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Bud set in poplar – genetic dissection of a complex trait in natural and hybrid populations

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Summary

- The seasonal timing of growth events is crucial to tree distribution and conservation. The seasonal growth cycle is strongly adapted to the local climate that is changing because of global warming. We studied bud set as one cornerstone of the seasonal growth cycle in an integrative approach.
- Bud set was dissected at the phenotypic level into several components, and phenotypic components with most genetic variation were identified. While phenotypic variation resided in the timing of growth cessation, and even so more in the duration from growth cessation to bud set, the timing of growth cessation had a stronger genetic component in both natural and hybrid populations.
- Quantitative trait loci (QTL) were identified for the most discriminative phenotypic bud-set components across four poplar pedigrees. The QTL from different pedigrees were recurrently detected in six regions of the poplar genome.
- These regions of 1.83–4.25 Mbp in size, containing between 202 and 394 genes, form the basis for further molecular-genetic dissection of bud set.

Introduction

The seasonal cycle of growth and dormancy is a distinct feature of perennial plants and represents one of the most basic adaptations of trees to their environment. Many events in the annual growth cycle, such as bud flush, growth, growth cessation, bud set, bud dormancy and release from dormancy through chilling, are regulated by aspects of local climate. The recurrent transitions of meristems between growth and dormancy are tightly linked to the yearly dates of bud flush and bud set. These two events delimit the growing season and are under strong selection to avoid cold injury by either early frost in autumn or late frost in spring. Bud flush and bud set show strong genetic differentiation along latitudinal and altitudinal clines, typically resulting in locally adapted ecotypes (Howe *et al.*, 2003; Savolainen *et al.*, 2007; Aitken *et al.*, 2008). Along large geographical clines, bud set is often more differentiated among populations than bud flush. Q_{ST} values, which estimate the proportion of the total trait variation between populations, are generally higher for bud set than for bud flush (Howe *et al.*, 2003).

In the light of climate change, bud flush and bud set have gained considerable attention. Changes in phenology have been noted during the past decades and informed the discussion on whether trees will cope with the expected speed of climate change (Saxe *et al.*, 2001; Aitken *et al.*, 2008; Marris, 2009). Because phenology is so crucial to tree distribution and conservation, research efforts to understand its genetic basis are of paramount importance.

Bud set is the net result of growth cessation and bud formation. Many broad-leaved trees cease growth in response to increasing night length, sometimes in combination with or completely dependent on decreasing temperature (Nitsch, 1957; Heide, 1974; Junttila, 1982). In poplar, increasing night length is perceived and mediated through phytochrome and the CONSTANS (CO)/FT regulon (Howe *et al.*, 1996; Olsen *et al.*, 1997; Böhlenius *et al.*, 2006). In addition to growth cessation, autumnal bud development is a composite of three processes, namely bud formation, simultaneous acclimatization to dehydration and cold, and acquisition of dormancy (Ruttink *et al.*, 2007).

Once a critical night length – the night length triggering growth cessation – is achieved, it takes several weeks until the completion of bud set. During this time dynamic changes take place that are reflected at both the molecular and phenotypic levels (Ruttink *et al.*, 2007). Traditional measurements of bud set have focused only on the timing of bud set, when all biological processes are completed (Frewen *et al.*, 2000). To better understand the genetic components and the plasticity of bud set, it is important to also describe the initiation of bud set at cessation of growth and its dynamics until bud set. These aspects may contribute significantly to genetic variation at both among- and within-population levels.

The aim of this study was to dissect bud set at the phenotypic level into its components, to identify those expressing a high level of genetic variation, and to detect genomic regions associated to this genetic variation by quantitative trait loci (QTL) mapping. First, we developed a new scoring system for bud set to measure all aspects of the phenotype, including the onset and the duration of autumnal bud development. Bud set was assessed in a wide collection of European riparian Black poplars (*Populus nigra*) and in four interspecific and intraspecific poplar pedigrees. The elaborate phenotypic data revealed that phenotypic variation resided in both the timing of cessation of growth and the duration from cessation of growth to bud set. Although the duration of bud formation varied at the phenotypic level, it contained, under the environmental conditions of this experiment, a smaller genetic component than the timing of growth cessation. Based on this information, quantitative trait loci for the most discriminative components of bud set, covering onset of growth cessation and duration of bud formation, were detected across four poplar pedigrees. The QTL from different pedigrees coincided in six regions of the poplar genome. Together, we integrate all information from phenotype, the contribution of its components to genetic variation, the genomic regions that control bud set variation and candidate genes. Because, this study is based on heterogeneous genetic material, the shared QTL regions provide a robust result to initiate the identification of the underlying genes.

Materials and Methods

Genetic material and field sites

A collection of European *Populus nigra* A total of 466 *P. nigra* genotypes were collected along riparian ecosystems in five European countries and planted in Geraardsbergen (Belgium) (Table 1; Supporting Information, Table S1). Bud set was scored for 437 genotypes with at least three ramets per genotype. The following populations had been previously sampled and characterized with neutral markers as true populations: Drôme1, Drôme6, TicinoSN, TicinoN, Kühkopf, Ebro1 and Ebro2. The overall genetic differentiation between populations (F_{ST}) is low to moderate with values between 0.03 and 0.28 (van Dam & Bordács, 2001; Imbert & Lefèvre, 2003; Smulders *et al.*, 2008). The regional collections consisted of the individual trees from narrow regions (Durance, LoireE, LoireW, Ijssel/Rhine, and Waal/Maas; Storme *et al.*, 2004). A few singular genotypes from France and the Netherlands were included (individual trees F and NL, respectively).

Pedigrees Four intraspecific and interspecific poplar F_1 hybrid families were used (Table 2; Table S1). POP2 with 330 progeny was derived from an interspecific cross of *Populus deltoides* '73028-62' (IL, USA; 37°32′ N 89°49′ W) and *Populus trichocarpa* '101-74' (Nisqually River, WA, USA; 47° N 123° W) (INRA, Orleans, France) (Jorge *et al.*, 2005). POP3a and POP3b were two hybrid F_1 interspecific poplar maternal half-sib families (INBO, Geraardsbergen, Belgium) (Dillen *et al.*, 2009). POP3a consisted of 180 progeny of an interspecific cross between *P. deltoides* 'S9-2' and *P. nigra* 'Ghoy' (Ghoy, Belgium; 50°43′ N 3°47′ E). *P. deltoides* 'S9-2' was derived from a cross of *P. deltoides* 'V1' (Ontario, Canada; 42°40′ N 80°10′ W) and *P. deltoides* 'V5' (Bellevue, IA, USA; 42°15′ N 90°45′ W). POP3b consisted of 182 progeny of an interspecific cross involving the same female parent *P. deltoides* 'S9-2' and *P. trichocarpa* 'V24' (Camas, OR, USA; 45°30′ N 122°40′ W). POP5 consisted of 165 progeny of an intraspecific cross of *P. nigra* '58-861' (Val Cenischia, Italy; 45°09′ N 7°01′ E) with *P. nigra* 'Poli' (Sinni river, Italy; 40°09′N 16°41′E) (DISAFRI, Viterbo, Italy) (Gaudet *et al.*, 2008).

Field sites A completely randomized block design with six blocks and one ramet per genotype and block was used for the establishment of experimental plantations in Belgium (Geraardsbergen; 50°46′ N 3°52′ E; *P. nigra* collection), Central France (Ardon, Loire valley; 47°46′ N 1°52′ E; POP2, POP3a, and POP3b), and Northern Italy (Cavallermaggiore, Po valley; 44°21′ N 8°17′ E; POP5). Trees were planted at 0.75 m by 2 m distance. A double border row of poplars was planted around the experimental

Table 1 The collection of *Populus nigra* accessions from Europe and means for growth cessation and duration of bud formation. A total of 437 genotypes (2217 individuals), collected in riparian ecosystems in Europe were evaluated in a common garden in Belgium (50°N). Bud set was evaluated twice a week from day 234 to day 318 (24 times)

Country	Name	Type	Location (nearest town or landmark)	F_{IS}[1]	Latitude at origin	Effective number of genotypes	Effective number of individuals	date2.5_cnl (mean ± SE)	subproc1_Δcnl (mean ± SE)	subproc2_Δcnl (mean ± SE)
Spain	Ebro1	Population	Near Novillas	0.0221	41°N	19	75	915.72 ± 6.67	138.08 ± 1.18	159.78 ± 1.89
Spain	Ebro2	Population	Near Alfranca	0.0127	41°N	32	136	931.40 ± 6.24	134.79 ± 1.09	160.44 ± 1.88
France	Durance	Regional collection	Along the Durance		43°N	10	41	751.99 ± 14.19	146.47 ± 2.02	154.95 ± 2.82
France	Drôme1	Population	Along the Drôme	0.0927	44°N	61	315	766.85 ± 4.54	149.31 ± 1.15	157.21 ± 1.01
France	Drôme6	Population	Along the Drôme	0.1321	44°N	63	346	803.61 ± 4.56	142.16 ± 0.84	158.98 ± 1.36
Italy	TicinoSN	Population	Near La Zelata		45°N	42	213	817.01 ± 4.69	135.76 ± 0.67	146.28 ± 0.96
Italy	TicinoN	Population	Inside Bosco Siro Negri		45°N	60	298	822.37 ± 4.45	137.73 ± 0.55	152.48 ± 0.97
France	Individual trees F	Individual trees			44°–47°N	5	29	832.08 ± 16.84	146.13 ± 0.76	162.53 ± 2.55
France	LoireE	Regional collection	Eastern part Loire		46°–47°N	22	103	621.57 ± 7.98	145.63 ± 2.41	156.22 ± 2.86
France	LoireW	Regional collection	Western part Loire		47°N	19	89	686.06 ± 13.89	141.83 ± 2.01	162.42 ± 1.79
Germany	Kühkopf	Population	Ginsheim/Kühkopf	0.0296	49°N	54	285	628.87 ± 3.03	143.17 ± 0.88	161.74 ± 1.09
The Netherlands	Waal/Maas	Regional collection			51°N	12	72	556.55 ± 2.86	109.74 ± 1.28	137.13 ± 1.17
The Netherlands	Individual trees NL	Individual trees			50°–52°N	7	38	602.88 ± 9.54	127.81 ± 2.59	146.91 ± 2.61
The Netherlands	Ijssel/Rhine	Regional collection			50°–52°N	31	177	577.17 ± 4.95	113.54 ± 1.08	138.05 ± 0.85

[1]F_{IS} based on 105 amplified fragment length polymorphism (AFLP) markers (Smulders *et al.*, 2008).

Table 2 Interspecific and intraspecific pedigrees and progeny means for growth cessation and duration of bud formation. Pedigrees POP2, POP3a and POP3b were evaluated in France and POP5 was evaluated in Italy

Pedigree	Female parent	Male parent	Latitudinal origin (female parent)	Latitudinal origin (male parent)	Latitude of progeny selection	Scoring period[1]	Effective progeny size[2]	date2.5_cnl (mean ± SE, *n*)	subproc1_Δcnl (mean ± SE, *n*)	subproc2_Δcnl (mean ± SE, *n*)
POP2	*P. deltoides* '73028-62'	*P. trichocarpa* '101-74'	37°N	47°N	47°N	240–279 (10)	330	649.73 ± 0.95 (*n* = 234)	107.96 ± 1.18 (*n* = 234)	143.42 ± 1.45 (*n* = 325)
POP3a	*P. deltoides* 'S9-2'	*P. nigra* 'Ghoy'	42°N	50°N	50°N	231–256 (8)	179	464.75 ± 2.07 (*n* = 64)	68.00 ± 1.65 (*n* = 64)	144.29 ± 2.00 (*n* = 138)
POP3b	*P. deltoides* 'S9-2'	*P. trichocarpa* 'V24'	42°N	45°N	50°N	244–262 (6)	179	592.74 ± 3.85 (*n* = 35)	71.88 ± 2.27 (*n* = 35)	106.72 ± 1.43 (*n* = 161)
POP5	*P. nigra* '58-861'	*P. nigra* 'Poli'	45°N	40°N	44°N	252–286 (17)	152	863.25 ± 2.66 (*n* = 151)	100.08 ± 1.44 (*n* = 151)	124.05 ± 2.22 (*n* = 139)

[1]Scoring period is given in days of the year. The number of measurements during this period of time is given within parentheses.
[2]Effective progeny size includes genotypes with no fewer than three individual trees with data.
[3]*n* corresponds to the number of genotypes with data for the trait. See also the Supporting Information, Table S1. For POP3a and POP3b, average performances related to date2.5_cnl and subproc1_Δcnl are biased at population level because information is available for a limited number of genotypes.

plot to reduce border effects. The field trials were established from hardwood cuttings in spring 2003 (France and Italy) and spring 2004 (Belgium). The sites were irrigated (only France and Italy), weed controlled and treated with insecticides and fungicides as necessary throughout the growing seasons. Trees were cut back in January 2005 and only the best-performing shoot was preserved after resprouting. Cumulative night length (cnl) was calculated from day-length data retrieved from the US Naval Observatory (http://aa.usno.navy.mil/) with the respective geographic coordinates of the field sites. Temperature sums after July 1 were calculated from the temperature records at the respective field sites. These considered either the daily mean temperature (cumulative mean temperature, cmt) or daily minimum temperature (cumulative daily minimum temperature when below 10°C, cmt10). When the daily minimum temperature was below 10°C, the minimum temperature was subtracted from 10°C to give lower temperature values a larger contribution to cmt10. July 1 was chosen to separate from the first growth flush that is typically accomplished before June 24. Both Δcnl and Δcmt10, used to describe the temperature sums for duration traits, were calculated by subtracting cnl and cmt10 at stage 1.5 from those at stage 2.5 (subproc1) or by subtracting cnl and cmt10 at stage 0.5 from those at stage 1.5 (subproc2).

Data collection, treatment and statistics

Bud set scoring and data analysis Bud set was scored in autumn 2005, in the main apical bud of the 1-yr-old shoot on 2-yr-old (*P. nigra* collection) and 3-yr-old (pedigrees) roots, applying the scoring scheme (Fig. 1). Local polynomial regression was used to smooth the bud-set curve of each individual and to estimate dates of bud-set stages that had not been observed in the field because of the speed of bud set. Observed scores were fitted to a local polynomial regression of degree 2 with, as predictors, the different dates of observation in the given site (Cleveland *et al.*, 1992). Date x was fitted using the 75% nearest data points and weighted least squares. High quality of fit was obtained for

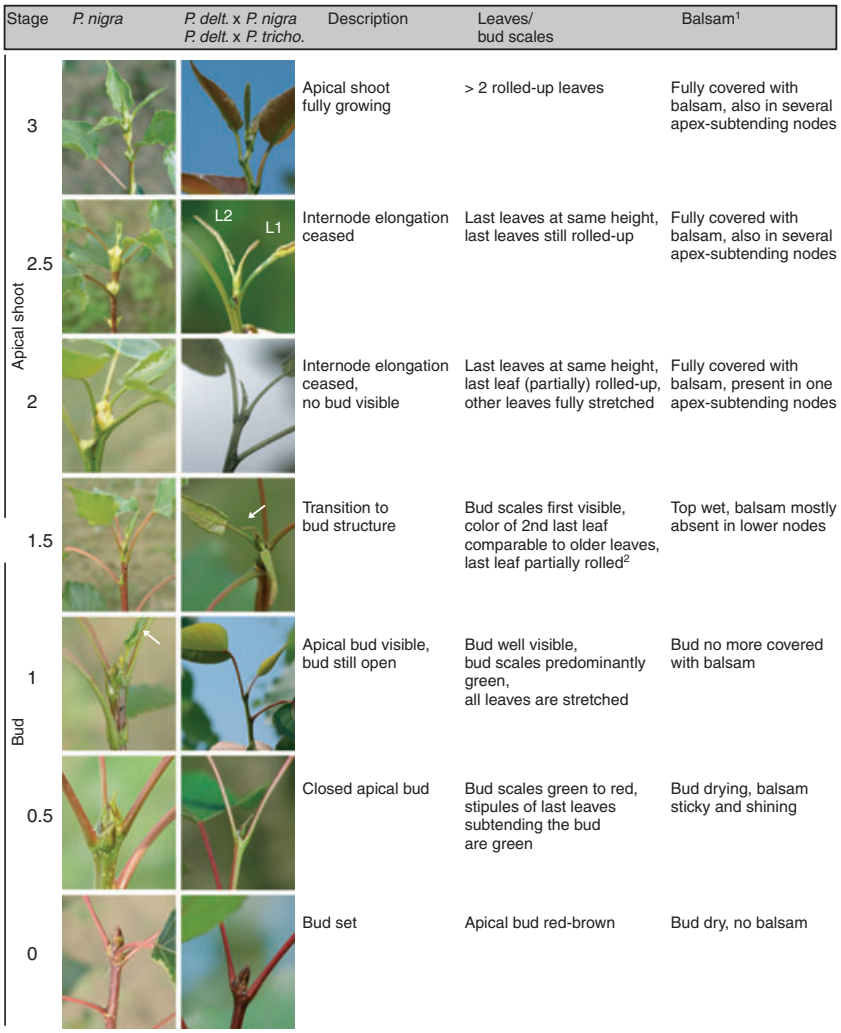


Fig. 1 Scoring scheme for bud set in poplar. Seven discrete stages were delineated to cover onset, dynamics and duration of bud set in different poplar species. Phenotypic aspects of the apical part, such as appearance of last emerged leaves, internode elongation, appearance of bud scales, color of the bud and presence of balsam, allowed the various stages to be distinguished. [1]Balsam is indicative, primarily in *P. nigra*; [2]Last leaf is not always reliable; sometime an atypical last small leaf sticks out from the bud (arrows) and dies after bud set. Not to be considered for scoring. L1, last emerged leaf; L2, one but last leaf; *delt., deltoides; tricho., trichocarpa*.

all individual plants with R^2 ranging from 0.885 to 0.999. Pearson correlations between observed and predicted dates surpassed 0.986 for all bud-set stages. From the fitted curve, discrete values for the day of the year were retrieved for all scores from 3 to 0, respecting the range defined by first and last observation. This data were analysed with a two-step ANOVA in all genotypes that had at least three ramets. The first model considered $Y_{ij} = \mu + B_i + G_j + \varepsilon_{ij}$, where μ is the general mean, B_i is the effect of block i (fixed), and G_j is the effect of genotype j. G_j and ε_{ij} are assumed to be normally distributed random variables with zero means and variance components, V_G and V_R, respectively. From the results, the individual estimates of each block were retrieved and used to correct Y_{ij} as follows: $Y'_{ij} = Y_{ij} - B_i$. ε_{ij} from the first ANOVA, plotted onto maps of the field sites, showed no additional unaccounted microspatial influences. Y'_{ij} was taken to the second ANOVA. The second ANOVA for the *P. nigra* collection considered $Y_{jkl} = \mu + P_k + G_{j(k)} + \varepsilon_{jkl}$, where μ is the general mean, and P_k and G_j the effects of population k and genotype j nested under population k (both considered as random). The second ANOVA for the pedigrees considered $Y_{jk} = \mu + G_j + \varepsilon_{jk}$, where μ is the general mean, and G_j the effect of genotype j, considered as random.

Principal component analysis Multivariate analyses using principal component analysis (PCA) were performed separately for the *P. nigra* collection and each mapping pedigrees on individual tree basis. For POP3a and POP3b, because a high proportion of trees had already reached stages 2 or 1.5 at the beginning of the experiment (Fig. 2a), multivariate analysis could be performed either on a limited progeny sample (POP3a) or on a limited set of traits (POP3b). All initial variables were standardized and orthogonal factors (PC1 and PC2 axis) were successively constructed as linear combinations of initial variables to maximize percentage of phenotypic variance explained.

Heritability, genetic variation, and population differentiation For the *P. nigra* collection, heritability, genetic variation and population differentiation were calculated from restricted maximum likelihood (REML) estimates of variance components using the block-adjusted phenotypic values (Y'_{ij}), considering genotype (nested under population) and population as random factors. P_k, $G_{j(k)}$, and ε_{jkl} were assumed to be normally distributed random variables with zero means and variance components σ^2_{pop}, σ^2_{geno} and σ^2_R, respectively. Using the estimates of variance components, individual broad-sense heritability (H_i^2) and genotypic heritability (H_g^2) were calculated for each trait using $H_i^2 = V_G/(V_G + V_R)$ and $H_g^2 = V_G/(V_G + V_R/n_i)$, respectively, where V_G is the genetic variance ($V_G = \sigma^2_{pop} + \sigma^2_{geno}$), V_R (σ^2_R) the residual variance, and n_i the number of ramets/genotype for this trait. Q_{ST} was calculated using $Q_{ST} = \sigma^2_{pop}/(\sigma^2_{pop} + 2 \times \sigma^2_{geno})$, considering the true populations and regional collections. For the pedigrees, genetic and residual variance components were calculated by equating observed mean squares to expected mean squares and solving the resulting equations according to the Henderson III procedure (Henderson, 1953; Searle *et al.*, 1992). For pedigrees, the coefficients of genetic and residual variation (CV_G%; CV_R%) were the genetic and residual standard deviation of a trait divided by the mean and expressed as a percentage.

QTL detection

Quantitative trait loci were determined for selected traits in four poplar breeding pedigrees with previously established genetic maps (framework markers with logarithm of odds (LOD) > 2) (Jorge *et al.*, 2005; Gaudet *et al.*, 2008; Dillen *et al.*, 2009) with MultiQTL 2.4 (http://www.multiqtl.com/; MultiQTL Ltd, Institute of Evolution, Haifa University, Haifa, Israel). Single traits were analysed with multiple interval mapping. The entire genome was first scanned with the one-quantitative trait locus and the two-QTL models. Permutation tests (1000 runs), comparing hypotheses H_1 (one quantitative trait locus in the chromosome) and H_0 (no QTL in the chromosome), were run to obtain chromosome-wise statistical significance. In a second step, the genome was scanned for QTL assuming a two-QTL model. For chromosomes for which a single quantitative trait locus was already detected, permutation tests (1000 runs) were run to compare the hypotheses H_2 (two linked QTL in the chromosome) vs H_1. Subsequently, when $P_{(H2vsH1)} < 0.05$, permutations were run to compare H_2 vs H_0. A two-linked QTL model was accepted only, when the two intervals were not adjacent and the one-quantitative trait locus model was significant. In a last step, multiple-interval mapping was carried out on all significant QTL ($P < 0.05$). Permutations were run per chromosome, using $P < 0.05$ as threshold per chromosome. Bootstrap analysis was done to estimate the 95% confidence interval. The option 'marker restoration' was used to reduce the effect of missing information. The Kosambi mapping function was chosen for recalculation of maps on genotypic data. The 2-LOD support intervals of QTL peaks were calculated from the exported LOD curve.

Integration of genetic maps with the poplar genome

Simple sequence repeat (SSR) markers that had been mapped in the various pedigrees were placed on the poplar genome with BLAST algorithms (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html; genome assembly version 1.1). In addition, amplified fragment length polymorphism (AFLP) markers from the genetic maps of POP3a and POP3b were sequenced and compared with the poplar

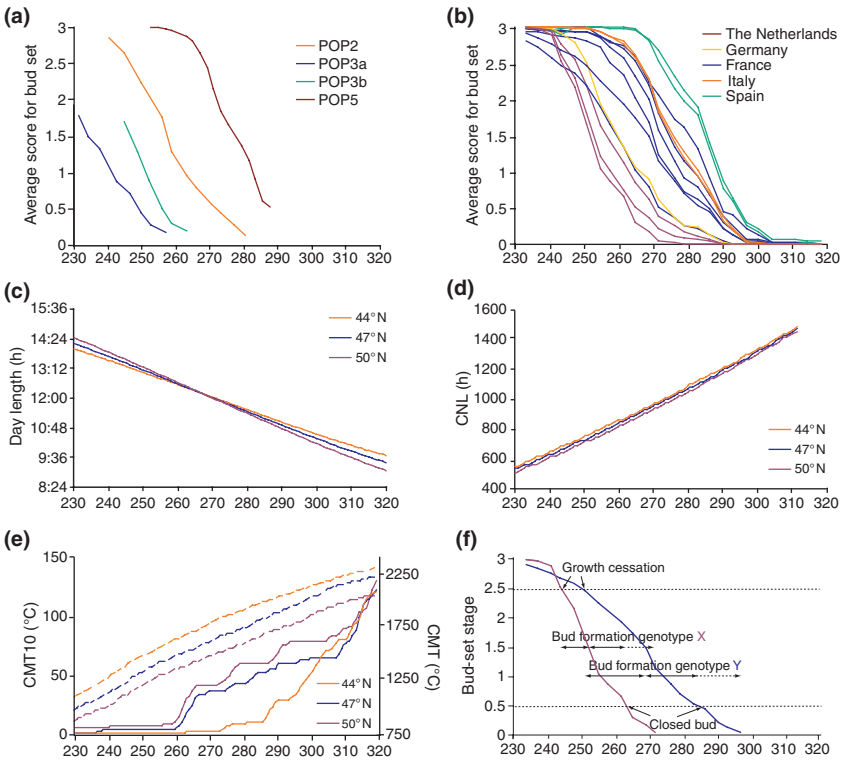


Fig. 2 Progression of bud development and climatic conditions in three field sites. The progression of bud development, expressed as mean stage of a population/pedigree at a given day of the year (DOY), and relevant climatic variables are given for the 90-d period between day 230 and day 320, including autumn equinox at day 267. (a) Bud development in four poplar F_1 pedigrees. POP2, POP3a and POP3b were evaluated in France and POP5 was evaluated in Italy. The lines represent the period and frequency of measurement that are also detailed in Table 2. (b) Bud development in 14 populations and regional collections of *Populus nigra*, evaluated in Belgium. All populations were evaluated twice a week from day 234 to day 318 (24 times). Populations are color-coded per country; more detailed results are available in the Supporting Information, Table S1. (c) Daylength progression for the three field sites situated at 44° N, 47° N, and 50° N. (d) Cumulative night length (cnl), calculated from July 1 for the three field sites. (e) Cumulative sums of daily mean temperature (cmt) and daily minimum temperature when below 10°C (cmt10), both calculated from July 1, for the three field sites. Dotted lines correspond to cmt, full lines to cmt10. See the Materials and Methods section for the calculation of cmt10. (f) Schematic situating bud-set traits relative to time. The progression through bud-set stages is shown for two hypothetical genotypes. Cessation of growth and bud formation together lead to bud set. From the description of stages, cessation of growth, the duration from cessation of growth to visible budscales (subprocess 1, depicted by first arrow-head line), the duration from visible bud scales to closed apical bud (subprocess 2, depicted by second arrow-head line) and duration until bud set (depicted by dashed line) can be delineated.

genome with BLAST. From these, 155 new AFLP markers, for which genetic and suggested genome positions were in agreement, were added to the analysis as genome-anchored markers. All genetic and QTL maps and genome sequence are visualized with the web-based cmap tool (http://www.gmod.org) that can be publicly accessed at http://services.appliedgenomics.org/poplar_budset.

Results

Definition of traits to dissect bud set

To dissect bud set, we developed a new detailed scoring system with seven stages that cover the different aspects of the progression from a growing apex to a closed bud (Fig. 1). Stage 3 corresponds to full, active growth. Stage 2.5 marks growth cessation and, thus, is the closest approximation for sensing the critical night length. Stage 1.5 pinpoints the important transition from shoot to bud (Fig. 1). Stage 0.5 (green, closed bud) or 0 (red–brown mature bud) correspond to bud set as used in most traditional analyses that have assessed accomplished bud set (Fig. 1). The identification of landmark stages throughout bud development offered the possibility to dissect the phenotype. Hence, the timing of growth cessation and the duration of bud formation could be considered as separate determinants of bud set.

Bud development was assessed with the new scoring system (Fig. 1) in poplar germplasm of natural and hybrid origin. A total of 437 accessions of riparian *P. nigra* populations and regional collections represented a wide range of previously characterized, autochthonous provenances across Europe (Table 1; Storme *et al.*, 2004; Smulders *et al.*,

2008). Together with additional hybrid material from four different poplar breeding pedigrees (Table 2), a total of 6847 individual trees were assessed and their bud development was expressed as stage over chronological time (Fig. 2a,b). At the same day of the year (DOY), trees in the three different field sites located at 44° N, 47° N, and 50° N experience different night lengths and temperatures. The actual night lengths differ by *c.* 15 min, a month before and after the autumn equinox per 3° change in latitude (Fig. 2c). To allow for the direct comparison of data obtained in different field sites, data were subsequently expressed to light- and temperature-based scales. These include the cumulative night length (cnl), and two different temperature sums: cumulative daily mean temperature (cmt) and cumulative daily minimum temperature when below 10°C (cmt10) (see the Materials and Methods; Fig. 2d,e). In all sites, the cnl metric roughly parallels the DOY scale and brings photoperiod evolution for the three field sites closer to each other (Fig. 2d). This metric is not biologically relevant because photoperiod perception is governed by a coincidence of internal circadian clock rhythms and external light. It is used to provide a decimal scale for easier statistic data treatment. Conversely, cumulative temperature is used for its biological relevance for phenological events and growth rates. The differences in mean (cmt) and minimum temperature (cmt10) sums are characteristic for the cooler northern and warmer southern locations (Fig. 2e).

Traits were derived from the stages to describe the onset and dynamics of the process (Fig. 2f). Traits include date-of-onset of a defined stage (as described by the scoring scheme; Fig. 1) and the duration of two biologically coherent processes. Subprocess 1 (subproc1), corresponding to the duration from onset of stage 2.5 to onset of stage 1.5, essentially describes the changes in the growth-ceasing apical shoot. Subprocess 2 (subproc2), covering the time from stage 1.5 to stage 0.5, refers to changes of the bud after the first visible signs of bud initiation. Depending on the scale relative to which traits are expressed, date-of-onset traits are extended by _cnl (cumulative night length), _cmt10 (cumulative daily minimum temperature when below10°C) and duration traits by _Δcnl and _Δcmt10.

Phenotypic variation in bud-set traits

Typically, cessation of growth is initiated above a critical night length in poplar (Vegis, 1964; Howe *et al.*, 1996). Accessions in the *P. nigra* collection, if ordered according to their latitude of origin, showed a roughly clinal response for growth cessation (date2.5_cnl; Fig. 3a). Based on the average date2.5_cnl for populations or regional collections, the critical night lengths were inferred to vary between 13:32 h (Waal/Maas, earliest) and 12:26 h (Ebro2, latest) within the collection, when assessed at 50°N (Figs 2b, 3a; Table 1).

In the poplar pedigrees, cessation of growth occurred first in the interspecific F1 families POP3a, then in POP3b, POP2 and finally in the intraspecific *P. nigra* family POP5 (Table 2). In the three pedigrees grown together in France, the female *P. deltoides* parent (and in POP3b also the male parent) originated south of the site of the progeny selection, thus carrying a critical-night-length character that delayed bud set relative to trees originating at the site. The order of pedigrees with respect to growth cessation reflected the extent of latitudinal shift, with the latest-setting POP2 shifted farthest north (10°-Δlatitude; Table 2).

In the *P. nigra* collection, the differences in the duration of bud formation were minor. Part of the variation might have gone undetected because of an exceptionally warm autumn in 2005. As little as 1.5 d constitute the difference for the duration of either subprocess 1 or subprocess 2 at the level of population means (data not shown). Expressing the duration to minimum temperature (cmt10) revealed, however, that early-setting populations (Dutch accessions) experienced less cold during bud formation than late-setting populations (Ebro1 and Ebro2) within a comparable period of time (Fig. 3b). Thus, the actual temperature sum for bud formation differed to a great extent. The effect of temperature was more pronounced for subprocess 1, when leaf primordia are formed and differentiated, than for subprocess 2, comprising the maturation of the bud (Fig. 3b). In the pedigrees, the phenotypic variation in the duration of bud formation is comparable to the variation in growth cessation and bud initiation (Table 2; Fig. 3c,d). Thus, duration of bud formation is yet another important factor determining the completion of bud set.

Genetic variation in bud-set traits

All traits showed relatively high broad-sense heritabilities, both at the individual as well as at the genotypic level (Fig. 4). In the pedigrees, genotypic heritabilities for onset-of-stage traits (0.5–0.9) were higher than those of the two-duration traits (0.25–0.5; Fig. 4). This difference in heritability will provide a higher precision for QTL detection for onset-of-stage traits. In the *P. nigra* collection representing a large range of geographical origins, genotypic heritabilities were even higher: > 0.9 for the onset-of-stage traits and *c.* 0.7 for the two-duration traits (Fig. 4). These values are comparable with, or in case of the *P. nigra* collection surpass the broad-sense heritabilities of 0.51 at individual level and 0.81 at genotype level found earlier for bud set in an F_2 poplar pedigree (Howe *et al.*, 2000).

Bud set typically is a highly differentiated trait in forest trees. In the *P. nigra* collection, the effects of genotype and population were highly significant ($P < 0.0001$) for all traits, as estimated with ANOVA. For the various onset-of-stage traits, between 76% and 86% of the phenotypic variance was partitioned to genetic variance (V_G) in the *P. nigra*

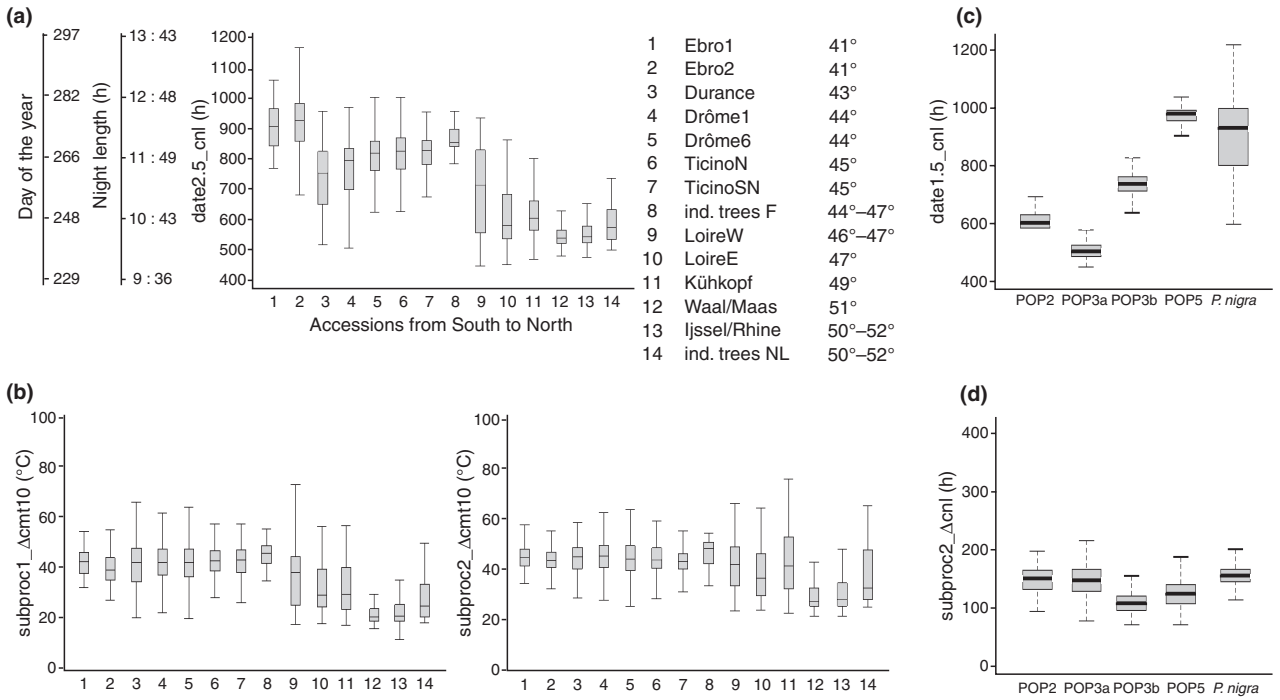


Fig. 3 Phenotypic variation in the cessation of growth and duration of bud formation. The date of cessation of growth or bud initiation, respectively, and duration of bud formation are given for 14 populations/regional collections of *Populus nigra* and the four pedigrees, based on block-adjusted genotypic means. Box plots represent the range of genotypic means between the 25% and 75% percentile by the box, the median by the middle horizontal line, and minimum and maximum observations by the whiskers. In each panel, populations or regional collections are numbered and ordered from southern to northern origin (see Table 1 for full information). (a) Population means for cessation of growth (date2.5_cnl). Additional informative scales on day of the year and night-length are given, but are not metric. (b) Population means for the duration of bud formation (subproc1_Δcmt10 and subproc 2_Δcmt10). (c) Pedigree means for bud initiation (date1.5_cnl). (d) Pedigree means for the duration of bud formation (subproc 2_Δcnl). The grand mean of the *P. nigra* collection is included for reference in (c) and (d).

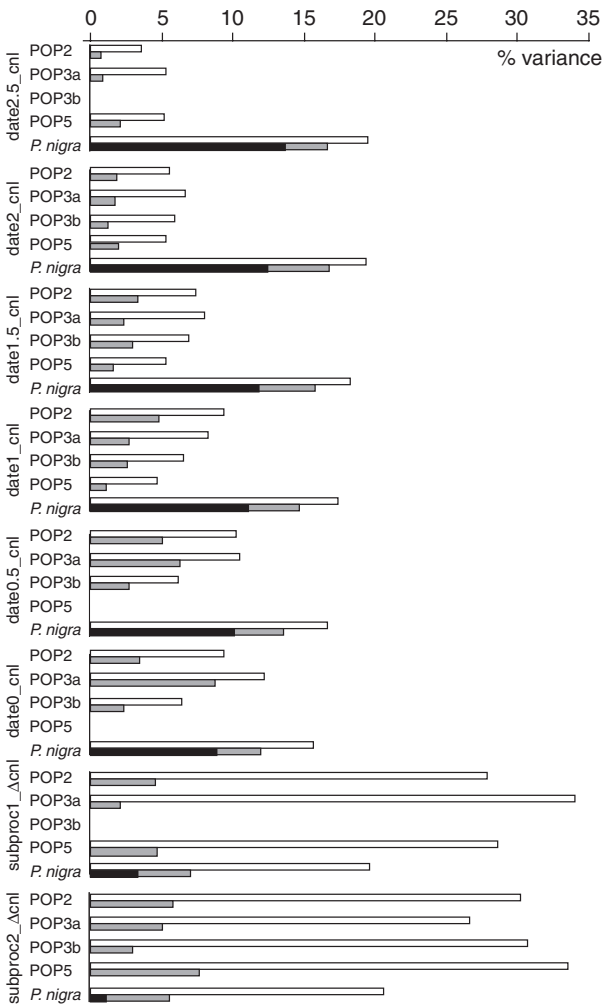
collection (Fig. 4). The V_G of duration traits reached only 27–36% of the phenotypic variance, underlining the higher influence of environmental conditions on duration traits (Fig. 4). Irrespective of the trait, most V_G, on average 75%, was partitioned to the factor population (Fig. 4). Consequently, population differentiation (Q_{ST} of *c.* 0.6) was quite high; only subprocess1_cnl showed less differentiation (Fig. 4). In a European aspen (*Populus tremula*) collection covering a steeper cline from 56° N to 66° N, Q_{ST} for bud set was within the same range (*c.* 0.75; Hall *et al.*, 2007).

Among all traits of the *P. nigra* collection, subprocess2_Δcnl, describing the maturation of the bud, appeared strongly influenced by environmental conditions (Fig. 4). Only the late-setting populations from the Ebro displayed significant genetic variation (Fig. S1). For cessation of growth (date2.5_cnl), the two populations from the Drôme displayed most phenotypic variation, but the two populations from the Ticino together with one population from the Ebro had a relatively higher genetic variation (Fig. S1). These examples of within-population genetic variation illustrate that natural selection can act at relatively small geographical scales.

In the four pedigrees, the contribution of genetic variance to phenotypic variance was generally lower than that of residual variance. However, the onset-of-stage traits showed a relatively higher genetic component in the total variance (Fig. 4). Some pedigrees presented specific genetic variation, for example POP3a for the last stages of bud maturation and bud set (date0.5_cnl and date0_cnl; Fig. 4). Most variation was found for the date of growth cessation in the *P. nigra* collection, whereas the four poplar pedigrees presented similar total amounts of genetic variance for onset-of-stage and duration traits.

Definition of the most descriptive traits based on phenotypic variation

The onset-of-stage and duration traits together describe a developmental sequence and therefore tend to be correlated. In particular, the onset-of-stage traits correlated highly with each other (Fig. 5a,b). Duration and onset-of-stage traits were, on average, less (*P. nigra*) or even negatively (POP3a, POP5; Table S2) correlated at the trait level. Hence, in the pedigrees, the later cessation of growth occurred, the shorter the duration of bud formation was. While all stages are important for a profound physiological dissection of bud development, many highly correlated traits will provide redundant information when genetic trait architecture is



	H_i^2	H_g^2	Q_{ST}
POP2	0.213	0.548	
POP3a	0.169	0.530	
POP3b	NA	NA	
POP5	0.417	0.766	
P. nigra	0.866	0.970	0.585
POP2	0.336	0.719	
POP3a	0.253	0.624	
POP3b	0.215	0.569	
POP5	0.364	0.722	
P. nigra	0.868	0.971	0.595
POP2	0.445	0.814	
POP3a	0.294	0.690	
POP3b	0.437	0.795	
POP5	0.310	0.672	
P. nigra	0.860	0.969	0.600
POP2	0.514	0.860	
POP3a	0.321	0.721	
POP3b	0.403	0.789	
POP5	0.252	0.605	
P. nigra	0.847	0.966	0.601
POP2	0.491	0.849	
POP3a	0.602	0.894	
POP3b	0.438	0.806	
POP5	NA	NA	
P. nigra	0.813	0.956	0.599
POP2	0.373	0.750	
POP3a	0.716	0.928	
POP3b	0.353	0.734	
POP5	NA	NA	
P. nigra	0.762	0.941	0.598
POP2	0.165	0.471	
POP3a	0.060	0.262	
POP3b	NA	NA	
POP5	0.163	0.470	
P. nigra	0.358	0.737	0.308
POP2	0.190	0.562	
POP3a	0.191	0.543	
POP3b	0.094	0.334	
POP5	0.228	0.555	
P. nigra	0.268	0.649	0.666

Fig. 4 Genetic variation of bud-set traits. Onset-of-stage and duration traits were defined from original data (Fig. 2f). The bars represent phenotypic variance components expressed as % of the trait mean: total phenotypic variance (open bars) and total genetic variance (tinted bars). For *Populus nigra*, the genetic variance is further partitioned to population (dark tinted bars) and individual within population (lighter tinted bars) components. Broad-sense heritability on individual (H_i^2) and genotype (H_g^2) basis and population differentiation (Q_{ST}) are given. Genetic variance and heritability were not calculated (NA, not available), when genetic variance estimation was biased owing a to limited number of genotypes with data.

investigated. Therefore, we used an unsupervised method to define those traits that are most discriminative in terms of phenotypic variation.

A principal component analysis using the six onset-of-stage and two duration traits allowed identifying those traits that differentiated most in the populations (Fig. 6). The phenotypic variation in *P. nigra*, the most comprehensive dataset, was partitioned into a major contribution from onset-of-stage traits (principal component 1, PC 1; 81.2%) and a minor contribution from the duration traits (PC 2, 18.7%) (Fig. 6). Similar to *P. nigra*, the phenotypic variation in the pedigrees is 50–75% partitioned to PC 1 and 20–35% partitioned to PC 2 (Fig. 6). In POP2 and POP3b, onset-of-stage traits (primarily date1.5_cnl and date0.5_cnl), determine the phenotypic spread along the axis of PC1 (Fig. 6). In POP5, date1.5_cnl has a major effect and subproc2_Δcnl a minor opposing effect on the phenotypic spread along the axis of PC1. subproc2_Δcnl contributes most to PC2 in POP2, POP3a and POP3b, while subproc1_Δcnl generates a distinct pattern along PC2 in POP5. Thus, without a priori assumptions, the onset of bud initiation (date1.5_cnl) and the duration from bud initiation to completed bud set (subproc2_Δcnl) were identified as major contributors to phenotypic variation. Based on this analysis, the following traits were selected for further analysis of genetic aspects. date0.5_cnl and subproc2_Δcnl cover most of the phenotypic variation. In addition, date1.5_cnl, subproc1_Δcnl were included for their specific importance in POP3a and POP5, respectively. However, we need to bear in mind that both date1.5_cnl and date0.5_cnl have a clearly higher genetic variation within the total variation (Fig. 4).

QTL for bud set in four poplar breeding pedigrees

Across the four pedigrees, 105 QTL were detected for all six onset-of-stage and two duration traits. All QTL were projected onto the respective genetic maps (Table S3) and can be publically viewed at http://services.appliedgenomics.org/poplar_budset. Not unexpectedly, QTL for various traits

(a) Trait correlation across *P. nigra* collection

	date2.5_cnl	date2_cnl	date1.5_cnl	date1_cnl	date0.5_cnl	date0_cnl	subproc1_Δcnl	subproc2_Δcnl
date2.5_cnl		**0.992**	**0.984**	**0.975**	**0.951**	**0.898**	0.118	0.208
date2_cnl			**0.997**	**0.991**	**0.972**	**0.926**	0.238	0.242
date1.5_cnl				**0.997**	**0.982**	**0.943**	0.293	0.269
date1_cnl					**0.991**	**0.960**	0.329	0.327
date0.5_cnl						**0.982**	0.368	0.446
date0_cnl							0.432	0.514
subproc1_Δcnl								0.508
subproc2_Δcnl								

(b) Average trait correlation across pedigrees

Total number of co-location events

	date2.5_cnl	date2_cnl	date1.5_cnl	date1_cnl	date0.5_cnl	date0_cnl	subproc1_Δcnl	subproc2_Δcnl
date2.5_cnl		**0.831**	0.682	0.532	0.446	0.175	−0.223	−0.615
date2_cnl	5		**0.888**	0.726	0.474	0.380	0.319	−0.397
date1.5_cnl	3	**8**		**0.880**	0.607	0.507	0.594	−0.393
date1_cnl	2	6	**11**		0.761	0.660	0.551	−0.151
date0.5_cnl	1	4	7	**9**		**0.898**	0.336	0.563
date0_cnl	0	0	1	3	4		0.205	0.524
subproc1_Δcnl								−0.354
subproc2_Δcnl	1	3	5	4	1	2		

(c)

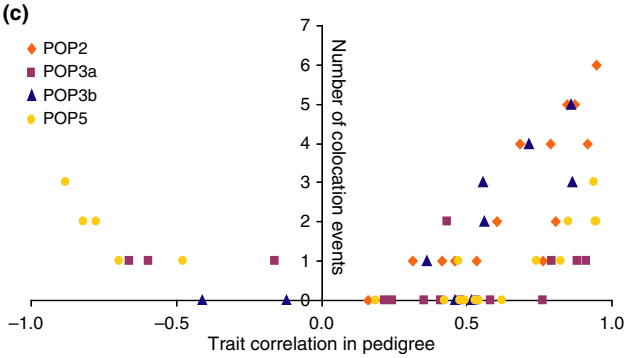


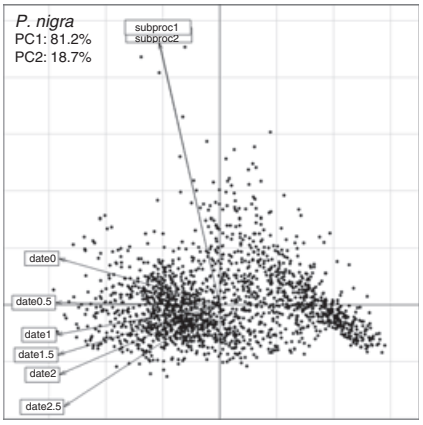
Fig. 5 Correlations between phenotypic traits of bud set and effect on trait-pair quantitative trait loci (QTL) colocalization. (a) Trait correlations in the *Populus nigra* collection, based on individual values. All correlations are significant at $P < 0.0001$. (b) Average trait correlation across the four pedigrees above the diagonal, and the total number of colocalization events of traits below the diagonal. Trait correlations above 0.8 and total number of colocalization above 7 are in bold; negative trait correlations are in red. Because of the limited data, QTL for subproc1_Δcnl were excluded from the colocalization analysis. Trait correlations are detailed for each pedigree in Table S2. (c) Colocalization events in function of trait correlation within the individual pedigrees.

tended to stack because of the correlated nature of phenotypic data. The higher the traits were correlated, the more the corresponding QTL colocated (Fig. 5c). Most colocation events, relative to the total number of QTL, were detected in POP2 and POP5 (Fig. 5c). Approximately 20% of the colocation events involved a colocation of duration and onset-of-stage traits. Most often, colocation of such negatively correlated traits was noticed in POP5 (Fig. 5c). If based on the same gene(s), the respective locus might be under evolutionary constraint owing to the opposite effects on the onset and duration of bud set.

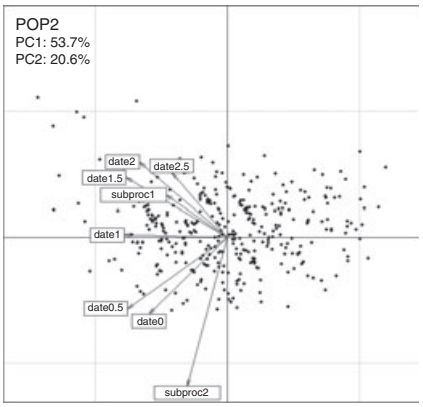
For the purpose of understanding genetic trait architecture, only 53 QTL of the four most descriptive traits chosen from PCA are discussed further (Table 3). Relatively more QTL were found in the two pedigrees involving inter-American crosses (POP2 and POP3b) than in the Euramerican (POP3a) and the intraspecific crosses (POP5) (Table 3). The strong segregation for bud-set traits might be based on the large difference in latitudinal origin of the parents for POP2 and POP3b (Table 2). A single QTL explains on average 8.6% of the phenotypic variation. The average sum of explained phenotypic variation per trait in a pedigree was 38% (Table 3). Consistent with the phenotypic and genetic results, QTL are revealed for both the onset-of-stage and the duration traits (Table 3). In most cases, a trait was influenced by a small number of loci. Given the progeny size, it is likely many QTL of smaller effect that contribute to phenotypic variation were not even detected. Together, these results underscore the quantitative nature of bud set.

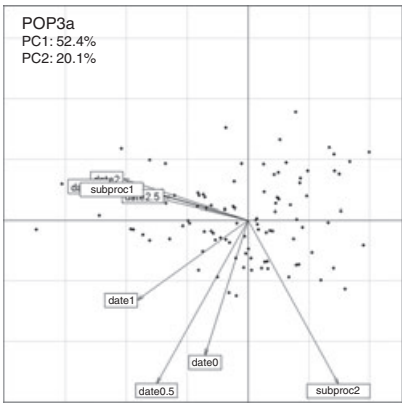
Six robust QTL regions detected across various pedigrees

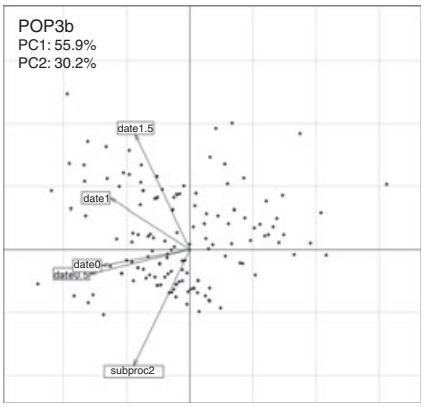
Tentatively similar QTL regions were observed in a number of chromosomes across two or more parental maps (Table S3). The investigation of the corresponding genetic and physical sequence regions appeared to be hampered, however, by problems in species with less-developed genetic



		Contribution to PC1	PC2	
P. nigra	date2.5	−0.950	subproc1	0.793
	date2	−0.975	subproc2	0.816
	date1.5	−0.986		
	date1	−0.994		
	date0.5	−0.999		
	date0	−0.979		
POP2	date1.5	−0.911	subproc2	−0.870
	date1	−0.929		
	date0.5	−0.898		
POP3a	date1.5	−0.979	date0.5	−0.721
			subproc2	−0.723
POP3b	date0.5	−0.960	date1.5	0.814
			subproc2	−0.821
POP5	date2	0.955	subproc1	−0.884
	date1.5	0.994		
	date1	0.952		
	subproc2	−0.874		







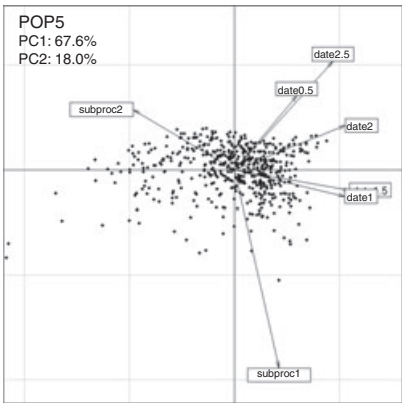


Fig. 6 Principal component analysis (PCA) of bud-set traits. The PCA was applied to delineate bud-set traits that covered most phenotypic variation. The individual block-adjusted phenotypic values of six onset-of-stage and two duration traits were included, except for POP3b (date2.5, date2, and subproc1 excluded). For each pedigree and the *Populus nigra* collection, the portion of variance association to the principal components (PC) is given. Contributions of all original traits to PC1 and PC2 are listed for *P. nigra*. For the pedigrees, only traits with high contribution are shown.

resources, such as low marker density, low marker sharing among genetic maps and the lack of marker sequence information to establish a link to the genome sequence. In consequence, a conservative approach using sequence-anchored flanking markers was adopted to determine whether QTL occurring in two or more parental maps identified the same region.

Out of the 53 QTL for the four most descriptive traits, 36 are linked to the physical genome sequence with at least two sequence-anchored microsatellite or AFLP markers flanking the LOD peak of the QTL (http://services.appliedgenomics.org/poplar_budset). Of these, 21 QTL from different parents identified six recurrent QTL regions in the poplar genome that are important for bud set (Fig. 7). These six robust QTL regions ranged from 1.83 to 4.25 Mbp in size and contained from 202 to 394 annotated genes (Figs 7, 8; Table S4).

QTL studies cannot reveal the specific genes underlying the traits. Still, for the sake of developing a strategy toward the cloning of genes carrying the causative polymorphisms, it is interesting to integrate the data with various genes known to be associated with bud set either through function or expression (Frewen *et al.*, 2000; Böhlenius *et al.*, 2006; Ruttink *et al.*, 2007; Ingvarsson *et al.*, 2008; Ruonala *et al.*, 2008; Ibáñez *et al.*, 2010). Of the 945 genes with differential expression during bud development in poplar (Ruttink *et al.*, 2007), 757 genes were located on assembled genome linkage

Table 3 Quantitative trait loci (QTL) for the four most descriptive bud-set traits

	Number of QTL					Total PVE			
Trait	POP2	POP3a	POP3b	POP5	Total	POP2	POP3a	POP3b	POP5
date1.5_cnl	10	3	5	3	21	0.649	0.260	0.335	0.429
date0.5_cnl	5	2	7	No QTL	14	0.373	0.168	0.474	–
subproc1_Δcnl	–	–	–	2	2	–	–	–	0.267
subproc2_Δcnl	4	6	3	3	16	0.218	0.666	0.316	0.392
Total	19	11	15	8	53				

Where no number appears, too few and/or skewed data were available. In POP5, phenotypic data of date0.5_cnl were subjected to QTL analysis but did not detect any QTL. A complete list of QTL and their characteristics is available in Table S3.

Parent	Trait	LOD	PVE	LOD peak cM	Flanking Markers M1, cM	M2, cM	Interval cM	bp
POP2_*P. delt.*	subproc2_Δcnl	1.96	0.056	47.2	22.8	49.9	27.1	4245795
POP3b_*P. delt.*	date0.5_cnl	2.39	0.052	12.7	8.0	38.5	30.5	5841457
POP2_*P. delt.*	date0.5_cnl	2.12	0.029	123.3	100.7	151.1	50.4	3242362
	date1.5_cnl	3.49	0.037	127.7	100.7	151.1	50.4	3242362
POP2_*P. trich.*	subproc2_Δcnl	5.01	0.118	146.1	131.5	197.9	66.4	5166648
	date0.5_cnl	8.99	0.234	152.6	131.5	197.9	66.4	5166648
	date1.5_cnl	11.07	0.258	151.5	131.5	197.9	66.4	5166648
POP3b_*P. delt.*	date1.5_cnl	2.21	0.057	88.8	30.3	125.5	95.2	9987199
	date0.5_cnl	2.02	0.065	103.0	30.3	125.5	95.2	9987199
POP3b_*P. trich.*	subproc2_Δcnl	2.66	0.161	37.2	0.0	48.0	48.0	1841904
POP5_*P. nigra* P	date1.5_cnl	3.54	0.136	152.5	119.6	152.9	33.3	6840365
	subproc2_Δcnl	3.11	0.159	152.5	119.6	152.9	33.3	6840365
POP3a_*P. nigra*	subproc2_Δcnl	5.60	0.148	104.0	91.1	193.5	102.4	6248840
	subproc2_Δcnl	5.60	0.148	156.0	91.1	193.5	102.4	6248840
POP3b_*P. delt.*	date0.5_cnl	1.93	0.055	47.7	26.2	59.2	33.0	4627349
POP5_*P. nigra* 58	subproc1_Δcnl	2.67	0.136	13.8	13.5	44.6	31.1	3658846
	subproc2_Δcnl	2.37	0.144	85.2	68.0	109.3	41.3	4539718
	date1.5_cnl	2.33	0.118	101.0	68.0	109.3	41.3	4539718
POP2_*P. trich.*	date0.5_cnl	2.05	0.020	0.8	0.0	121.3	121.3	9369173
	date1.5_cnl	1.82	0.016	6.3	0.0	121.3	121.3	9369173
POP5_*P. nigra* P	subproc1_Δcnl	2.09	0.131	6.5	0.0	30.1	30.1	2501104

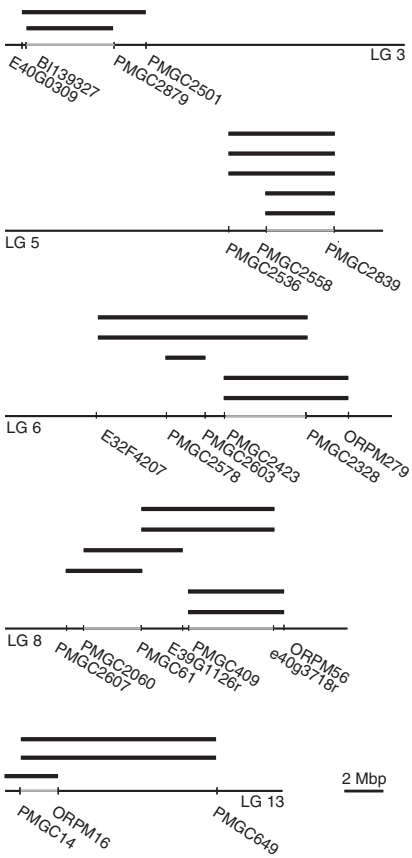


Fig. 7 Robust quantitative trait loci (QTL) regions for bud set. The QTL regions were linked to the physical genome sequence by sequence-anchored markers flanking the LOD peak. Twenty-one QTL fall into recurrently detected regions across pedigrees and are depicted here by their physical regions above the respective genome linkage group (in Mbp). Only the flanking markers considered are shown; additional markers can be viewed through the cmap application at http://services.appliedgenomics.org/poplar_budset. Genomic regions (highlighted in gray) were delineated to the smallest overlapping regions. The features of the 21 QTL (LOD, PVE, LOD peak, genetic and physical size) are given. One quantitative trait locus on chromosome 6, detected in *Populus trichocarpa* of POP3b, turned out to have no overlap with the quantitative trait locus detected in *Populus deltoides* of POP3b (region therefore not highlighted). A size reference in Mbp is given in the lower right corner. M1, marker start; M2, marker stop; LOD, logarithm of the odds; PVE, phenotypic variance explained; LG, genome linkage groups; *P. delt.*, respective *P. deltoides* parent; *P. trich.*, respective *Populus trichocarpa* parent; *P. nigra* P, *Populus nigra* 'Poli'; *P. nigra* 58, *P. nigra* '58-861'.

groups (Fig. 8). These expressional candidate genes were evenly distributed across the genome. Fifty-two expressional candidate genes (6.9%) resided within the six highlighted QTL regions for bud set. The proportion of these expressional candidate genes amounted, on average, to 2.8% of all genes contained in the respective regions (Fig. 8).

Next, we asked whether functional candidate genes were colocalizing with the six genomic regions. *CO* (gw.1.123.49.1), involved in the transduction of daylength signals for growth cessation (Böhlenius *et al.*, 2006), was an obvious candidate but resided on an unassembled scaffold of the poplar genome. *CO* was mapped to chromosome 17 of *P. nigra* (POP3a) through two single nucleotide polymorphisms (SNPs). Because a very strong QTL was located close to this position in *P. deltoides* (POP3a; Table S3), additional single-marker *t*-tests (Kruskal–Wallis) between phenotypic data and markers adjacent to the *CO*-SNPs (and similarly contained in the *P. deltoides* map) were carried out. No significant correlations of marker polymorphisms with any bud-set trait were revealed, rejecting the possibility that a segregation of *CO* alleles would have caused the QTL effect in the POP3a pedigree.

The *FT* gene, similarly involved in the transduction of daylength signals (Böhlenius *et al.*, 2006), colocated with a robust QTL region on chromosome 8, thereby constituting a good candidate to be tested for segregation in the pedigrees (Fig. 8). Also, *GIGANTEA* (*GI*), adjusting the expression of the photoperiod-regulated *CO* gene to the circadian clock, colocates to a robust region on chromosome 5 (Fig. 8). *PHYTOCHROME B2*, of which two SNPs explained part of the phenotypic variation in timing of bud set in European aspen (Ingvarsson *et al.*, 2008), did not coincide with any of the robust QTL regions (Fig. 8). None of the poplar homologs of dormancy-associated MADS-box genes, that were functionally characterized in peach (Jiménez *et al.*, 2009; Li *et al.*, 2009), colocalized with a robust QTL region (Fig. 8).

The QTL for bud set had been previously identified on linkage groups 3 (F), 6 (PY), and 10 (J) (the linkage groups within brackets correspond to Frewen *et al.*, 2000; Fig. 8). The QTL on chromosome 6 most probably corresponds to a robust region identified again in two different parental maps (Fig. 8).

In conclusion, the pedigrees displayed segregating genetic variation at various loci, of which six recurrently detected QTL regions define new, highly interesting and reasonably small areas (Fig. 8). These regions contain yet to be identified new causative genes and/or might in part be based on segregation in known functional genes, such as *FT* or *GI*, colocating with them (Fig. 8).

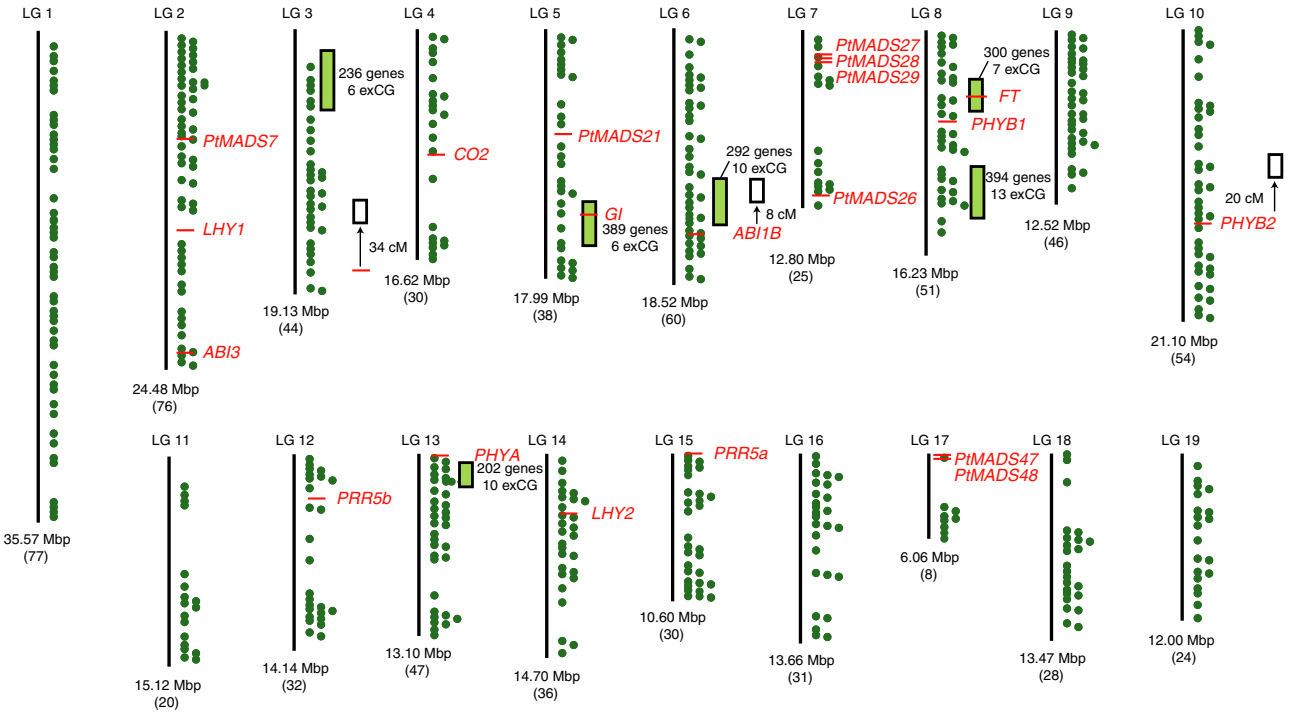


Fig. 8 Six new genomic regions, previously identified quantitative trait loci (QTL) and candidate genes for bud set across the poplar genome. The 19 genome linkage groups are drawn to scale, with their total size in Mbp given below. Expressional candidate genes, as identified by Ruttink *et al.* (2007), are drawn at their respective positions with green dots. The total number of expressional candidate genes per linkage group is given within parentheses below each linkage group. The six robustly detected QTL regions from Fig. 7 are drawn to scale and position with green rectangles. The identities of the genes contained in each of the six QTL regions are given in Table S4. Functional candidate genes are drawn at their respective genome position, following previous studies (Frewen *et al.*, 2000; Rohde *et al.*, 2002; Böhlenius *et al.*, 2006; Ingvarsson *et al.*, 2008; Ruonala *et al.*, 2008; Jiménez *et al.*, 2009 and Li *et al.*, 2009; Ibáñez *et al.*, 2010). Three QTL identified in family 822 (Frewen *et al.*, 2000) are drawn with open rectangles (not to scale) at their tentative location, by giving the genetic distance in cM from the respective anchoring marker with an arrow (on LG3, the simple sequence repeat (SSR) name of the anchoring marker is suppressed). *TFL1/CENL1*, *TOC1* and *CO* are located on unassembled genome scaffolds.

Discussion

Dissecting the bud set phenotype

For an understanding of bud set and its rational exploration in forestry, it is of primary interest to disentangle the conundrum of various overlapping processes that co-occur in time and/or space. The in-depth analysis of bud set, as applied in the current study, offers the possibility of dissecting the phenotype into stages and to estimate their relative contribution to the accomplishment of bud set (Fig. 1). Most importantly, the time of cessation of growth and the duration of bud development can be considered as separate and crucial determinants of bud set, which is a major improvement compared with the commonly used date of completed bud set.

Bud set proved to be determined by the timing of the cessation of growth and the duration of bud formation. Phenotypic variation was split, using PCA, into the onset of cessation of growth and duration of bud formation in the natural and hybrid populations (Fig. 6). However, the timing of the cessation of growth had a relatively higher contribution of genetic variance to total phenotypic variance than the duration of bud formation (Fig. 4).

Night length is the most stable predictor of coming winter hazards in many environments. Accordingly, the latitude of origin is often strongly correlated with the timing of bud set (Pauley & Perry, 1954). Here, cessation of growth triggered by an increasing night-length was the most decisive factor for bud set (Figs 3, 4, 6). The timing of the cessation of growth differed by approx. 27 d, equaling a difference of 2 h night-length, in all *P. nigra* accessions covering origins over 11° latitude (Fig. 3a; Table 1). Similarly, in the four poplar pedigrees, the timing of the cessation of growth contributed most to the timing of bud set (Fig. 6).

The duration of bud formation varied by only a few days in the *P. nigra* collection. Northern ecotypes set bud slightly quicker than southern (Table 1; Fig. 3b), as was also observed in birch (*Betula alba*) under controlled growth conditions (Junttila *et al.*, 2003). The duration of bud formation, because of its lower dependence on genetic factors (Fig. 4), probably allows for the accommodation of environmental influences and might be particularly important in the adaptation to short-term fluctuations or year-to-year variation in temperature, or even in the adaptation to different environments. Together, the timing of the cessation of growth as well as the duration of bud formation determine, albeit not to the same extent genetically controlled, the timing of bud set.

The unknown contribution of phenotypic plasticity to variation in bud set

For a rational selection of ecotypes in breeding programs as well as for the deployment of seeds or other propagules in forest plantation programs, phenology is of primary importance. Phenology largely determines to which degree trees will synchronize growth to the local climate. Quite rapid changes in climate might uncouple populations from the local environment to which they had strongly adapted. Adequate genetic adaptation through migration and evolution *in situ* are considered rather unlikely scenarios for forest trees (Aitken *et al.*, 2008). Another short-term option for adaptation, namely phenotypic plasticity, remained largely uncharacterized for phenology traits.

Genetic determinants of phenotypic plasticity, the degree to which the expression of characters is changed by different environments, are not well understood (Via & Lande, 1985). Variation of phenotypic response at population level ideally draws from large within-population variation and results in low clinal and/or ecotypic differentiation. Phenology traits, however, usually exhibit less within-population phenotypic variations than many growth-related traits (Howe *et al.*, 2003; Aitken *et al.*, 2008). The within-population genetic variation, an indicator for the potential adaptive response to natural selection, remained below 25% of the total genetic variation in the *P. nigra* populations (Fig. 4). A small, but significant within-population genetic variation for critical night length at bud set was also noted in a collection of European aspen (Ingvarsson *et al.*, 2006). Because variation in phenotypic plasticity can contribute to short-term adaptation, replicated clonal experiments in several field sites need to be carried out to study the genetic variation for phenotypic plasticity.

The genetic architecture of bud set with small-effect genes implies a strong genetic redundancy (Table 3). The fairly steep phenotypic clines of adaptive traits, such as bud set, may not correlate with allelic clines in single genes. Many genes of small effect determine and perpetuate the cline. Selection acts to reinforce phenotypic (and QTL) effects through generating covariance between individual alleles of several loci (Le Corre & Kremer, 2003). Many loci (genes or QTL) stay as undifferentiated as neutral markers; a few loci, at best, might show signs of differentiation, which do not need to be the same across populations or pedigrees. This genetic architecture argues for sufficient loci that alone and through epistatic interactions can contribute to phenotypic plasticity, despite the limited within-population variation.

Other, yet little investigated, mechanisms might also enable a quick adaptation to the local climate. In Norway spruce, the prevailing temperature during embryo development determines in each seed, probably epigenetically, the timing of bud set of the future plant (Kvaalen & Johnsen, 2008). Such a mechanism, if widespread among forest trees, would not only enable a quick (within one generation) adaptation to changes in climate variables, but also generate variability within a population, depending on warm or cold temperatures during seed set from year to year (Rohde & Junttila, 2008).

In addition to phenotypic plasticity, bud set is clearly influenced by a number of phenotype-confounding factors, such as growth rate, branching system and drought stress or pathogen infection at the end of the growing season. Foliar rust infection by *Melampsora larici-populina* can significantly advance or delay bud set (C. Bastien, unpublished). These current biotic or other abiotic (drought) constraints on phenology might become more important cofactors for bud set when trees need to adapt to changing climatic variables.

Toward the causative genes for genetic variation in bud set

Quantitative trait loci mapping remains challenging in forest trees, primarily because of the limited manageable progeny sizes that impede the resolution of genetic maps. However, additional robustness can still be achieved through searching QTL in different genetic backgrounds, implying a multiplicity of epistatic effects. Here, four different poplar pedigrees were investigated for bud-set QTL (Table 3) and a number of QTL regions coincided across different parents (Fig. 7). The number of coinciding genomic regions is most probably underestimated: only 36 of 53 considered QTL could be investigated for physical overlap because of a lack of genome-anchored markers across the parental maps. Nevertheless, the six genomic regions with coinciding QTL from several parents are considered as more robust than those found in one parent only (Fig. 8). Still, it remains to be seen whether the same causative genes underlie the QTL in the different parents.

In addition to the ability to detect meaningful quantitative variation, the delineation of narrow QTL regions for the identification of the genes carrying the causative polymorphisms remains extremely challenging in forest trees. Through the comparative approach, we have established reasonably sized regions in which to start looking for the causative genes (Fig. 8). The best-supported genomic region, detected in three different parents on chromosome 8, still contains 394 genes (region 8b; Figs 7, 8). Genes within this or the other region(s) can be scanned for allelic variation to start pinpointing the gene(s) that underlie(s) the QTL and to identify the relevant alleles for adaptation. To estimate their putative significance for adaptation under the climate change scenarios, genes with demonstrated function at bud set will need to be characterized not only for their functional polymorphisms, but also for plasticity of their expression in ecologically relevant, natural environments.

Together, the data from bud-set-related gene expression studies (Ruttink *et al.*, 2007) and the QTL presented here indicate that probably many genes are involved in the regulation of bud set and, thereby, in the adaptation to local climate in general. The need to consider many different genes underscores the difficulties in predicting complex interactions of trees with their environment. However, the diversity in the genes with a functional role during bud set will determine how rapidly populations will respond to selective environmental pressures. Combining genomics, functional and developmental approaches with ecological mechanisms will advance our understanding of phenotypic adaptation in the context of global warming.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Total and genetic variation within the seven populations *sensu stricto* contained within the *Populus nigra* collection.

Table S1 Trait means per pedigree/*Populus nigra* collection

Table S2 Phenotypical trait correlations based on individual fitted and block-adjusted values

Table S3 Bud set quantitative trait loci (QTL) with logarithm of the odds (LOD) value, LOD peak position, 95% confidence interval, 2-LOD support interval, phenotypic variance explained (PVE) and phenotypic effect for four poplar pedigrees

Table S4 Genes contained within the six highlighted quantitative trait loci (QTL) regions for bud set

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