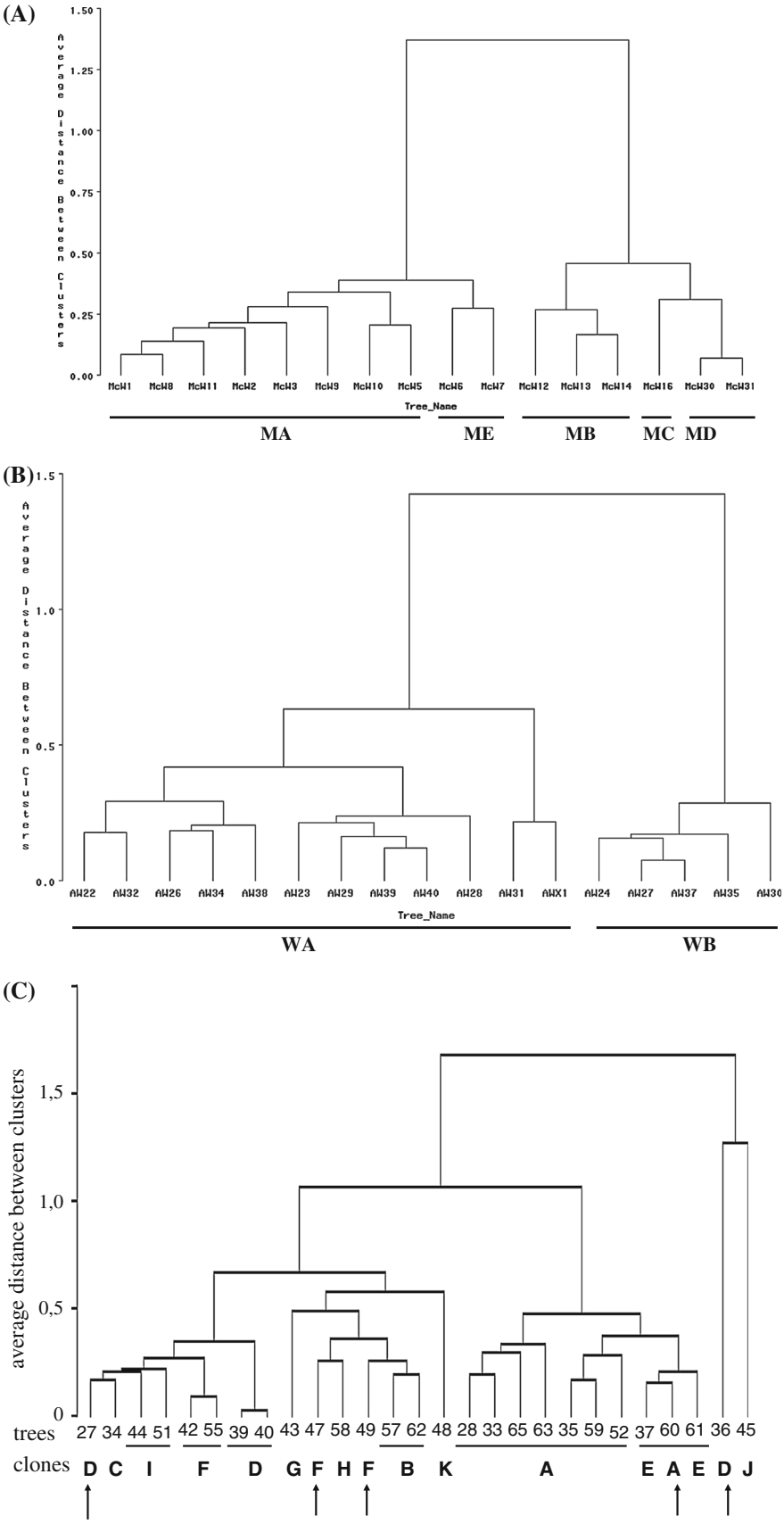

Fig. 4 Cluster analysis dendrograms derived from a matrix of mean Euclidian distances based on principal components calculated from nEFDs for 80 harmonics. Outliers are marked by *arrows*. Clones defined by microsatellite markers are *underlined* and marked by *letters*. **a** McWatters, **b** Aiguebelle, and **c** Kanasuta site

Table 4 Summary of canonical variates analyses of aspen leaf shape based on principal components of elliptic Fourier coefficients

Site	No. of hrm.	Between clones within sites	Within clones (λ_{Clone})	Between ramets within clones	Within ramets (λ_{Ramet})
Kanasuta	20	88.10	11.90	7.65	4.25
	40	95.91	4.09	2.93	1.16
	80	98.54	1.46	1.30	0.16
McWatters	20	93.21	6.79	5.02	1.77
	40	94.67	5.33	4.09	1.24
	80	97.17	2.83	2.53	0.30
Aiguebelle	20	81.68	18.32	13.11	5.21
	40	81.08	18.92	15.51	3.41
	80	86.54	13.46	12.54	0.92

Groups were successively defined as clones and ramets and individual leaves were used as replicates. Partitioning of the total variation based on Wilks' λ [%]

All Wilks' lambdas were significant ($P < 0.05$)

differentially flushing ramets had root connections to other ramets of the same or different clones.

Discussion

Trembling aspen is noted for marked variations in leaf shape. Differences in leaf morphology were reported between populations across North America (Barnes 1966, 1969; Gom and Rood 1999). Here, we demonstrated that even at a very fine scale, among trees growing in close proximity, quite a remarkable variation in leaf shape could be observed (Figs. 2, 3). Most variation was contained by the first component expressing the blade width to length ratio and distance from the insertion point to the blade maximum width (Table 3; Fig. 1). Similar features of complex leaf shape were identified to carry most variation in other species of the genus *Populus* (Lexer et al. 2009). Besides, these measures were traditionally included in morphometrics (Barnes 1966, 1969; Barnes and Han 1993; Suvanto and Latva-Karjanmaa 2005) and were, thus, likely to capture a large proportion of the total variation. Other characteristics such as the shape of apex and base contained within the second component were traditionally assessed only categorically.

The automated quantitative leaf shape evaluation allowed us to make a quick assessment of 480 leaves from 60 trees in 3 sites. The cluster analysis grouped similar foliar types which in sites McWatters and Aiguebelle corresponded to the clones defined by microsatellites (Fig. 4). Foliar morphology worked poorly in site Kanasuta. We suppose that the lack of discriminatory power in this site was most likely caused by the juvenile character of the trees. This aspen stand was 23 years old and even though some trees produced catkins in the spring of the assessment, the trees were probably immature in some phenotypic traits. This was also supported by the fact the no difference in the bark type and color could be distinguished at this site (Table 6).

Even though the ramets from all the clones in the two mature sites were clustered correctly, in accordance with the microsatellite-defined clones, it was not possible to set a clear threshold that would allow clone separation. Such a threshold would express a minimum distance between two individuals so one could say that they still belong to two different clones. In other words, it would express the maximum distance by which two individuals can differ while still belonging to the same clone. For instance, according to the largest clone WA from Aiguebelle site (Fig. 4b), this maximum distance could be set to 0.6. However, under this precondition, clones MA and ME and MB, MC, MD from McWatter site would not be differentiated (Fig. 4a). On the other hand, setting this distance lower would divide the large clones WA and MA into a few other clones. This was a consequence of a varying range of leaf shape variability among clones and small distances separating the clones (Fig. 3).

While <1 % of leaf shape variation was located within ramets (Table 4). This indicated that there was either little within tree variation or it could be efficiently reduced by selection of leaves of the same age and from the same

Table 5 Test of the discriminatory power of the EFDs

	Hrm.	MA	MB	MD	ME	WA	WB	KA	KB	KD	KE	KF	KI	Total (%)
Error rate (%)	20	0	0	0	100	8	0	0	33	33	0	0	100	13
	40	0	0	0	0	0	0	0	33	0	0	0	100	6
	80	0	0	0	0	0	0	0	0	0	0	0	100	4
Misclassified as	20				WA, KI	ME			WB	MA			KF	
	40								WB				KF	
	80												KF	

Ramets defined by leaf averages of CVs were reassigned into clones identified by molecular markers. Clone discriminant variables were calculated from nEFDs for 20, 40, and 80 harmonics. Single-ramet clones were excluded from the analysis

Table 6 Number of clones identified by microsatellite markers and number of morphotypes defined by spring phenology and bark characteristics

Sites	Nb. of clones	Nb. of bark phenotypes	Nb. of phenotypes according to spring phenology	Nb. of morphotypes (ramets differing in bark and/or spring phenology)
McWatters	5	3	4	6
Aiguebelle	2	2	3	5
Kanasuta	11	1	3	3
Total	18	6	10	14

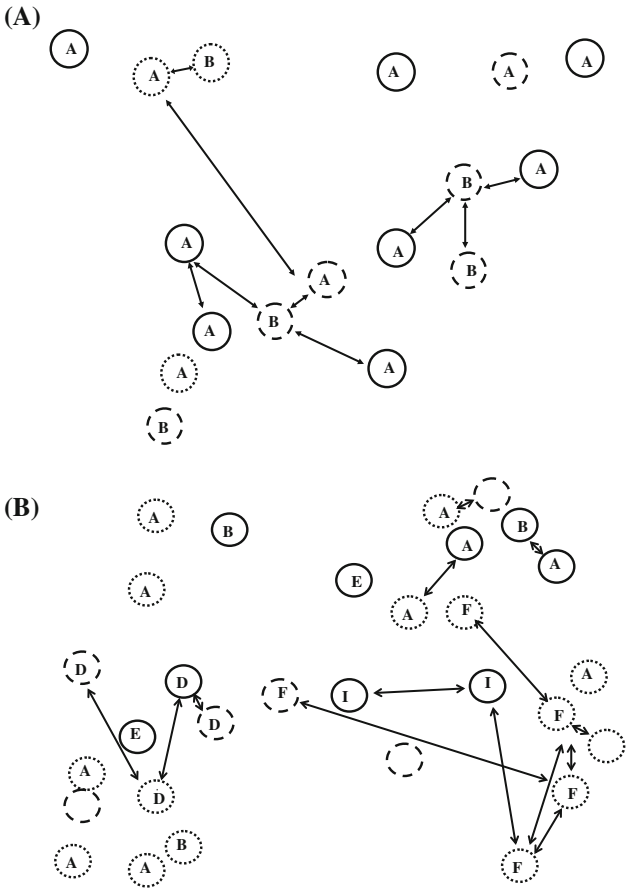

Fig. 5 Scheme of sites Aiguebelle (**a**) and Kanasuta (**b**) showing the leaf out times and clones defined by microsatellite markers. The microsatellite-defined clones are marked by *letters*, single ramet clones are marked by *empty circles*. *Full line*, *dotted*, and *dashed circles* mark the leaf out time: part **a** *full line*—15 May, *dotted*—24 May, *dashed*—after 24 May, **b** *full line*—before 19 May, *dotted*—19 May, *dashed*—23 May; *Black arrows* show root connections among ramets

position in the crown. Small within plant component of leaf shape variation was also reported in other clonal species by Rumpunen and Bartish (2002) in the genus *Chaenomeles* (Rosaceae), Persson and Gustavsson (2001) in lingonberry (*Vaccinium vitis-idaea* L.). Their observations indicated that leaf shape variation was little effected by environmental factors. Thus, little variation would be expected to be found among identical individuals. This should be particularly true when separating symmetrical and asymmetrical features of leaf shape variation as in this study since the asymmetrical component is more likely to be a consequence of environmental effects (Iwata et al. 2002b). By contrast, all symmetrical features of leaf shape studied by Lexer et al. (2009) in *Populus* species had heritable components. Nonetheless, despite the exclusion of asymmetrical features from our analysis, over 12 % of variation was observed among ramets within clones at Aiguebelle site. This number showed that the among ramet proportion of leaf shape variation may not be negligible (Table 4). To finally clarify the genetic × environmental relationship, direct common garden trials would be needed.

When using EFDs, one has to decide how many harmonics should be used. Employing too few could result in low precision of contour extraction, while using too many may lead to introduction of random errors. In general, 10–40 harmonics were sufficient for most purposes of leaf shape analysis, however, up to 100 harmonics have also been used (McLellan and Endler 1998; Mancuso 1999; Persson and Gustavsson 2001; Rumpunen and Bartish 2002; Menesatti et al. 2008). We found 20 harmonics sufficient to suggest grouping of similar foliar types (Fig. 4). Nonetheless, a greater proportion of ramets was assigned correctly to microsatellite-defined clones when employing a higher number of harmonics (Table 5). By doing so, the clonal discriminatory power of nEFDs could be increased from 87 to 96 %. Similar misclassification rates were reached when using EFDs for plant species identification (Neto et al. 2006). Discriminant analysis allowed for distinguishing 11 out of 12 multi-ramet clones (Table 5). Nonetheless, it is worth noticing that the discriminant function requires prior knowledge of well-defined classes that are created with the help of reference samples. In this study, we applied the discriminant analysis to find out if there were features of leaf shape that can delineate the clones and not as a main method for clone identification.

Spring phenology turned out to be the least reliable trait to discriminate between clones. Many ramets in our sites that actually belonged to the same clone flushed at different times (Fig. 5). For instance, trees from the large clone WA in site Aiguebelle leafed out at three different times separated by more than 9 days. Timing of leaf flushing was considered to be among phenotypic traits that are less reliable for clone identification as it can be affected by light exposure or site orientation (Kemperman and Barnes 1976). Nonetheless, it has never been explicitly shown that leaf out time can differ so dramatically among trees

growing under very similar conditions at the same microsite. Some of differentially flushing ramets were obviously suppressed stems and their delayed leaf flush could be a sign of their approaching decline. Nonetheless, among the differentially flushing trees, there were even dominant trees with large diameters at breast height that showed no reduction of growth (growth ring measurements not shown). Rather, it was interesting to notice that some of differentially flushing trees were root grafted to trees from different clones (Jelínková et al. 2009). Even though little is known about the transport of substances through root grafts (Baret and DesRochers 2011), it is tempting to speculate that even the leaf out time could be modulated by the transfer of hormones through these root connections. As natural root grafting was found to be frequent in aspen stands (DesRochers and Lieffers 2001; Jelínková et al. 2009), we think that this phenomenon should be included among other environmental factors such as light exposure that make spring phenology an unsuitable trait for clone identification.

Conclusion

Foliar diversity analyzed by automated image processing has rarely been used as a tool for differentiation among genotypes of the same species (Persson and Gustavsson 2001; Menesatti et al. 2008). This is the first study in aspen that shows its quantitative measurement by normalized EFDs. In the two mature sites, the cluster analysis of leaf shape characteristics grouped together similar foliar types and reflected well clones defined by microsatellite markers. The reclassification test indicated that leaf shape contains features according to which very similar clones can be differentiated with low error rates. However, because it was not possible to set a threshold for maximum distances within clones, application of this approach of clone identification in natural aspen stands with a high number of multi-ramet clones and many singletons is difficult.

Acknowledgments We thank to everybody who has participated in this project namely M. Baret, E. Whitfield, and C. Pack. This work was supported by the Centre for Forest Research, National Science and Engineering Research Council, Industrial Chair NSERC-UQAT-UQAM in Sustainable Forest Management, Fonds de recherche sur la nature et les technologies, and the government of Quebec.

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