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Phylogenetic relationships in *Betula* (Betulaceae) based on AFLP markers

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Abstract The genus *Betula* comprises various species in boreal and temperate climate zones of the Northern Hemisphere. The taxonomy of *Betula* is controversial and complicated by parallel evolution of morphological traits, polyploidization events, and extensive hybridization and introgression among species. Multilocus molecular data from AFLPs were used to provide phylogenetic information. A large number of polymorphic markers (321 variable bands) were produced in 107 *Betula* accessions from 23 species and 11 hybrids. The AFLP results were largely congruent with the results from previously examined nuclear DNA markers. Four distinct subgenera were identified within the genus *Betula*. These subgenera were partly in disagreement with the traditional (but disputed) division of the genus. In addition, the results indicated several groups of conspecific taxa. The majority of the species fell within subgenus *Betula* and shared a high degree of similarity with *B. pendula*. All hybrids were associated with this group, and the AFLP data contained signals on putative parents for some of the interspecific hybrids. Subgenus *Chamaebetula* and part of the *Neurobetula* species should be merged with *Betula*. The subgenera *Betulenta*, *Betulaster*, and the remaining part of *Neurobetula* are distinct and well supported. Although our results indicate that four major taxonomic groups can be recognized within the genus *Betula*, the relationship between them remains unclear. This may be due to the occurrence of hybridization and introgression, which would have a homogenizing effect on the relationships between species. Naturally occurring *Betula* species of hybrid origin may explain the low bootstrap values within the *Betula* clade.

Keywords *Betula* · Birch · AFLP · Amplified fragment length polymorphism

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Introduction

The genus *Betula* contains trees and shrubs from diverse habitats in boreal and temperate climate zones of the Northern Hemisphere. Estimates of the number of species range from 30 to 60 (De Jong 1993; Furlow 1990). The genus is placed within the Betulaceae family of the order Fagales. In Northern Europe, pollen of birch is a major cause of hay fever complaints, as is pollen of the Fagales species hazel and alder (Breiteneder et al. 1989, 1992; Lüttkopf et al. 2002). The major allergen involved is Bet v 1 of which several variants exist that may differ in their

allergenicity (Ferreira et al. 1996, 1997; Schenk et al. 2006). Given the socioeconomic impact of hay fever, birch represents a relevant target for the development of allergy prevention strategies. Selection and breeding of potential hypoallergenic birch trees requires knowledge on the genetic background of the available birch species, as the evolution of allergenic proteins is linked to the evolution of the species in which they are found. Therefore, phylogenetic relationships among *Betula* species may be used to predict allergenicity of birch species.

The taxonomy of *Betula* is controversial, and various classifications have been proposed. The first monographer who provided an extensive review of the genus was Regel (1865), who divided the genus into subgenera *Betulaster* and *Eubetula*. Subgenus *Betulaster* contains only one section, the *Acuminatae*. Subgenus *Eubetula* comprises six sections, namely C*ostatae*, *Lentae*, *Nanae*, *Albae*, *Fruticosae*, and *Dahuricae*. Winkler (1904) proposed a slightly different division, lowering the status of the sections to that of subsections, merging the *Fruticosae* and the *Dahuricae* with the subsection *Albae* and merging the *Lentae* with the *Costatae*. More recently, De Jong (1993) proposed a division into five subgenera, namely *Betulenta*, *Betulaster*, *Neurobetula*, *Betula* and *Chamaebetula*. Subgenus *Betulenta* is considered the most primitive subgenus, followed by *Betulaster* and *Neurobetula*. *Neurobetula* is considered a very heterogeneous and partly artificial group (De Jong 1993). The subgenera *Chamaebetula* and *Betula* are considered to be more derived.

The basic chromosome number of *Betula* is $n=14$, and the species form a series of polyploids with chromosome numbers of $2n=28$, 56, 70, 84, 112, and 140 (Furlow 1990). Polyploidy is a common feature among *Betula* species, and its presence within at least four of the five recognized subgenera suggests several independent polyploidization events. Hybridization and introgression are common in situations where the natural distributions of birch species overlap, for example among the European birch species *B. pendula*, *B. pubescens*, and *B. nana* (Palme et al. 2004). Moreover, several of the recognized *Betula* species have a hybrid origin (Nagamitsu et al. 2006). Hybrids generally show a morphology intermediate between the parental species but are not always morphologically distinct as a group (Thórsson et al. 2001). This overlap in morphological features complicates species and hybrid identification. Introgression appears to be bidirectional (Williams and Arnold 2001) but asymmetrical (Palme et al. 2004). Hybridization and introgression are further facilitated by the introduction and distribution of artificially propagated cultivars outside the natural distribution range. The simultaneous occurrence of polyploidization, extensive hybridization, and introgression complicates taxonomical studies in the genus *Betula*. In addition, several morphological characters are likely to have evolved independently more than once or have experienced parallel evolution (Li et al. 2005).

Given the difficulties with morphological characters in reconstructing species relationships within the genus *Betula*, alternative markers were explored, e.g., flavonoid composition (Keinänen et al. 1999), nuclear deoxyribonucleic acid (DNA) sequences (Järvinen et al. 2004; Li et al. 2005; Nagamitsu et al. 2006), a microsatellite (Nagamitsu et al. 2006), and chloroplast DNA sequences (Järvinen et al. 2004). These markers have provided useful information on the evolution of the genus, but relationships between species remain largely inconclusive due to their limited variation. For example, the chloroplast *matK* sequences examined by Järvinen et al. (2004) differentiated only three North American species from the other species. Moreover, trees constructed from different nuclear DNA markers display incongruences, e.g., nuclear ribosomal internal transcribed spacers (ITS) versus microsatellite sequences (Nagamitsu et al. 2006) and ITS versus *ADH* sequences (Järvinen et al. 2004; Li et al. 2005).

In the present study, we examined the use of AFLPs as an alternative for morphological markers, chloroplast DNA sequences, and nuclear DNA sequences. AFLP is a DNA-fingerprinting technique that generates large numbers of highly reproducible fragment markers with a genome-wide distribution. The technique is relatively fast and cost efficient and requires no prior knowledge of the genome (Jones et al. 1997; McGregor et al. 2000; Russell et al. 1997; Vos et al. 1995). Relative to morphological markers, AFLPs have the advantage that they are not under direct selection pressure, since most of the fragments represent noncoding parts of the genome (Vos et al. 1995). AFLPs are more variable than chloroplast sequences (Koopman et al. 2008). Moreover, AFLPs represent both paternal and maternal lineages because they are almost entirely derived from the nuclear genome (Althoff et al. 2007). Compared to nuclear DNA sequences such as ITS, AFLPs have the advantage that they are more variable and that they are sampled across the entire genome rather than in a specific location (Koopman 2005). However, AFLPs also have drawbacks that potentially may hamper their use as phylogenetic characters (reviewed in Koopman, 2005), most notably a possible lack of homology between fragments across taxa (Althoff et al. 2007). Several studies have shown that homology assignment between AFLP fragments decreases with increasing evolutionary distance between taxa (Althoff et al. 2007; Koopman 2005). Koopman (2005) contrasted AFLP variation with ITS sequence divergence in a large number of taxa and concluded that AFLPs are reliable phylogenetic markers for plant taxa with ITS sequences differing up to 30–35 nucleotides. A GenBank survey for the species in the present study

revealed that ITS sequence differences among ingroup species ranged from 0 to 22 nucleotides, which is well within the range defined by Koopman (2005). Therefore, it is expected that the AFLP marker variation in our data set is a suitable indicator of *Betula* relationships. Arens et al. (1998), Cervera et al. (2005), Ziegenhagen et al. (2008), and Smulders et al. (2008) demonstrated in poplar that the AFLP pattern of hybrid offspring contains bands of both parental species. Therefore, the comparison of AFLP patterns of taxa may serve to identify hybrids.

The objectives of the present study were (1) to reconstruct the phylogeny of *Betula*, while positioning and identifying hybrid taxa and cultivars, and (2) to evaluate the (sub)sections proposed by Regel (1865) and Winkler (1904), and the subgenera proposed by De Jong (1993). The division of De Jong (1993) will be used as a starting point. Species from all sections and subgenera proposed by the abovementioned authors were included, as were several hybrid taxa.

Materials and methods

Plant material

We collected young leaves from 62 *Betula* accessions in the botanical collections of Applied Plant Research, Unit Nursery Stock (Boskoop, The Netherlands), the Botanical Garden of Wageningen University (Wageningen, The Netherlands), and the Von Gimborn Arboretum (Doorn, The Netherlands). In addition, leaves were collected from ten accessions of *B. pendula* in a birch seed orchard in Urk (The Netherlands) and 31 cultivated *Betula* accessions growing as lane trees in Ede (The Netherlands) and Munich (Germany; Table 1). The accessions were originally attributed to 23 species and five interspecific hybrids based on descriptions and names available from the botanical collections. The phenetic analysis revealed that 11 accessions, cultivars mostly, did not group with the expected taxon. Nine suspected misclassified accessions were labeled as hybrids after the evaluation of the ploidy levels (Table 1). The morphology of the remaining two accessions did not match with the taxon suggested by the original label, and in one of these accessions, the ploidy level did not match either. Both accessions were tentatively assigned to the correct species (Table 1). All (sub)sections and subgenera proposed by Winkler (1904) and De Jong (1993) were represented by at least two species, except for subgenus *Betulaster* that was represented by a single species. Based on the results of Chen et al. (1999) and Li et al. (2005), two *Alnus* and two *Corylus* accessions were included as outgroups. Taxonomical names of (sub)sections and subgenera follow De Jong (1993).

Flow cytometry

Fresh leaf samples were sent to Plant Cytometry Services (Schijndel, The Netherlands) to determine the ploidy level. Ploidy levels were estimated by flow cytometry as described in Koopman (2000). Diploid (*B. pendula*) and tetraploid (*B. pubescens*) controls were included.

AFLP genotyping

For DNA extraction, young leaves of approximately 1 cm^2 were collected, immediately frozen in liquid nitrogen, and subsequently freeze dried for storage. Total genomic DNA was extracted with the DNeasy 96 Plant Kit (Qiagen, Venlo, The Netherlands) from grinded leaf tissue according to the manufacturer's instructions. The AFLP assay (Vos et al. 1995) was performed after digestion/ligation with the 6-bp cutting enzyme *Eco*RI and the 4-bp cutting enzyme *Mse*I, followed by a two-step polymerase chain reaction (PCR) amplification protocol (Arens et al. 1998) with the modification of using IRD700 fluorescence-labeled primers instead of ^{33}P-labeled primers. We used three selective primer combinations (Bonin et al. 2004): *Eco*RI 5′-GACT GCGTACCAATTC*AGT*-3′/*Mse*I 5′-GATGAGTCCTGAG TAA*CTC*-3′, *Eco*RI 5′-GACTGCGTACCAATTC*ATG*-3′/ *Mse*I 5′-GATGAGTCCTGAGTAA*CAC*-3′, and *Eco*RI 8 5′-GACTGCGTACCAATTC*ATG*-3′/*Mse*I 5′-GATG AGTCCTGAGTAA*CAA*-3′. Amplified fragments were separated on 6.5% denaturing polyacrylamide gels and analyzed on a LI-COR 4300 DNA analyzer (LI-COR Biosciences, Lincoln, NE, USA). Three accessions failed to produce a scorable AFLP pattern due to incomplete digestion. The decaploid species *B. medwediewii* was excluded because it showed an excessive number of bands, which would hamper a reliable analysis.

AFLP data analysis

Li-COR TIFF images were imported into QUANTAR software (Keygene, Wageningen, The Netherlands). Two standard samples were run on each gel to allow automatic positioning of marker bands. Presence (1) or absence (0) of polymorphic AFLP bands was scored for all accessions in the range from 100 to 450 bp. Only intense and well-separated bands were scored. The primer combinations yielded 119, 113, and 89 AFLP markers, respectively (321 in total). Eight duplicate accessions were included as controls.

Several accessions were present in duplicate in our data set. The vast majority of these were identical, but occasionally, one band was scored differently. The calculated Dice similarity was, however, always above the 98.5% limit that was indicated by Arens et al. (1998) to allow for an error in duplicated samples. All accessions displaying more than

Table 1 Plant material

	Accession code[a]	Species	Subspecies, variety, or cultivar	Ploidy[b]	Subsection (Winkler 1904)	Subgenus (De Jong 1993)	Remarks
1	W003	*B. albosinensis*	Var. *albosinensis*	4*n*	Costatae	Neurobetula	
2[d]	B004	*B. albosinensis*	Fascination	n.d.	–	Neurobetula	
3	D202	*B. alleghaniensis*		6*n*	Costatae	Betulenta	
4	W005	*B. alleghaniensis*		6*n*	Costatae	Betulenta	
5	W009	*B. chichibuensis*		2*n*	–	Neurobetula	
6	D085	*B. costata*		2*n*	Costatae	Neurobetula	
7	W012.3	*B. costata*		2*n*	Costatae	Neurobetula	
8	W013	*B. davurica*[f]		8*n*	Albae	Neurobetula	
9	D092	*B. ermanii*	Blush	4*n*	Albae	Neurobetula	
10	W021	*B. ermanii*	Var. *ermanii*	4*n*	Albae	Neurobetula	
11	D089	*B. ermanii*		4*n*	Albae	Neurobetula	Original label: *B. papyrifera* subsp. *humilis*[e]
12	W022	*B. grossa*		4*n*	Costatae	Betulenta	
13	W023	*B. humilis*		8*n*	Nanae	Chamaebetula	
14	D087	*B. korshinskyi*		4*n*	Albae	Betula	
15	D203	*B. lenta*	Subsp. *lenta*	n.d.	Costatae	Betulenta	
16	W027.1	*B. lenta*	Subsp. *lenta*	2*n*	Costatae	Betulenta	
17	W029	*B. litwinowii*		4*n*	Albae	Betula	
18	D082	*B. maximowicziana*		2*n*	Acuminatae	Betulaster	
19	W032	*B. medwediewii*		10*n*	Costatae	Betulenta	
20	B003	*B. nana*		2*n*	Nanae	Chamaebetula	
21	B002	*B. nigra*		2*n*	Costatae	Neurobetula	
22	D093	*B. nigra*		2*n*	Costatae	Neurobetula	Identical to B002
23	D097	*B. nigra*		2*n*	Costatae	Neurobetula	Identical to B002
24	E021.1	*B. nigra*		2*n*	Costatae	Neurobetula	Identical to B002
25	E021.2	*B. nigra*		2*n*	Costatae	Neurobetula	Identical to B002
26	E022.2	*B. nigra*		2*n*	Costatae	Neurobetula	Identical to B002
27	W037	*B. papyrifera*		6*n*	Albae	Betula	
28	W038	*B. papyrifera*		6*n*	Albae	Betula	
29	W044	*B. papyrifera*	Subsp. *cordifolia*	6*n*	Albae	Betula	
30	W047	*B. papyrifera*	Var. *commutata*	4*n*	Albae	Betula	
31	M040	*B. papyrifera*		n.d.	Albae	Betula	
32	W075	*B. papyrifera*		n.d.	Albae	Betula	Original label: *B. populifolia*[e]
33	B006	*B. pendula*	Laciniata	2*n*	Albae	Betula	
34	D094	*B. pendula*	Fastigiata	2*n*	Albae	Betula	
35	D095	*B. pendula*	Tristis	2*n*	Albae	Betula	
36	W058	*B. pendula*	Youngii	2*n*	Albae	Betula	
37	W059	*B. pendula*	Youngii	2*n*	Albae	Betula	Identical to W059
38	D096	*B. pendula*	Obelisk	2*n*	Albae	Betula	Identical to D094
39	W051	*B. pendula*	Dalecarlica	2*n*	Albae	Betula	
40	W057.2	*B. pendula*	Tristis	2*n*	Albae	Betula	
41	E015.1	*B. pendula*	Youngii	2*n*	Albae	Betula	Identical to W059
42	E016	*B. pendula*		2*n*	Albae	Betula	
43[c]	E017	*B. pendula*		2*n*	Albae	Betula	
44	E019	*B. pendula*		2*n*	Albae	Betula	
45	E020	*B. pendula*		2*n*	Albae	Betula	
46	E023	*B. pendula*		2*n*	Albae	Betula	
47	U001	*B. pendula*		n.d.	Albae	Betula	
48	U002	*B. pendula*		n.d.	Albae	Betula	
49	U003	*B. pendula*		n.d.	Albae	Betula	
50	U004	*B. pendula*		n.d.	Albae	Betula	
51	U005	*B. pendula*		n.d.	Albae	Betula	
52	U006	*B. pendula*		n.d.	Albae	Betula	
53	U007	*B. pendula*		n.d.	Albae	Betula	

Table 1 (continued)

	Accession code[a]	Species	Subspecies, variety, or cultivar	Ploidy[b]	Subsection (Winkler 1904)	Subgenus (De Jong 1993)	Remarks
54	U008	*B. pendula*		n.d.	Albae	Betula	
55[c]	U028	*B. pendula*		n.d.	Albae	Betula	
56[c]	U029	*B. pendula*		n.d.	Albae	Betula	
57	M001	*B. pendula*		n.d.	Albae	Betula	
58	M003	*B. pendula*		n.d.	Albae	Betula	
59	M004	*B. pendula*		n.d.	Albae	Betula	
60	M005	*B. pendula*		n.d.	Albae	Betula	
61[c]	M006	*B. pendula*		n.d.	Albae	Betula	
62	M007	*B. pendula*		n.d.	Albae	Betula	
63	M008	*B. pendula*		n.d.	Albae	Betula	
64[c]	M028	*B. pendula*		n.d.	Albae	Betula	
65[c]	M029	*B. pendula*		n.d.	Albae	Betula	
66	D084	*B. platyphylla*	Subsp. *mandshurica*	2*n*	Albae	Betula	
67[c]	W060	*B. platyphylla*		2*n*	Albae	Betula	
68	W068	*B. platyphylla*	Subsp. *szechuanica*	2*n*	Albae	Betula	
69	D086	*B. populifolia*		2*n*	Albae	Betula	
70	W080.2	*B. pubescens*	Subsp. *tortuosa*	4*n*	Albae	Betula	
71[c]	E015.2	*B. pubescens*		4*n*	Albae	Betula	
72	M022	*B. pubescens*		n.d.	Albae	Betula	
73	M031	*B. pubescens*		n.d.	Albae	Betula	
74	M023	*B. pubescens*		n.d.	Albae	Betula	
75	M220	*B. pubescens*		n.d.	Albae	Betula	
76	M014	*B. pubescens*		n.d.	Albae	Betula	
77	D090	*B. pumila*	Subsp. *pumila*	8*n*	Nanae	Chamaebetula	
78	W085	*B. schmidtii*		2*n*	Costatae	Neurobetula	
79	D091	*B. utilis*	Subsp. *utilis*	4*n*	Costatae	Neurobetula	
80	D100	*B. utilis*	Doorenbos	4*n*	Costatae	Neurobetula	
81	E010	*B. utilis*	Subsp. *jacquemontii*	4*n*	Costatae	Neurobetula	Identical to D100
82	E011	*B. utilis*	Subsp. *jacquemontii*	4*n*	Costatae	Neurobetula	Identical to D100
83	M039	*B. utilis*	Doorenbos	4*n*	Costatae	Neurobetula	Identical to D100
84[d]	B012	*B. utilis*		n.d.	Costatae	Neurobetula	
85[d]	E009	*B. utilis*	Subsp. *jacquemontii*	n.d.	Costatae	Neurobetula	
86	D098	*B.*×*caerulea*		n.d.	–	–	
87	W001	*B.*×"Edinburgh"		4*n*	–	–	
88	D201	*B.*×*fetisowii*		n.d.	–	–	
89	W024	*B.*×*koehnei*		4*n*	–	–	
90	W036	*B.*×*obscura*		4*n*	–	–	
91	B007	Unknown hybrid		4*n*	–	–	Original label: *B. ermanii*[e]
92	E013	Unknown hybrid		4*n*	–	–	Original label: *B. ermanii*[e]
93	E001	Unknown hybrid		4*n*	–	–	Original label: *B. ermanii*, identical to B007[e]
94	W077	Unknown hybrid		4*n*	–	–	Original label: *B. pubescens*, identical to B007[e]
95	D099	Unknown hybrid		4*n*	–	–	Original label: *B. papyrifera* subsp. *papyrifera*[e]
96	W052	*B.*×"Elegans pendula"		3*n*	–	–	Original label: *B. pendula* "Elegans pendula"
97	W020	*B.*×"Holland"		4*n*	–	–	Original label: *B. ermanii* "Holland"

Table 1 (continued)

	Accession code[a]	Species	Subspecies, variety, or cultivar	Ploidy[b]	Subsection (Winkler 1904)	Subgenus (De Jong 1993)	Remarks
98	D088	Unknown hybrid		2*n*	–	–	Original label: *B. albosinensis*
99	B011	*B.*×"Long Trunk"		4*n*	–	–	Original label: *B. pendula* "Long Trunk"
100	W204	*Alnus avellana*	Aurea	–		Corylus	
101	W203	*Alnus colurna*		–		Corylus	
102	W202	*Corylus incana*		–		Alnus	
103	W201	*Corylus rubra*	Oberon	–		Alnus	

The birch accessions analyzed in this AFLP study
[a] Accession number; *W* Botanical garden Wageningen (The Netherlands), *B* Botanical garden Boskoop (The Netherlands), *D* Botanical garden Doorn (The Netherlands), *U* Seed orchard Urk (The Netherlands), *E* Lane tree Ede (The Netherlands), *M* Lane tree Munich (Germany)
[b] Ploidy level; *n.d.* not determined
[c] Samples from these accessions were run in duplicate.
[d] Accessions excluded because of incomplete digestion
[e] Accessions in which the morphology conflicted with the original label
[f] Also written as *B. dahurica*

98.5% similarity potentially represent clones and were removed from further analyses (Table 1). The phenetic analyses were conducted on a data set containing 87 unique accessions, including hybrids. Similarity matrices of Jaccard distances and Dice distances were calculated using NTSYSpc 2.10j (Applied Biostatistics, Setauket, NY, USA). Dendrograms were subsequently constructed using neighbor-joining (NJ) analysis. Cophenetic matrices were calculated from the resulting dendrograms and the product–moment correlation between cophenetic and similarity matrices was calculated to test the goodness of fit of the cluster analysis. The Mantel test for matrix correspondence was performed with 1,000 permutations. The combination with the best fit (Dice+NJ) was chosen for phenetic analysis. To allow bootstrapping, the analysis was repeated in Paup 4.0b10 (Swofford 2002) using Nei–Li distances (=1−Dice similarity) and 1,000 bootstrap replicates.

The relationships among species classified in subgenus *Betula* (cluster IV, see "Results") were examined in detail with a principle coordinate analysis (PCO). The PCO was based on Dice distances and carried out in NTSYSpc. A further classification of these accessions was made in a Bayesian analysis using STRUCTURE 2.2 (Falush et al. 2003; Pritchard et al. 2000). The objective of this analysis was to test whether species form separate clusters or species groups and whether hybrids are classified within or between these groups. STRUCTURE was developed for studies on populations, in which individual samples are assumed to be able to exchange genetic material. This is clearly not the case for the genus *Betula* as a whole, but may be realistic for the subgenus *Betula*, in which hybridization and introgression are common features. Ploidy levels in this subgenus vary from diploid to octoploid. As STRUCTURE does not contain models that can deal with this situation, all accessions were treated as diploid (explained below). Dominant AFLP marker data were entered by coding both alleles as "1" when a band was present and as "0" when a band was absent, while specifying "0" as a recessive allele for all markers. Estimates were obtained under the admixture model using the correlated allele frequencies option. Version 2.2 accommodates genotypic uncertainty in dominant marker data by sampling present bands as homozygotes or heterozygotes according to their posterior probabilities (Falush et al. 2007). This does not fully account for the fact that, given the dominant nature of AFLP markers, higher ploidy levels would contain a higher level of genotypic uncertainty. This may distort the absolute genetic distances, with polyploids ending up genetically more similar to one of the parents, but this will not interfere with the goal of identifying hybrid accessions. The number of inferred groups was evaluated at values of K ranging from 1 to 17, in which the maximum of $K=17$ corresponds to the number of sampled species in subgenus *Betula* (excluding hybrids). Three replicate runs were performed for each value of K. A burn-in of 50,000 cycles and data collection for 100,000 cycles was used. The admixture model estimates the proportion of each accession's genome that descended from each of the K inferred groups.

Phylogenetic analyses were conducted on two data sets. The first set contained a maximum of four accessions per species because otherwise some species would be overrepresented in the data set. Accessions that were initially misclassified were preferentially excluded, followed by accessions that had the most missing values. Hybrid cultivars were also excluded. The second set was a subset of the first set, containing only the diploid species. Phylogenetic signal in

the data set was quantified with the g_1 statistic (Hillis and Huelsenbeck 1992). Parsimony analyses were conducted in PAUP as heuristic searches with 100,000 random additions (holding one tree at each step), tree–bisection–reconnection (TBR) branch swapping, multrees switched off, and ACCTRAN for character optimization. The initial search was followed by additional branch swapping on the most parsimonious trees (MPTs) with the multrees option switched on. Branch support was assessed by bootstrap analysis comprising 10,000 replicates consisting of ten random addition sequences with TBR branch swapping.

Results

AFLP similarities

The three AFLP primer combinations produced 321 variable bands in 99 *Betula* and four outgroup accessions. Dendrograms obtained by unweighted pair group method with arithmetic mean (UPGMA) and NJ using Jaccard and Dice distances were highly similar (data not shown). Correlations between Dice and Jaccard similarity matrices and cophenetic matrices from the dendrograms were high (0.96–0.98), with a one-tailed probability of 0.001 at 1,000 permutations. This indicates that the dendrograms provided a good fit to the similarity matrices. The highest correlation was found for Dice distances in combination with NJ (0.98). Similarity values between the outgroups (*Corylus* and *Alnus*) and ingroup (*Betula*) ranged from 0.14 to 0.33, while ingroup similarity values all exceeded 0.32. The NJ tree shows a clear structure, although the support values for most branches were quite modest (Fig. 1). Four major clusters were present: (I) the *B. schmidtii/B. chichibuensis* cluster, (II) the *B. nigra* cluster, (III) the subgenus *Betulenta* cluster, and (IV) the subgenus *Betula* cluster. The latter contained all accessions from subgenus *Betula* and additional accessions from other subgenera (discussed below). Group IV was by far the largest group and contained several supported subgroups, such as the cluster with both *B. costata* accessions, the cluster with *B. davurica* and *B. humilis*, the cluster with all *B. papyrifera* accessions, and the cluster with *B. utilis* subsp. *jacquemontii* and *B.*×"Long Trunk." Mean similarity values among *Betula* species are provided as electronic supplementary material (Table S1).

Relationships within the subgenus *Betula*

To allow a detailed analysis of the relationships within the *Betula* cluster (IV), we performed a PCO on the accessions within this group. The first three components had Eigenvalues of more than 1.0 and explained 29.6% of the variation (16.8, 7.4, and 5.3, respectively). The first two components of the PCO are plotted in Fig. 2a, in which five groups are distinguished. Group A is represented by a large number of accessions comprising the species *B. pendula*, *B. plathyphylla*, and *B. populifolia*. These species are not separated from each other on the first three components. Group B in the PCO plot comprised six species: *B. pubescens*, *B. litwinowii*, *B. korshinskyi*, *B. papyrifera*, *B. pumila*, and *B. nana*. With the exception of *B. nana*, these species are hardly separated on the first two components. The third component did separate *B. papyrifera* and *B. pubescens* and to a lesser extent *B. pumila* and *B. pubescens* (not shown). Betula *maximowicziana* is placed in group C. Although the PCO puts *B. maximowicziana* close to group D, *B. maximowicziana* has the most basal position in cluster IV and a relatively low similarity to the other accessions in subgenus *Betula* (Fig. 1). The species *B. costata*, *B. davurica*, *B. humilis*, and *B. ermanii* made up group C. These species branch off sequentially at more derived positions in cluster IV (Fig. 1) as do the species in group E, comprising *B. grossa*, *B. utilis*, and *B. albosinensis.*

Most cultivars of hybrid origin are placed in between the groups A to E. Their hybrid origin was confirmed by comparing the AFLP profile and/or ploidy level. Notable exceptions were the triploid hybrid "Elegans Pendula" and *B.*× *koehnei* (a hybrid between *B. pendula* and *B. papyrifera*), which was located among the *B. pubescens*-like accessions in group B. The cultivars "Long Trunk" and "Edinburgh" were positioned within group E, close to *B. utilis* and *B. albosinensis*. "Long Trunk" was originally described as a *B. pendula* cultivar, but clusters with *B. utilis* "Doorenbos" in the NJ dendrogram and, based on the AFLP profile, appears to represent either a hybrid between *B. utilis* and *B. pendula* or a true *B. utilis* cultivar. The parental species of the hybrid cultivars could be established for some accessions, although the close relatedness among the species in subgenus *Betula* and the presence of species with a hybrid origin complicated this analysis. For example, the AFLP profile of *B.*×"Elegans Pendula" shared most bands with *B. pendula* (Dice similarity with *B. pendula* accessions of 0.7 or more). The position in the PCO suggests that the other parent may be *B. pubescens* or a related species. The largest number of bands not shared with *B. pendula* was shared with two accessions of *B. pubescens* and the accession from *B. litwinowii*.

To test the separation into groups and the presence of admixture in hybrid accessions within subgenus *Betula*, we used a Bayesian population clustering approach implemented in the program STRUCTURE (Falush et al. 2003; Pritchard et al. 2000). The STRUCTURE analysis provided strong support for three species groups, with large and consistent improvements in the probability function [Ln $P(D)$] for runs with K=3 relative to K=2. Values of Ln $P(D)$

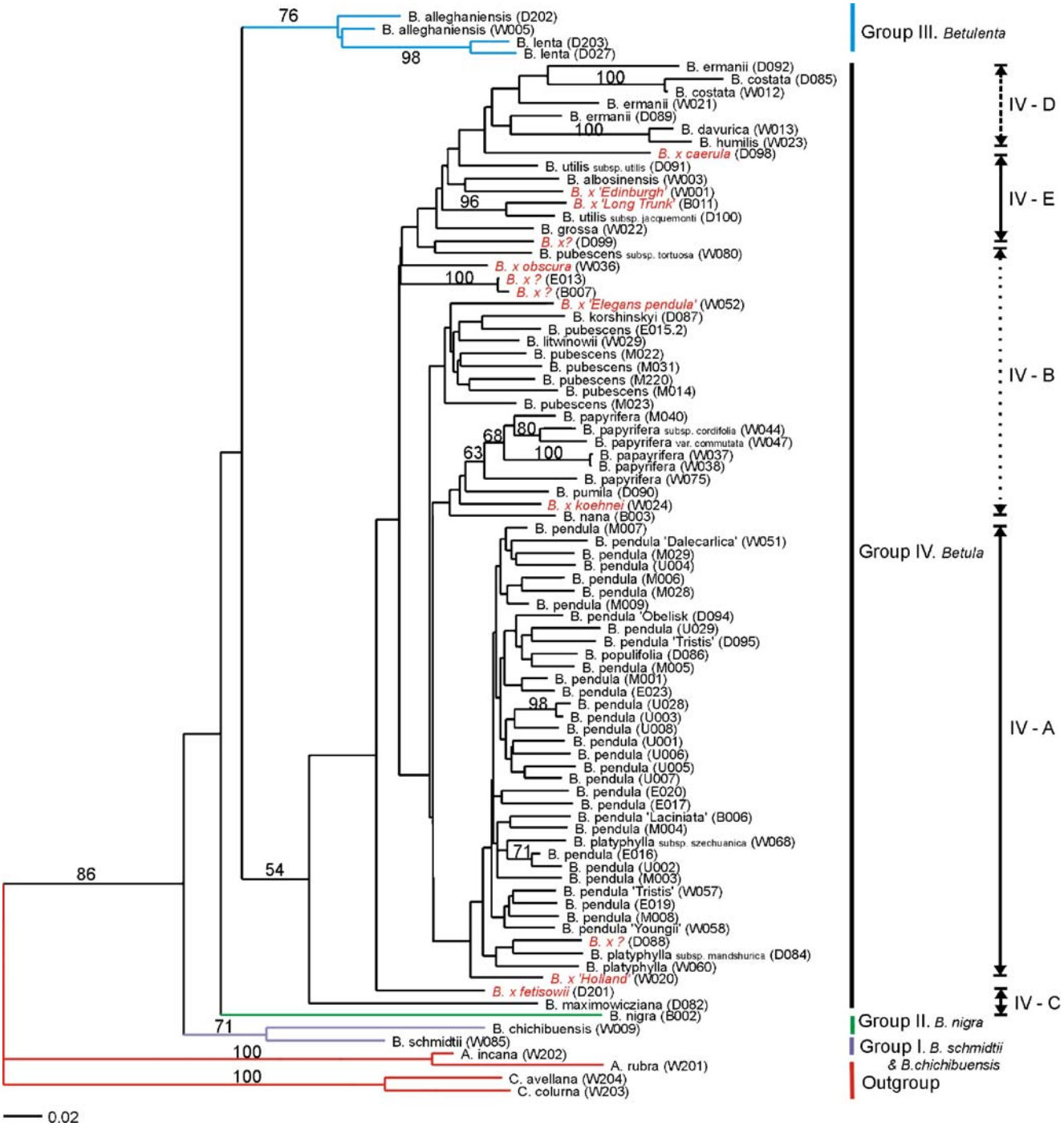

Fig. 1 Neighbor-joining tree of Dice similarities among 22 *Betula* species and ten interspecific hybrids (shown in *red italics*) based on 321 AFLP markers. Bootstrap percentages greater than or equal to 50 are shown on the *branches*. The groups within subgenus *Betula* as found in the PCO (Fig. 2) are shown on the *right*

improved modestly for K=4 relative to K=3, while for K>4 runs, the variation between replicate runs became very large, so that there was no consistent further improvement. This may be due to the fact that the first two groups identified (marked green and blue in Fig. 2) are rather homogeneous, while the third group includes even single accessions of some species. The average values were: K=1: −5,836.8, K=2: −5,175.2, K=3: −4,944.8, K=4: −4,873.9. The three species groups that were distinguished were largely in line with the groups IV-A, IV-B, and IV-C+D that were distinguished by the PCO (Fig. 2a,b). Some hybrids were actually clustered within the three distinguished groups. For example, *B.*×*koehnei*, *B.*×"Elegans Pendula," and *B.*×*obscura* fell within group IV-B, while

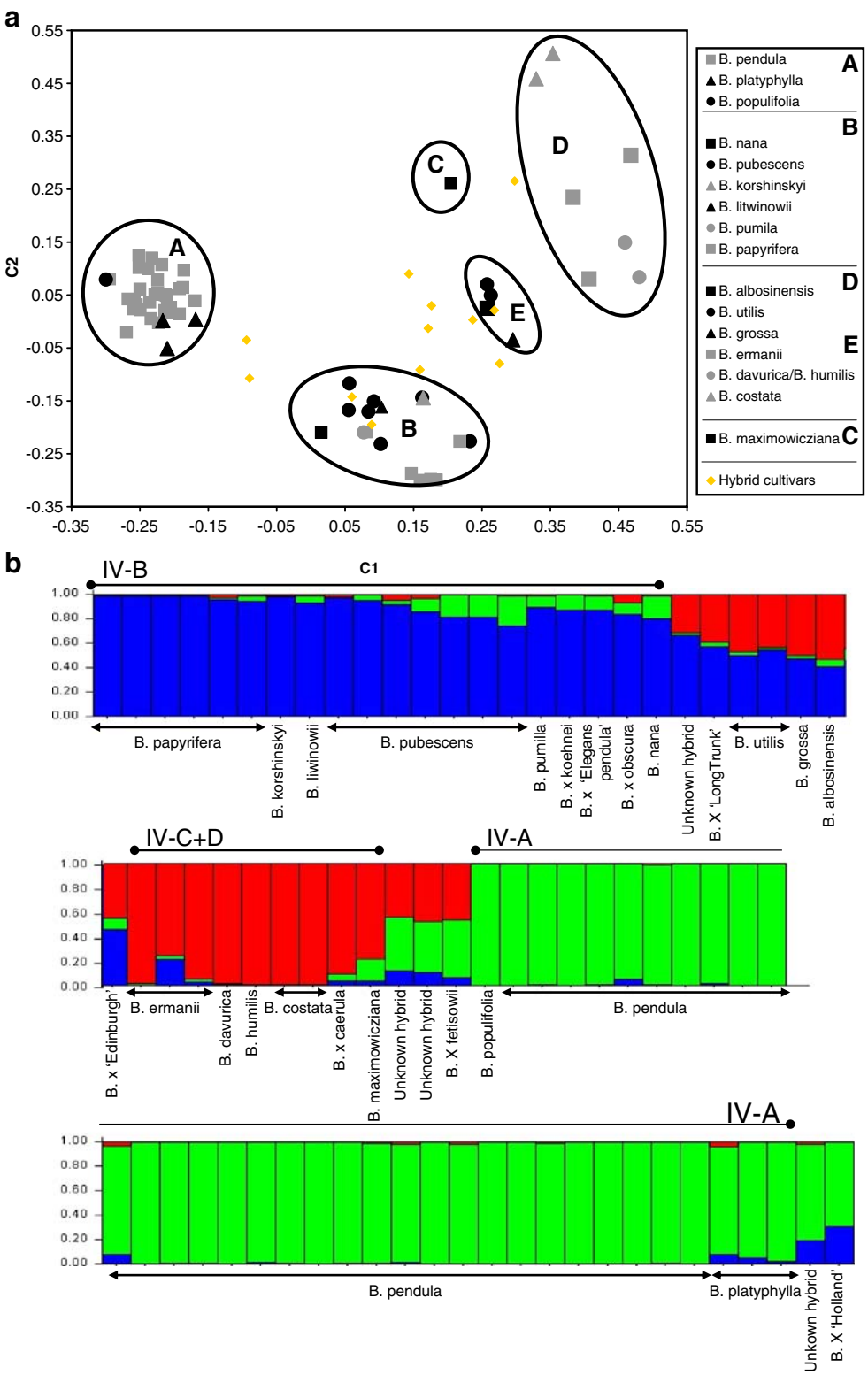

Fig. 2 **a** Principal coordinates plot of the *Betula* accessions in subgenus *Betula* (group IV; see Fig. 1) for the first two principal components estimated with 234 AFLP markers. **b** STRUCTURE analysis of the *Betula* accessions in subgenus *Betula* (group IV) inferred from AFLP markers. In this figure, each accession is represented by a *vertical bar* partitioned into $K=3$ colored segments (*green*, *blue*, and *red*). At $K=2$, the accessions in *green* were separated from the rest. At $K=3$, the *blue* group was separated from the rest (in *red*). The corresponding groups (IV-A, B, and C+D) from **a** are displayed *above the bars*

B.×*caerulea* fell within group IV-C+D. The other hybrids showed clear signs of admixture between groups. Notably, the species *B. albosinensis*, *B. utilis*, and *B. grossa* that were attributed to group IV-E in the PCO plot were not distinguished as a separate group in the cluster analysis and showed admixture between group IV-B and IV-C+D, which is consistent with their position relative to these groups in the PCO plot (Fig. 2a).

Phylogentic analysis of AFLP data

Cultivars with a mixed species background (interspecific hybrids, listed in Table 1) were excluded from the phylogenetic analysis, which was performed with 43 accessions. The data set included 297 variable bands, 211 of which were parsimony informative. The g_1 statistic for the data set was −0.52. This value is considerably lower than the corresponding critical value of −0.09 (p=0.01; Hillis and Huelsenbeck 1992) indicating the presence of ample phylogenetic signal. The initial parsimony analysis resulted in 12 MPTs of 721 steps on 11 different islands. The trees had a consistency index (Kluge and Farris 1969) of 0.412 and a retention index (Farris 1989) of 0.585. Additional branch swapping did not yield any extra trees. The strict consensus of the MPTs is shown in Fig. 3.

The consensus tree from the data set without hybrids (Fig. 3) and the NJ tree of the data set including hybrids (Fig. 1) have a similar topology regarding the accessions that are present in both trees. Several groups can be identified when both trees are considered: (I) *Betula schmidtii* and *B. chichibuensis* form a distinct and supported cluster in both the NJ (71% bootstrap support) and MP tree (85%). Both are Asian species from subgenus *Neurobetula.* These species are relatively divergent from the other *Betula* accessions, with similarities between 0.33 and 0.48. (II) *Betula nigra*, a North American species, is classified in subgenus *Neurobetula* and is clearly separated from all other *Betula* species. It was the most divergent accession in the NJ tree with a similarity of 0.32 to 0.39 relative to the other *Betula* accessions. (III) *Betula lenta* and *B. alleghaniensis* are two closely related North American species that are classified in subgenus *Betulenta*. *B. lenta* is diploid, while *B. alleghaniensis* is hexaploid. The clade is supported in both the NJ (76%) and the MP tree (99%).

B. maximowicziana is an Asian species that is the only representative of subgenus *Betulaster* included in our study. It has a basal position in group IV in both the NJ and MP tree and is placed in group IV-C (Figs. 1 and 3). *B. costata* is the next species to branch of in the MPT, while being placed among the other representatives of group IV-D in the NJ tree. The remaining species of group IV-D and IV-E branch off sequentially within group IV. The groups D and E do not form supported groups in the MPT. The species *B. ermanii*, *B. davurica*, *B. utilis*, and *B. albosinensis* were previously classified in subgenus *Neurobetula. B. grossa* (subgenus *Betulenta*) is also placed within this group. In addition, *B. humilis* (subgenus *Chamaebetula*) is shown to be closely related to *B. davurica*. The clade with groups IV-A and IV-B includes all examined species from subgenus *Betula*, namely *B. pendula*, *B. plathyphylla*, *B. populifolia*, *B. pubescens*, and *B. papyrifera*, and two species from subgenus *Chamaebetula*, namely *B. nana* and *B. pumila*. *B. pumila* clusters with *B. papyrifera* in both the NJ tree and the MPT. Group IV-A from the PCO contains the *Betula* species *B. pendula*, *B. plathyphylla*, and *B. populifolia* and also forms a separate clade in the MPT.

Species with higher ploidy levels may represent natural interspecific hybrids. To exclude the effects of hybridization as much as possible, we repeated the analysis with only the diploid species. In the resulting NJ tree and MPTs (not shown), five groups could be identified. The first three groups (I to III) were similar to the groups discussed above, while the clustering within group IV was slightly different. In Fig. 3, *B. maximowicziana* is the most basal clade in group IV. In the diploid tree, *B. maximowicziana* and *B. costata* clustered together and had a basal position relative to *B. nana*, *B. pendula*, *B. plathyphylla*, and *B. populifolia*. The grouping of *B. maximowicziana* with *B. costata* was also suggested by the STRUCTURE analysis.

Discussion

AFLP markers for phylogeny

The taxonomy of the genus *Betula* is controversial. Although ample morphological variation exists in characters such as leaf shape, bark color, and shape of the catkins, attempts to reconstruct species relationships using morphological characters failed to produce a reliable classification. The occurrence of polyploidization (Nagamitsu et al. 2006), hybridization, and introgression (Palme et al. 2004; Thórsson et al. 2001; Williams and Arnold 2001) and the fact that morphological characters may have evolved independently more than once in *Betula* (Li et al. 2005) may account for this. Up to now, *Betula* taxonomy had been studied using morphological characters, flavonoid composition, and nuclear and chloroplast DNA sequences. The main limitations of morphological markers are selection pressure on morphological markers and hybrid morphology not always being intermediate (Thórsson et al. 2001). The main limitation of the sequence markers is their limited variation. We applied AFLP as an alternative, because AFLP generates polymorphic markers at a high frequency, has a high reproducibility, and has genome wide sampling and its markers are not under direct selection pressure (Jones et al. 1997; McGregor et al. 2000; Russell et al. 1997).

More than 200 parsimony informative AFLP markers were generated in a data set of 87 unique *Betula* accessions. The AFLP data distinguished four subgenera and four groups within the largest subgenus (*Betula*). However, we could not unambiguously resolve relationships among these groups. Due to the extensive hybridization and introgression within the genus *Betula*, good support for the

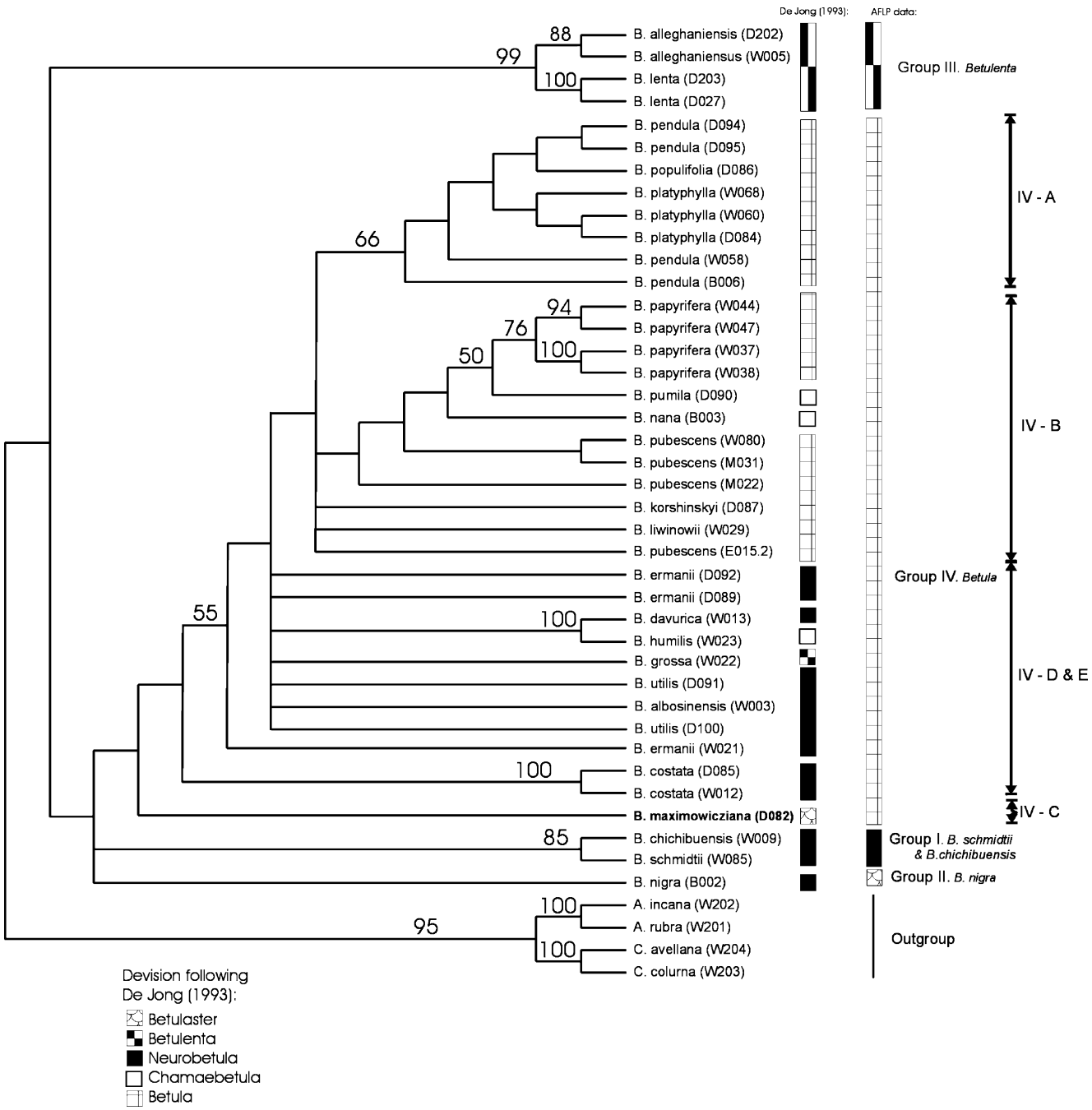

Fig. 3 Strict consensus of 12 MPTs based on 297 AFLP markers and 22 *Betula* species. Bootstrap percentages greater than or equal to 50 are shown *on the branches*. The subgeneric division as proposed by De Jong (1993) is shown on the *left vertical bar*; the groups suggested by the AFLP data are shown on the *right bar*

relationships is not always to be expected, although bootstrap support was generally higher for the AFLP-derived groups compared to groups identified based on ITS sequences (Li et al. 2005; Nagamitsu et al. 2006), and AFLP was able to provide resolution on clades that were unresolved with ITS data. Apart from differences in support and resolution, congruence between ITS data and AFLP data was high for the genus *Betula*. This general congruence between AFLP and ITS tree topologies is found across a wide range of taxonomic groups (Koopman 2005). Besides yielding detailed information on *Betula* relationships, AFLPs provided complementary information on hybridization events. Such events were reflected in the AFLP profiles by bands shared between the hybrid and parental species and for some hybrids in the STRUCTURE analysis. In ITS sequences, the parental information may be

lost or misleading (Álvarez and Wendel 2003), even when fragments are cloned before sequencing (Nagamitsu et al. 2006).

Phylogeny of the genus *Betula*

In summary, the AFLP results indicate the presence of four subgenera in *Betula*, as opposed to the five subgenera originally recognized by De Jong (1993). Each of the original subgenera is discussed separately below.

Subgenus *Betulenta* (De Jong 1993), which is synonymous with the *Lentae* of Regel (1865), was represented by *B. lenta*, *B. alleghaniensis*, *B. medwediewii*, and *B. grossa* in our study. Winkler (1904) merged this group with subsection *Costatae*, but our results support the division of De Jong (1993) and show that the *Betulenta* sensu Winkler (1904) would be paraphyletic. *B. lenta* and *B. alleghaniensis* grouped together with good support in both the AFLP NJ and maximum parsimony analysis. The close relationship of *B. lenta* and *B. alleghaniensis* (together with *B. medwediewii*) is supported by nuclear ITS and chloroplast *matK* sequence data (Järvinen et al. 2004; Li et al. 2005). The decaploid *B. medwediewii* was not included in our analysis due to an excess of bands in the AFLP profile. Our AFLP results are not in line with data on ITS sequences that indicated a close relationship of *B. alleghaniensis* with the non-*Betulenta* species *B. costata* (Li et al. 2005). Morphologically, *B. costata* and *B. alleghaniensis* are very distinct, and we therefore consider the AFLP results to be more reliable. The position of *B. grossa* in the *Betulenta* is supported by data on phenolic variation (Keinänen et al. 1999). However, both our AFLP data and data on ITS sequences (Nagamitsu et al. 2006) suggest that *B. grossa* is not positioned within *Betulenta*. The position of this species is thus uncertain. *B. lenta*, *B. alleghaniensis*, and *B. medwediewii* are maintained within *Betulenta*.

Subgenus *Betulaster* was represented by a single accession of *B. maximowicziana*. According to the AFLP data, this accession is positioned at the periphery of subgenus *Betula* in group C. Li et al. (2005) pointed out that *B. maximowicziana* did not cluster with other representatives of subgenus *Betulaster*. Given that *B. alnoides* is the type species of subgenus *Betulaster*, the status of *Betulaster* would depend on the position of *B. alnoides*, which was not included in our study. *B. nigra* did cluster with *B. alnoides* according to Li et al. (2005). In our AFLP trees, *B. nigra* formed a separate and well-supported group. The above therefore suggests that subgenus *Betulaster* may, in fact, be a distinct group whose position in our AFLP trees is represented by *B. nigra*. The observation of Li et al. (2005) may be related to the fact that the distribution area of the two Asiatic *Betula* species is quite different: *B. maximowicziana* is distributed in the eastern part of Japan while *B. alnoides* is distributed in southeast Asia (from India to Vietnam and southern China).

In our data set, subgenus *Neurobetula* was represented by seven species (excluding *B. nigra*) that separated in two major groups. Group I (*B. chichibuensis* and *B. schmidtii*) was well defined and well supported, while the other group (Group IV-D+E in Figs. 1, 2, and 3) was more loosely defined and contained the species *B. costata*, *B. ermanii*, *B. davurica*, *B. utilis*, and *B. albosinensis*. Previous studies concluded that subgenus *Neurobetula* is a heterogeneous and polyphyletic group (De Jong 1993; Li et al. 2005). However, an alternative division was not proposed, because conflicts in morphological markers and low variation among DNA markers hampered an unambiguous conclusion. The position of *B. schmidtii* as a close relative to *B. chichibuensis* was in line with studies on ITS sequences (Li et al. 2005; Nagamitsu et al. 2006) and a recent morphological study by Skvortsov (2002). On the other hand, Keinänen et al. (1999) and Järvinen et al. (2004) found that *B. schmidtii* was closely related to subgenus *Betula*. Our results indicate that *B. costata*, *B. ermanii*, *B. davurica*, *B. utilis*, and *B. albosinensis* should be merged with subgenus *Betula*. The common existence of hybrids between the above species and species from subgenus *Betula* also support placement in a single subgenus. All these species are of Asian origin and a more extensive sampling from their natural range will be needed to resolve interspecific relationships and to disentangle phylogenic relationships from geographical components if gene flow (hybridization and introgression) occurred between certain species in overlapping parts of their distribution area.

Subgenus *Chamaebetula* (De Jong 1993) was represented in our study by *B. humilis*, *B. nana*, and *B. pumila*. According to De Jong (1993), subgenus *Chamaebetula* is polyphyletic and artificially grouped based on the single morphological character of having a shrubby habitus. Our results indicate that these species should be placed within subgenus *Betula*. For *B. nana*, a close relationship to subgenus *Betula* is supported by sharing of chloroplast haplotypes between *B. nana* and *B. pendula* (Maliouchenko et al. 2007; Palme et al. 2004). *B. nana*, *B. pumila*, and *B. humilis* are placed in different groups (IV-B vs. IV-D) within subgenus *Betula*, confirming the polyphyletic nature of the *Chamaebetula*. The above suggests that subgenus *Chamaebetula* is superfluous.

Subgenus *Betula* was represented in our study by *B. pendula*, *B. plathyphylla*, *B. populifolia*, *B. pubescens*, *B. litwinowii*, *B. korshinsky*i, and *B. papyrifera*. Although not supported by high bootstrap values, the species originally placed within subgenus *Betula* do consistently group together in both NJ tree (Fig. 1) and MPTs (Fig. 3). The PCO analysis distinguished four species groups in the subgenus *Betula* (Fig. 2a), while the STRUCTURE analysis

recognized three groups. The species *B. pendula*, *B. plathyphylla*, and *B. populifolia* clustered together in group A and showed hardly any genetic differentiation. In fact, the AFLP data failed to differentiate between these species, suggesting that they are conspecific. Their morphology is also very similar, and Skvortsov (2002) already considered *B. platyphylla* to be synonymous with *B. pendula*. Only one *B. populifolia* accession was included, so further sampling within the natural range of this species will be necessary to confirm its status. Group B consisted of the potentially conspecific *B. pubescens*, *B. litwinowii*, and *B. korshinskyi*, *B. papyrifera*, and two species from the *Chamaebetula*, namely *B. nana* and *B. pumilla*. *B. pubescens*, *B. litwinowii*, and *B. korshinskyi* do not separate in the PCO, but more extensive sampling is required to establish their status. Group D comprised *B. maximowicziana* as discussed above, while groups D and E comprised several species that were originally attributed to subgenus *Neurobetula*. The groups C and D formed a single group in the STRUCTURE analysis. Group E was shown to have an intermediate position between the groups B and D. This group contained only polyploid species, consistent with a potential hybrid origin. In summary, all previously assigned species were retained in subgenus *Betula*, while subgenus *Chamaebetula*, part of the species from *Neurobetula*, and *B. maximowicziana* were also placed in this subgenus.

Evolution

Although our results indicate that four major taxonomic groups can be recognized within the genus *Betula*, the relationship between them remains unclear. The most obvious explanation for the lack of support is the occurrence of hybridization and introgression, which would have a homogenizing effect on the relationships between species. Several types of hybrids may occur, and they can be classified as "newly formed (F1) hybrids," "later generation hybrids," and "hybrid species" (Vriesendorp and Bakker 2005). Hybrid cultivars are likely to fall within the first two groups, and we excluded the cultivated hybrids from our maximum parsimony analysis. However, naturally occurring hybrid species may also exist and may in fact make up a significant proportion of all *Betula* species. Their presence in the data set may explain the low bootstrap values within the *Betula* clade. Species such as *B. ermanii*, *B. humilis*, *B. utilis*, and *B. pubescens* each have ploidy levels higher than 2*n* and to some extent contain AFLP bands that can be regarded as diagnostic bands for *B. pendula*. However, we cannot determine to which extent this reflects shared evolution or shared parental species in a natural hybridization process. An alternative explanation for the lack of support relates to a situation in which the major speciation events took place within a very short time frame. This would result in so called bush-like clades that are characterized by short stems relative to the length of the branches (Rokas and Carroll 2006). Under these circumstances, homoplasy may limit the phylogenetic resolution by overwhelming the true phylogenetic signal. This could also explain why we cannot determine the relationships between the subgenera. If this is the case, these relationships may remain unresolved.

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