TITLE:

Seasonal changes in nitrate assimilation of boreal woody species: Importance of the leaf-expansion period

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ABSTRACT

Temporal changes in leaf nitrate assimilation with leaf growth were intensively investigated in boreal tree species to demonstrate the contribution of nitrate as a N source and to determine temporal changes in the contribution of nitrate during leaf ontogeny. Leaf area, mass, nitrate reductase activity (NRA), N concentration, and $\delta^{15}N$ were repeatedly measured in developing leaves of naturally grown *Alnus crispa*, *Betula neoalaskana*, and *Populus tremuloides* during their leaf expansion period. *Alnus crispa* and *B. neoalaskana* showed distinct peaks in NRA during leaf expansion, whereas *P. tremuloides* did not. The highest peak in NRA occurred for *A. crispa*, whereas it had low NRA during the summer. Peak NRA in *B. neoalaskana* was lower than that of *A. crispa* ($p < 0.01$, ANOVA), although it showed higher NRA during summer ($p < 0.01$, ANOVA). All species showed clear decrease in N concentration through the leaf expansion period, but total N content per leaf increased. Only the N-fixing species *A. crispa* showed a rapid change in $\delta^{15}N$ during the leaf expansion, and the decline indicated the changes in N source during the leaf development. The results indicate leaves of target species assimilated nitrate during the leaf expansion period, consuming immense energy, although leaves were considered a carbon sink during the early leaf expansion period. We suggest the early onset of leaf growth due to climate warming could influence plant nutrition via asynchrony between supply and demand for energy during spring.

KEYWORDS

boreal forest; bud break; green up; leaf expansion period; nitrate reductase

KEY MESSAGE

Nitrate served as an important nitrogen source for dominant deciduous tree species, especially during their leaf expansion period, even in boreal forests where nitrate availability was assumed to be low.

DECLARATIONS

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Conflicts of interest/Competing interests

The authors have no conflicts or competing interests associated with this manuscript.

Availability of data and material (data transparency)

Data will be made available in the Bonanza Creek LTER database.

Code availability

Not applicable

Authors' contributions

LAK and KK planned and designed the research. LAK mainly conducted fieldwork, laboratory assay, and data analysis with help of KK. LAK and KK interpreted data, and LAK wrote the manuscript in consultation with KK.

INTRODUCTION

The leaf expansion period is a spectacular season, not only from the view of the rapid increase in leaf biomass but also considering invisible physiological changes that occur in the rapidly growing leaves. Leaves are carbon sinks which receive carbon translocated from other tissues during the first half of their expansion period. They become carbon sources during the latter half of the expansion period as their photosynthetic capacity increases with their growth (Šesták et al. 1985). Nitrogen concentration very quickly increases in breaking buds and decreases relatively slowly during the leaf expansion, followed by a steady state in fully expanded leaves (Millard 1994; Kielland et al. 1998; Koyama et al. 2008).

The changes in nutrient acquisition and translocation during the leaf expansion period have been investigated in less detail than carbon balance with leaf ontogeny. With respect to N sources for newly developing leaves, N remobilization has been investigated using ^{15}N tracers and intensive leaf and xylem sap sample collection during leaf expansion in seedlings or grafted nursery plants of species, such as apple (*Malus domestica* Borkh.), cherry (*Prunus avium* L.), walnut (*Juglans nigra* × *regia*), and birch (*Betula pendula* Roth.) (Millard et al. 1998; Frak et al. 2002; Grassi et al. 2002; Guak et al. 2003). In general, these studies showed that N remobilized to leaves from other tissues contributed greatly to leaf expansion in the early stage. In total, the dependence of newly developing shoots on N translocated from the other tissues ranged from 14 % to 87 % in these deciduous tree species. However, it remains to be shown how the remainder of the necessary N was acquired by new leaves; i.e., what form of N was used and how foliar N acquisition activity changed with the development of leaves. In a temperate region, two deciduous and an evergreen species were investigated at intervals of two to three days at the most, to observe the temporal changes in nitrate assimilation during leaf expansion (Koyama et al. 2008). All three of the studied species showed apparent peaks in nitrate reductase activity (NRA), the activity of an enzyme that catalyzes the rate-limiting process in nitrate assimilation, during the leaf expansion period. In boreal regions, where both the growing season and leaf expansion periods are shorter than in temperate regions, there have been no high-temporal resolution studies of the physiological changes in leaves during leaf expansion, such as in the study by Koyama et al. (2008). Climate change influences the foliation in boreal regions more

readily than in temperate regions, and the spring phenology of leaves is advanced in boreal forests (Linkosalo 2009). This suggests that elucidation of the physiological progress during leaf expansion and how it is altered under warmer climate conditions is urgent. Nevertheless, the relationship between leaf ontogeny and other physiological changes is not fully understood.

Nitrate is an important N source for terrestrial plants, and nitrate assimilation has been investigated in a variety of plant species using *in vivo* NRA as an index (e.g., Smirnoff et al. 1984, Gebauer et al. 1988, Koyama et al. 2020). The advantage of NRA as an index for nitrate assimilation is that plant nitrate use can be estimated without disturbing the soil N condition, which is not the case for experimental manipulations such as the application of ^{15}N tracers. Previous studies revealed significant variability in the capacity to use nitrate as a N source among plant species, and some species lacked the capacity to produce nitrate reductase (NR), an essential enzyme to assimilate nitrate. In boreal regions, nitrate is not considered a major N source for plants (Valentine et al. 2006), because of low microbial activity caused by low temperature and acidic soils. However, recent studies on the capacity of plants to use nitrate suggest it can be important in certain northern ecosystems such as in riparian forests and non-acidic arctic tundra (Liu et al. 2018; Koyama and Kielland 2019).

Species dependence on nitrate or the role of nitrate as a N source for a species should be evaluated not only based on momentary NRA but also the time course of NRA; i.e., the potential of the species to use nitrate and the continuity of high NRA after leaf expansion. In the present study, we investigated the changes in NRA, N content, and $\delta^{15}N$ in the leaves of boreal tree species during leaf expansion. Our research questions were: 1) Does nitrate serve as a N source for boreal tree species during leaf expansion? 2) Does nitrate assimilation (and the roles of nitrate as a source of N) change temporally during leaf expansion of the boreal species? 3) Does the pattern of plant nitrate assimilation exhibit a different time course between the boreal species and the temperate species studied by Koyama et al. (2008)? To address these questions, we repeatedly collected leaf samples from boreal species in the interior of Alaska, USA, throughout the leaf expansion period. Three deciduous species that are dominant in the interior of Alaska, *Alnus crispa*, *Betula neoalaskana*, and *Populus tremuloides*, were selected as target species. Based on the results, we discuss the possible influences of

136 climate change on boreal forests focusing on the altered timing of leaf expansion and
137 physiological activities during leaf growth.
138
139 MATERIALS AND METHODS
140 ***Study site***
141 The study was conducted in a mixed forest adjacent to the campus of the
142 University of Alaska, Fairbanks, USA (64°51′29.7″N, 147°51′15.4″W). The climate is
143 strongly continental, and the area lies within a rain shadow created by the Alaska Range
144 approximately 100 km to the south. Temperature extremes range from -40 °C in winter
145 to ≥30 °C during the summer, with an average of -3.3 °C. The average annual
146 precipitation is 269 mm, 37% of which falls as snow. Snow covers the ground for six to
147 seven months of the year, from mid-October until early or mid-April. Soil pH in the
148 nearby US LTER site were 7.2 ± 0.4, 5.6 ± 0.7 and 5.9 ± 0.6 in the sites with alder, birch
149 and aspen, respectively (Yarie 1998).
150 The mixed forest was dominated by some deciduous tree species, such as *Alnus*
151 *crispa*, *Betula neoalaskana*, and *Populus tremuloides*, and an evergreen coniferous
152 species, black spruce (*Picea mariana*). Dominant understory species included
153 *Vaccinium vitis-idaea*, *Vaccinium uliginosum*, *Rubus chamaemorus*, *Rosa acicularis*,
154 and *Epilobium angustifolium*.
155
156 ***Study species and sample collection***
157 Five mature trees from each of three deciduous tree species, *A. crispa*, *B.*
158 *neoalaskana*, and *P. tremuloides*, were sampled regularly about 10 times during the leaf
159 expansion period. Prior to the investigation of the leaf expansion period, we collected
160 leaf samples during summer (for comparison to newly expanded leaves) on July 22,
161 2009. In the following year, leaf samples were collected from the same individuals to
162 investigate their size (area and mass), N concentration, $\delta^{15}N$, and NRA during the leaf
163 expansion period. The sample collection began just after the bud break, which occurred
164 from early- to mid-May and ended June 7, 2010. Samples were collected at intervals of
165 3–4 days throughout this period. At each sampling day, 10 leaves were collected for leaf
166 size measurement regardless of leaf growth stage, and a minimum of 5-10 leaf samples
167 were collected and bulked for assay of N concentration, $\delta^{15}N$, and NRA from the

various part of canopy to reduce the effects of within-individual variation. The samples were collected from the surface of the canopy at various heights from 10:00 to 14:00, and the sampled leaves were exposed to adequate light because of low canopy density. The samples were stored in dark until assay on ice and in 4 °C in the field and the lab, respectively. The storage time of all samples before analysis was about 1 hour; thus, storage effects were similar for all samples, since the change in NRA is very slow after an initial decline in the first 30 min after collection (Högberg et al. 1986).

Assays

Leaf area and mass were measured to determine the degree of leaf expansion. To determine NRA, N concentration, and $\delta^{15}N$ of the leaves, aliquots of the sampled leaves were then assayed.

Ten leaves collected from a tree were weighed and scanned to determine leaf area using the image analysis software ImageJ (version 1.43m; https://imagej.nih.gov/ij/index.html; Schneider et al. 2012). The average area and mass of the 10 leaves from each tree were used as an indicator of leaf area and mass for each tree, respectively. To clarify the growth stage of leaves on each sampling day, leaf growth was fitted to a logistic curve (Koyama et al. 2008):

$$L_e = \frac{L_f \cdot L_0 \cdot \exp(r \cdot d)}{L_f - L_0 + L_0 \cdot \exp(r \cdot d)}$$

where L_e = estimated leaf size, L_f = estimated leaf size at full expansion, L_0 = estimated leaf size at the beginning of bud break, r = leaf growth rate, and d = number of days after bud break. Fitting was applied for both leaf area and leaf mass as a measure of size.

We measured two modes of activities of NR, NRA($+NO_3$) and NRA($-NO_3$), as indices of plant NO_3^--N assimilation. NRA($+NO_3$) is a measure of the nitrate reduction capacity with a non-limiting nitrate supply, whereas NRA($-NO_3$) is the reduction rate of nitrate absorbed by plants, which is considered to be the closest approximation of the *in situ* NO_3^--N assimilation rate (Thomas and Hilker, 2000). Both NRA assays were conducted with modified versions of the Jaworski procedure (Jaworski, 1971; Koyama and Kielland, 2011). NRA($+NO_3$) was measured as the rate of nitrite (NO_2^--N) production in an incubation buffer containing a non-limiting concentration of NO_3^--N.

NRA(−NO_3) was determined with parallel measurements using an incubation buffer without additional NO_3^--N, which allowed us to examine the relative magnitude of *in situ* NO_3^--N assimilation. Approximately 100 mg (fresh weight) of leaves were cut into small discs (D = 2.5 mm) and transferred to test tubes. The incubation buffer (5 mL) was added to the leaves, and the tube contents were vacuum infiltrated. The composition of the incubation buffer for NRA(+NO_3) was as follows: 0.1 mol L^{-1} KNO_3, 0.1 mol L^{-1} KH_2PO_4, and 1.5 % 1-propanol. The pH was adjusted to ca. 7.5 using a NaOH solution. The incubation buffer for NRA(−NO_3) contained all of the reagents other than KNO_3. The samples were incubated at 30 °C for 1 h in darkness. Enzyme activity was terminated by placing the sample vials in hot water (>80 °C). The concentration of NO_2^--N in the incubation buffer was determined colorimetrically following diazotization and azo coupling (Keeney and Nelson, 1982), by measuring absorbance at the wavelength 545 nm (Lambda 25, PerkinElmer Inc., MA, USA). The confounding effects of plant pigments were accounted for by subtracting the absorbance of controls to which N-naphthylethylene diamine dihydrochloride was not added (Gebauer et al. 1998). A fraction of each leaf sample was oven-dried at 60 °C and then weighed to calculate activity per unit dry weight. Leaf NRA was calculated per unit leaf, too.

Leaf samples were dried at 60 °C for longer than 48 h and ground for $\delta^{15}N$ analysis. The stable N isotope ratio of samples was analyzed using an isotope ratio mass spectrometer (Delta S, Finnigan MAT, Bremen, Germany) coupled with an elemental analyzer (EA1108, Fisons, Milan, Italy) via Conflo II as an interface. The stable isotope ratios were expressed in δ notation as the differences in parts per thousand (‰) of the $^{15}N/^{14}N$ ratio of the samples from the $^{15}N/^{14}N$ ratio of the standard (atmospheric nitrogen). The precision of the on-line procedure was better than ±0.2 ‰. The amount of N in the standard reagent, DL-Alanine, calculated by the weight and N concentration were regressed with the Thermal Conductivity Detector (TCD) area ($R^2 > 0.999$, n = 23) obtained by the elemental analyzer. Hence, N concentration in the samples was calculated based on the TCD area, and N content per leaf were also calculated.

Statistical analysis

Pearson's correlation coefficients for estimated leaf area, estimated leaf area growth

rate, leaf N content (per dry weight and per leaf), leaf NRA(+NO_3) (per dry weight and per leaf), and $\delta^{15}N$ were calculated for each tree sample. The leaf growth curve was fitted for each tree to avoid the influence of individual differences in leaf growth stages at each sampling date, and the leaf area growth rate was calculated as the difference between leaf area on a certain day and that on the next day, as calculated for the leaf growth curve. Since the timings of bud breaks differed among species, it was unlikely to be appropriate to compare species at the same date especially to discuss the species potential for each trait. Therefore, we extracted peak timing for each of plant traits (leaf area, mass, leaf mass per area, NRA(+NO_3) and NRA(-NO_3) per dry weight and per leaf, N concentration, N content per leaf, and $\delta^{15}N$), and compared the peak values among species. To determine peak timing of plant traits during leaf expansion, repeated measures ANOVA was applied for each species. Then, peak values were compared among species with one-way ANOVA with post hoc Tukey HSD test. In addition, the values during summer were also compared among species with one-way ANOVA with post hoc Tukey HSD test. All statistical analyses were conducted using the statistical platform R (ver. 3.5.3; http://www.R-project.org).

RESULTS

Bud break and leaf expansion of *B. neoalaskana* and *P. tremuloides* began and ended earlier than that of *A. crispa* (Table 1, Fig. 1 and S1). Leaf mass increased for a longer period than did leaf area for all three species, and the peak rate of leaf mass increase occurred later than that of leaf expansion rate, except for *A. crispa* (Fig. S1**j**-**l**). This indicated that leaf mass per area (LMA) decreased with leaf expansion in the early expansion stage, and increased during the latter stage (Fig. S1**g**-**i**).

Leaf NRA(+NO_3) rapidly increased after bud break and declined after the peaks in *A. crispa* and *B. neoalaskana* (Fig. 1**g**-**i**, Table S4), indicating that these species were capable of assimilating nitrate in leaves during leaf development. However, the relationship of NRA(+NO_3) to leaf area or area growth rate differed among species (Table 2). A positive correlation between NRA(+NO_3) and area growth rate was observed for *A. crispa*, whereas *B. papyridera* showed a positive correlation between NRA(+NO_3) and leaf area. On the other hand, the increase after bud break was not clear in *P. tremuloides* leaf NRA(+NO_3), which was relatively low throughout the study

period (Table S5), suggesting low dependence on nitrate for this species during this time period. NRA($-NO_3$) was lower than NRA($+NO_3$) by as much as an order of two or was not detected in all three species.

Leaf N concentration showed a peak at the beginning of the leaf expansion period, and then monotonically decreased in all three species (Fig. 1**d-f**, Table S4), which reflected a typical growth dilution of initial N concentration in leaves. On the other hand, N content per leaf at the end of leaf growth was 8.2, 9.2, and 5.9 times larger than that at the beginning of leaf expansion for *A. crispa*, *B. neoalaskana*, and *P. tremuloides*, respectively. These were equivalent to the increase of 5.7 ± 1.2 mg N per leaf in 23 days, 2.2 ± 0.5 mg N per leaf in 27 days, and 2.3 ± 0.8 mg N per leaf in 22 days for *A. crispa*, *B. neoalaskana* and *P. tremuloides*, respectively, indicating that newly acquired N was more than the offset by the diluted N concentration (Figs. 2**d-f**, S1**a-c**). All three species showed a strong negative correlation between leaf N concentration and leaf area (Table 2; See also Table S1 for leaf mass). N concentration in *P. tremuloides* leaves apparently reached a steady state after the decline, while in the two other species the concentration of N continued to decrease until the end of the study period (Fig. 1**d-f**). However, the comparison between leaf N concentrations in the previous summer and at the end of study period showed that leaf N concentrations very likely reached steady states around the end of study period in both *A. crispa* and *B. neoalaskana*. Relations between leaf NRA and leaf N concentration or N contents per leaf differed among species (Table S2; Fig. S2), reflecting species difference in temporal changes of NRA.

Leaf $\delta^{15}N$ was stable throughout the study period in *B. neoalaskana* and *P. tremuloides* (Fig. 1**b**, **c**; Table S4), indicating that the composition of N sources did not change over time. On the other hand, $\delta^{15}N$ in *A. crispa* leaves exhibited the highest value which is compatible with reliance on N-fixation just after bud break, then rapidly decreased to a steady state (Fig. 1**a**, Table S4), implying a shift in N sources during leaf expansion. $\delta^{15}N$ and other leaf traits did not show consistent responses among individuals (Table S3; Fig. S2**a-c**).

There was a positive relationship between N content per leaf and the degree of leaf expansion; i.e., the percentage of the estimated leaf area at the day of sampling (L_e) to the maximum leaf area (L_f) based on their growth curve, that showed leaf N contents

monotonically increased with the growth of leaf area in all three species (Fig. 2**d-f**). On the other hand, leaf N concentration almost monotonically decreased with the increase in the degree of leaf expansion, indicating N dilution with leaf expansion (Fig. 2**a-c**). Following full leaf expansion then the N content per leaf reached a steady state (See also Fig. S1**a-c**).

Changes in NRA($+NO_3$) per weight and NRA($+NO_3$) per leaf with the degree of leaf expansion also showed different patterns among species (Fig. 2**g-l**). *Alnus crispa* exhibited peaks of NRA($+NO_3$) per weight and NRA($+NO_3$) per leaf, when the leaves reached approximately half of the full expansion. By contrast, *B. neoalaskana* did not peak until the leaves were nearly (ca. 90 %) fully expanded. NRA($+NO_3$) per leaf showed a pattern different from that shown by NRA($+NO_3$) per weight in *P. tremuloides*. NRA($+NO_3$) per leaf increased when leaves approached full expansion in *P. tremuloides*, whereas NRA($+NO_3$) per weight generally decreased with leaf expansion. Increased NRA($+NO_3$) per leaf after full leaf expansion in *P. tremuloides* implied that nitrate played a more important role as the N source later in summer after leaves were fully developed, although a relatively low NRA($+NO_3$) in this species suggested low overall dependence on nitrate as a N source.

DISCUSSION

Role of nitrate as a N source for the target species

Nitrate reductase is a substrate inducible enzyme, and the capacity to induce NR markedly differs among species (Smirnoff et al. 1984, Gebauer et al. 1988, Koyama et al. 2020). The target species in the current study also possessed NRA during the study period (Fig. 1**g-i**), indicating that all three of these boreal species had the capacity to induce NR and use nitrate as a source of N. Moreover, even though previous studies have indicated that woody species generally assimilate nitrate in roots especially under low N availability (Andrews 1986), the results of current study indicate that leaves assimilated nitrate in boreal tree species. The results suggested that nitrate was an important N source in boreal forests, as well as in arctic tundra where recently Liu et al. (2018) demonstrated the contribution of nitrate as a plant N source. The highest enzyme activity was detected in *A. crispa*, which exhibited higher NRA($+NO_3$) than the species with the lowest activity, *P. tremuloides*, by a degree of magnitude ($p < 0.04$, ANOVA;

Table S5). This observation is consistent with the observed higher rates of nitrification of alder stands in interior Alaska (Kielland et al. 2006).

The detection of NRA(+NO_3) in the study period indicated that the leaves of these species assimilated N themselves during the leaf expansion period in which N concentration drastically decreased due to growth dilution (Fig. 1). Increase in N content per leaf indicated that leaves acquired N during expansion (Fig. 2**d**-**f**, Fig. S1**a**-**c**). Because NRA values showed the potential activity but not the assimilated amount of N, we cannot quantitatively evaluate the contribution of nitrate assimilation in leaves to the increase of N content in leaves compared with N translocated from the other tissues. Regardless, our results indicated that leaves of boreal tree species assimilated nitrate themselves during expansion to at least partly compensate for the diluted N concentration and total increase in N content.

The patterns of temporal change in leaf NRA(+NO_3) differed among the three species (Fig. 1**g**-**i**, Table S4). The highest NRA(+NO_3) per dry weight was detected when the leaf size reached approximately half of the full expansion in *A. crispa* (Fig. 2**g**), and it was consistent with the significant positive correlation between NRA(+NO_3) per dry weight and area growth rate (Table 2). Both NRA(+NO_3) per unit weight and that per unit leaf mostly increased with the degree of leaf expansion in *B. neoalaskana* (Fig. 2**h**,**k**). Furthermore, the significant positive correlation between leaf area and NRA(+NO_3), regardless of the unit (Table 2), suggested that during leaf expansion period, nitrate use increased with time in this species. By contrast, *A. crispa* may have relied on nitrate later in the season, perhaps in response to increased nitrification rates as soil temperatures increased over the summer.

The relative dependence of the species on variant N sources should be reflected in $\delta^{15}N$ values of plant tissues (Koba et al. 2003, Liu et al. 2018). The $\delta^{15}N$ values of soil nitrate were lower than that of soil ammonium, and thus plants with lower tissue $\delta^{15}N$ are very likely to depend more on soil nitrate as a N source than plants with higher $\delta^{15}N$ values. In the present study, *P. tremuloides* clearly had a higher $\delta^{15}N$ value than that of the other species (Fig. 1**a**-**c**, Table S5 and S6), suggesting its dependence on the N source with high $\delta^{15}N$, which supposedly was not nitrate. This is consistent with the results wherein *P. tremuloides* showed a constantly low NRA(+NO_3) throughout the study period (Fig. 1**i**, Table S5). However, the current results were inconsistent with

early studies on nitrate use by *P. tremuloides* (Chapin et al. 1986; Kielland et al. 1998). Leaf $\delta^{15}N$ in *P. tremuloides* was much lower in the study of Kielland et al. (1998) than that in the current results, and were comparable to that in *B. neoalaskana*. It is still possible that soil nitrate availability differed between the sampling sites, and was reflected in leaf $\delta^{15}N$, although data regarding soil nitrate availability in the current sampling site were not available. Plant responsiveness to nitrate availability is species specific (Koyama et al. 2003), and it was observed that species that were less responsive and more flexible to nitrate availability had a wider distribution range with respect to nitrate availability (Koyama et al. 2013). Thus, we surmise that *P. tremuloides* are sufficiently physiologically flexible to change their N acquisition according to the soil conditions.

The depletion of $\delta^{15}N$ in *A. crispa* leaves in the early stage of leaf expansion (Fig. 1**a**, Table S4) suggest that the N source for leaves of *A. crispa* rapidly changed in the early leaf expansion season. *Alnus crispa* was the only symbiotic N-fixing species among the three target species, but $\delta^{15}N$ values in N-fixing plants vary in reliance on N_2 fixation. $\delta^{15}N$ values led to approximately 0 ‰ when the plants thoroughly depended on N_2 fixation (Craine et al. 2015). The $\delta^{15}N$ values of *A. crispa* were closer to 0 ‰ at the beginning of leaf expansion and deviated from 0 ‰ during the latter stage. The results suggested that leaves of *A. crispa* depended on N from N_2 fixation when they began to expand, and that the rapid increase in NRA($+NO_3$) synchronized with the decline of $\delta^{15}N$ suggest that nitrate was likely to increasingly contribute as N source in the early leaf expansion period (Fig. 1 **a, g**, **j**).

The decrease in N concentration per weight showed that N with a high concentration in leaves at bud break was strongly diluted during leaf expansion (Figs. 1 **d-f**, 2**a-c**; Table 2). On the other hand, the increase in N content per leaf represented that newly acquired N was more than the offset by the diluted N (Figs. 2**d-f**, S1**a-c**; Table 2), and that N was translocated to leaves from other tissues that had stored N beforehand or from roots that proximately absorbed N during the period, although the ratio of N origins was unidentified. Koyama et al. (2008) showed that N concentration was apparently reduced in 1-year old leaves during leaf expansion while N concentration in current buds increased in an evergreen temperate species, suggesting N was translocated from the 1-year leaves to the current buds. Previous researches on fruiting trees of

orchard species showed generally low contribution of newly absorbed N in comparison with stored N (orange: Feigenbaum et al. 1987; walnut: Deng et al. 1989; apple: Neilsen et al. 1997). Millard and Grelet (2010) summarized that the contribution of N remobilized to newly developing shoots from the storage tissues ranged from less than 10 % to 80–100 % in various deciduous woody species, although these estimates targeted relatively young individuals.

Temporal changes in nitrate use by boreal species compared with that of temperate species

In temperate region, it was shown that three broad-leaved species (including an evergreen species) had distinct peaks of NRA during leaf expansion period, and their NRA decreased after leaf expansion to a relatively steady state for each species, which continued throughout the following summer until the leaves fell (Koyama et al. 2008). The value of NRA in temperate species showed a positive correlation with leaf expansion rate, and it was concluded that the leaf growth stage was an important regulating factor of leaf nitrate assimilation. Additionally, nitrate assimilated in the current leaves at least partly compensated for the decreased N concentrations in leaves during leaf expansion. In this study, we found similar increases of NRA in boreal broad-leaved species during their leaf expansion (Fig. 1**g**-**i**), although the relationship of NRA to the leaf growth stage varied among species (Table 2; Table S1). With this small number of examples, however, one cannot generalize that plant species have different temporal patterns of NRA according to climatic regions; i.e., that temperate species have relatively synchronized patterns whereas boreal species have fluctuating patterns.

In temperate species, Koyama et al. (2008) revealed the positive correlations between NRA($+NO_3$) and leaf growth rate in individual trees of all three species with few exceptions. Likewise, NRA($+NO_3$) per unit dry weight showed a positive correlation with leaf area growth rate in *A. crispa* in this study (Table 2). On the other hand, NRA($+NO_3$) in *P. tremuloides* and *B. neoalaskana* showed different relationships with leaf growth. NRA($+NO_3$) in *P. tremuloides* was constantly low throughout the study period, and consequently, no clear relationship was observed in NRA($+NO_3$) with leaf area or growth rate. NRA($+NO_3$) of *B. neoalaskana* showed a positive correlation with leaf area but not with leaf growth rate, reflecting a peak NRA($+NO_3$) in the later

stage of leaf expansion (Table 2; Fig. 1**,** 2**g**-**i**). Moreover, NRA(+NO_3) per unit leaf in *B. neoalaskana* did not decrease even after the leaves fully expanded, and the summer reference showed a significantly higher NRA(+NO_3) than *A. crispa* in summer (Figs. S1**d**-**f**, Table S6). On the other hand, NRA(+NO_3) per unit leaf of *A. crispa* decreased after the peak in the middle of the growth stage, and the summer reference exhibited a very low NRA(+NO_3). The results suggested that the momentary NRA was not an adequate index to describe species characteristics regarding plant nitrate use.

Ecological implication – the influence of changing bud break timing

The results of this study revealed that the three boreal deciduous tree species had the capacity to assimilate nitrate as a source of N, and indeed assimilated nitrate during their leaf expansion period. However, questions remain regarding the uptake of nitrate from the soil, because, unlike ammonium, nitrate can be stored in plant tissues after uptake. Nitrate assimilation is a highly energy-consuming process, which consumes as much as 25 % of the energy from photosynthesis (Solomonson and Barber 1990). In addition, the nitrite reduction process that occurs in chloroplast immediately after nitrate reduction requires reducing power through photosynthetically reduced ferredoxin, which is a part of the mechanism for light requirement in nitrate assimilation processes (Lillo 2008). Thus, leaves that assimilated nitrate during the period with high growth rate must invest the energy for nitrate assimilation and not only their growth; i.e., current carbon acquisition. Earlier studies have revealed that the rate of light-saturated net photosynthesis per unit leaf area reached its maximum at or slightly before full leaf area expansion, and leaves received carbon as a sink before net photosynthesis reached its maximum (Sesták 1985). Considering that leaves are unlikely to have surplus carbon storage especially in the early leaf growth period, it must be cost effective for plants to have absorbed and incorporated N into organic N compounds consuming available energy in advance and translocate the organic N compounds to newly developing leaves. Nevertheless, the results of this study and that of Koyama et al. (2008) indicated that the leaves assimilated inorganic nitrate consuming energy during leaf growth, which suggested nitrate was proximately absorbed before assimilation.

The relationship between leaf development stage and leaf nitrate assimilation implies that leaf N acquisition is influenced by climate change in several ways. Global

warming could directly influence soil N availability by changing snow cover and the soil freeze-thaw cycle (Templer 2012) and, accordingly, plant N use. Moreover, the spring leaf expansion period will be advanced by warmer temperatures, causing a mismatch between the leaf expansion period and solar energy status (Makoto et al. 2014). As stated above, nitrate assimilation is a highly energy consuming process, and the energy is directly provided by photosynthesis (Solomonson and Barber 1990; Yanagisawa 2014). This suggests the possibility of energy deficiency for nitrate assimilation during leaf expansion in the case where leaves begin to grow earlier in lower light conditions and shorter daylight length, especially in boreal forests in high latitudes. Linkosalo et al. (2009) evaluated the bud burst of some boreal forest species, and found them advanced by 7.6–8.0 days per century, corresponding with a temperature increase of 1.5 °C in the same period. The meta-analysis by Menzel et al. (2006) revealed that leaf unfolding in Europe was advanced by 2.5 days per decade on average over the last three decades. It was observed that a single but significantly extreme warm event during winter could advance the phenology of nearly half the tree and shrub species in 101 observed temperate species (Ladwig et al. 2019). The ecological implication of the shift in plant phenology caused by warming was discussed with respect to spring frost risk (Hanninen 2006; Linkosalo et al. 2000) and synchrony between pollinator emergence and flowering (Kudo et al. 2008; Kudo and Cooper 2019). However, we are unaware of any studies that have examined the effects of early onset of leaf expansion on leaf nutrition, especially from the viewpoint of energy sources. A limited number of previous studies have focused on temporal changes in physiological activities related to nutrient acquisition during leaf ontogeny. What is now required is an integrated discussion on the effects of warming on plant phenology, seasonal change in soil nutrient availability, and energy availability—with a focus on the leaf expansion period.

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636

637 FIGURE LEGENDS

638

639 Figure 1. Temporal changes in leaf traits regarding N acquisition and growth for *Alnus*
640 *crispa* (left; (**a**), (**d**), (**g**), and (**j**)), *Betula neoalaskana* (middle; (**b**), (**e**), (**h**), and (**k**)),
641 and *Populus tremuloides* (right; (**c**), (**f**), (**i**), and (**l**)) during the greening season 2010 in
642 comparison with that of summer 2009. (**a**)-(**c**) leaf $\delta^{15}N$; (**d**)-(**f**) leaf N concentration;
643 (**g**)-(**i**) leaf NRA($+NO_3$) (closed circle) and leaf NRA($-NO_3$) (open circle); and (**j**)-(**l**)
644 leaf area (open diamond) with estimated growth curve of leaf area (solid line; left axis)
645 and leaf expansion rate (broken line; right axis). Average ± s.d. are shown for five trees.

646

647 Figure 2. Relationship between the degree of leaf expansion and leaf traits regarding N
648 acquisition for *Alnus crispa* (left; (**a**), (**d**), (**g**) and (**j**)), *Betula neoalaskana* (middle; (**b**),
649 (**e**), (**h**) and (**k**)), and *Populus tremuloides* (right; (**c**), (**f**), (**i**) and (**l**)) during the greening
650 season 2010. (**a**)-(**c**) N concentration per weight; (**d**)-(**f**) N content per leaf; (**g**)-(**i**)
651 NRA($+NO_3$) per leaf dry weight; and (**j**)-(**l**) NRA($+NO_3$) per leaf. The degree of leaf
652 expansion was defined as the percentage of the estimated leaf area (open symbols) or,
653 mass (closed symbols) on the day of sampling relative to the maximum leaf area based
654 on the growth curve. Average ± s.d. are shown for five trees.

655

SUPPORTING INFORMATION

The following Supporting Information is available for this article:

Fig. S1 Temporal changes in leaf traits regarding N acquisition and growth of *Alnus crispa* (left; (**a**), (**d**), (**g**), and (**j**)), *Betula neoalaskana* (middle; (**b**), (**e**), (**h**), and (**k**)), and *Populus tremuloides* (right; (**c**), (**f**), (**i**), and (**l**)) during the greening season 2010 in comparison with those of summer 2009. (**a**)-(**c**) N content per leaf; (**d**)-(**f**) NRA($+NO_3$) (closed square) and NRA($-NO_3$) (open square) per leaf; (**g**)-(**i**) leaf growth rate in area (broken line; left axis), leaf growth rate in mass (solid line; right axis) and LMA (leaf mass per area. cross; right axis); (**j**)-(**l**) leaf area (open diamond; left axis) and mass (closed diamond; right axis). Average ± s.d. are shown for five trees. Leaf growth rates were calculated as the difference of estimated leaf area on a day and the following day based on the growth curve. Note that the Y-axes are not identical among species to clearly show the intraspecies temporal changes.

Fig. S2 Relationship between leaf NRA($+NO_3$) and other leaf traits in individuals of *Alnus crispa* (left; (**a**), (**d**), (**g**), (**j**), (**m**) and (**p**)), *Betula neoalaskana* (middle; (**b**), (**e**), (**h**), (**k**), (**n**) and (**q**)), and *Populus tremuloides* (right; (**c**), (**f**), (**i**), (**l**), (**o**) and (**r**)) during the greening season 2010. (**a**)-(**c**) leaf $\delta^{15}N$; (**d**)-(**f**) leaf N content per leaf; and (**g**)-(**i**) leaf N content per area; (**j**)-(**l**) leaf N concentration; (**m**)-(**o**) NRA($-NO_3$) per leaf dry weight and (**p**)-(**r**) LMA (leaf mass per area). Different symbols indicate different individuals.

Table S1 Relationship between leaf N traits and leaf mass or leaf mass growth. Pearson's correlation coefficients were calculated between physiological traits regarding N use such as N concentration, N content per leaf, $\delta^{15}N$, NRA($+NO_3$) per dry weight, and per leaf in leaves and leaf growth traits; i.e., estimated leaf mass or its growth rate representing leaf growth stage in view of mass.

Table S2 Relationship among leaf N traits. Pearson's correlation coefficients were calculated between NRA per unit dry weight or per leaf and N content per unit dry weight or per leaf.

Table S3 Relation between $\delta^{15}N$ and other leaf traits regarding N assimilation. Pearson's correlation coefficients were calculated between $\delta^{15}N$ and other traits of leaves such as N concentration, N content per leaf or NRA (per unit dry weight or per leaf).

Table S4 Table S4 Changes in ten plant traits in three species during the leaf expansion period (mean ± s.d). Repeated measures ANOVA was applied and followed by post hoc Bonferroni test for multiple comparison to determine peak sampling days for each trait in each of three species. Values with the same letters are not significantly different ($p < 0.05$).

Table S5 Table S5 Results of ANOVA for the comparison among three species at the peak of each plant trait followed by tukey HSD test for multiple comparison. The peak of each trait was determined by repeated measures ANOVA (data shown in bold in Table S4 were used in the analysis), and the peak values of each species were compared by one way ANOVA.

Table S6 Table S6 Results of ANOVA for the comparison among three species of each plant trait in summer. The data collected during summer on July 22 2009 were analyzed by one way ANOVA followed by tukey HSD test for multiple comparison.

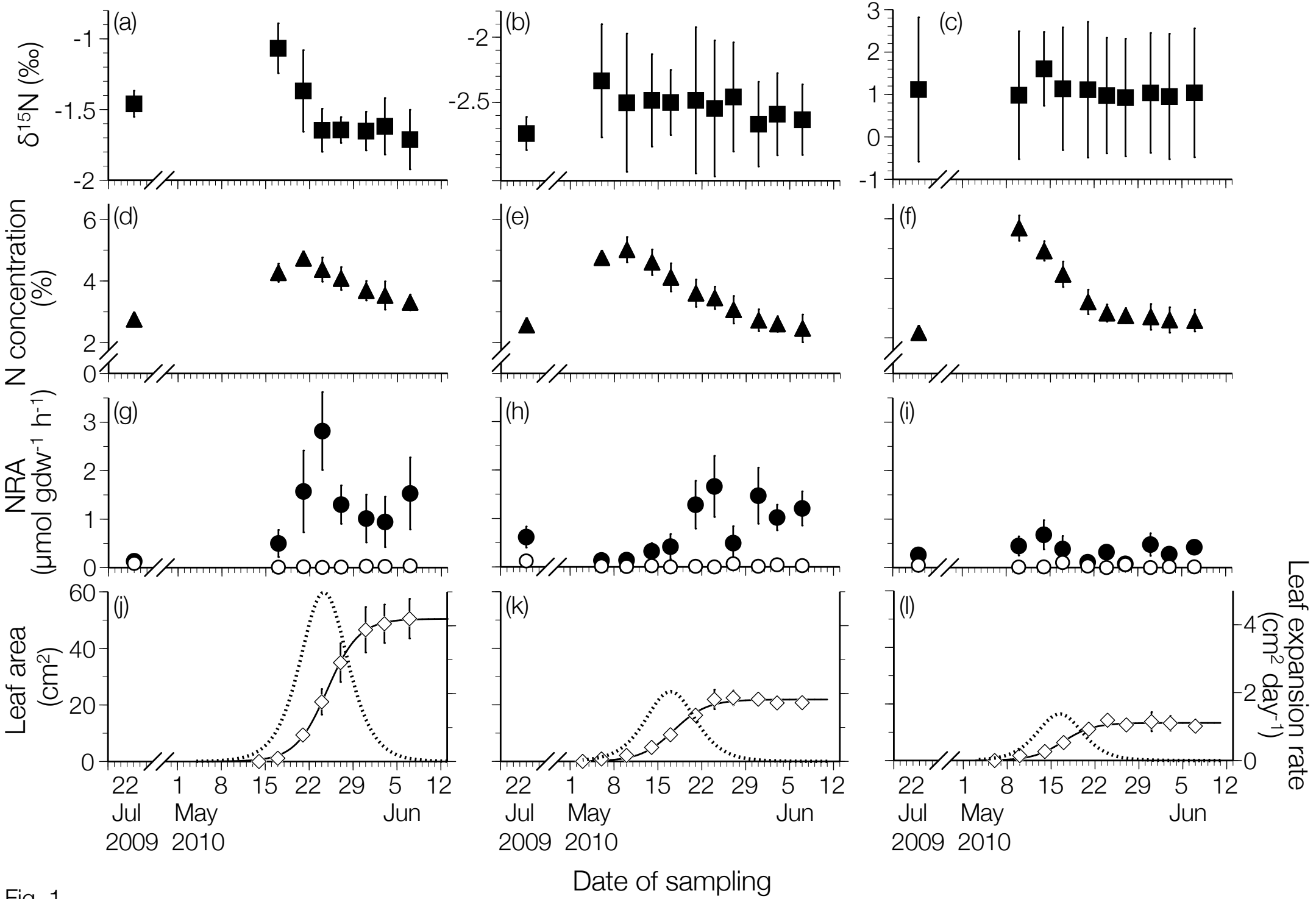

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
δ15N (‰)
N concentration (%)
NRA (μmol gdw-1 h-1)
Leaf area (cm2)
Leaf expansion rate (cm2 day-1)
22 Jul 2009
1 May 2010
8
15
22
29
5 Jun
12
Date of sampling

Fig. 1

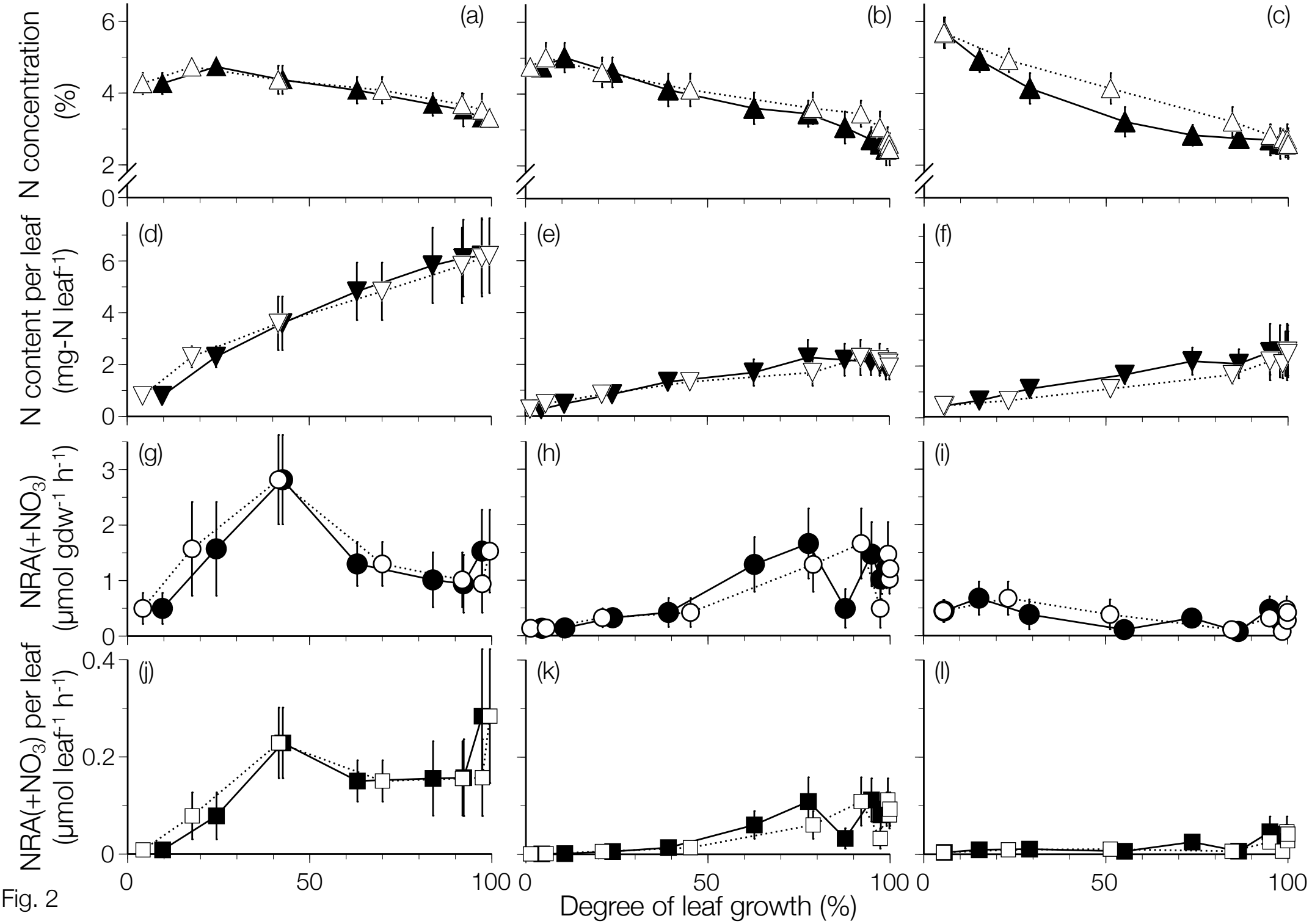

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
N concentration (%)
N content per leaf (mg-N leaf-1)
NRA(+NO3) (μmol gdw-1 h-1)
NRA(+NO3) per leaf (μmol leaf-1 h-1)
Degree of leaf growth (%)

Fig. 2

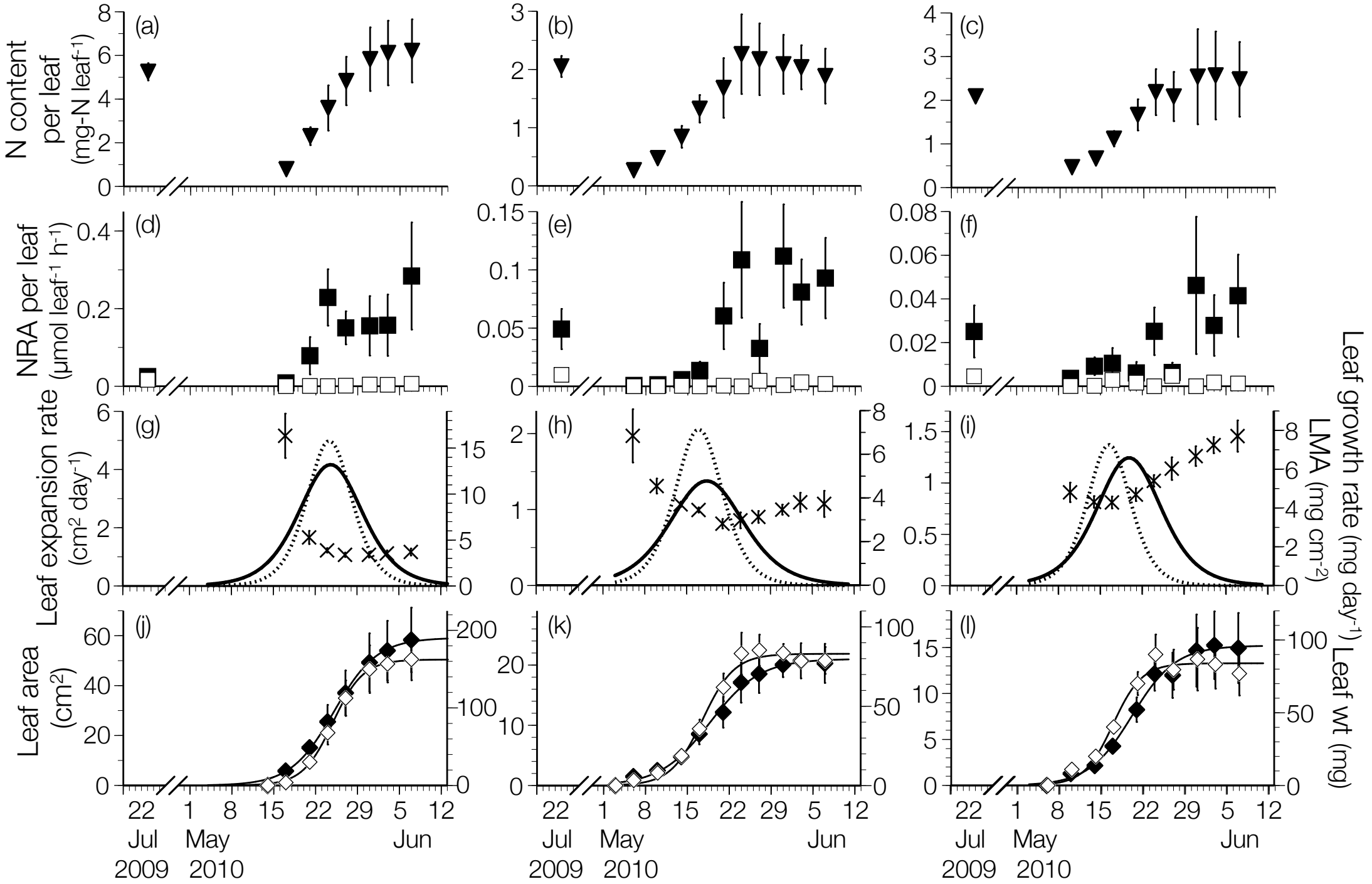
Table 1 The beginning and end dates, and duration of leaf expansion in the three target species in 2010. The day of full leaf expansion was defined as the day on which the estimated leaf area or mass reached to 99% of maximum based on their growth curve.

	Bud break	Full expansion in area	Duration for area growth (days)	Full expansion in leaf mass	Duration for mass growth (days)
Alnus crispa	May 14	June 6	23	June 11	28
Betula neoalaskana	May 3	May 30	27	June 7	35
Populus tremuloides	May 6	May 28	22	June 6	31

Table 2 Relationship between leaf N traits and leaf area or leaf growth rate. Pearson's correlation coefficients were calculated between physiological traits regarding N use such as N concentration, N content per area or leaf, $\delta^{15}N$, NRA($+NO_3$) per dry weight, area, leaf or N content in leaves and leaf expansion traits; i.e., estimated leaf area or its growth rate representing leaf growth stage, during leaf expansion.

	Tree	*A. crispa*						*B. neoalaskana*						*P. tremuloides*					
		Estimated leaf area (cm^2)			Area growth rate (cm^2 day^{-1})			Estimated leaf area (cm^2)			Area growth rate (cm^2 day^{-1})			Estimated leaf area (cm^2)			Area growth rate (cm^2 day^{-1})		
		R	P	n	R	P	n	R	P	n	R	P	n	R	P	n	R	P	n
N concentration	1	-0.817	0.01	8	0.831	0.01	8	-0.966	<0.01	11	0.532	0.09	11	-0.991	<0.01	10	0.633	0.05	10
(%)	2	-0.870	<0.01	8	0.682	0.06	8	-0.943	<0.01	11	0.53	0.09	11	-0.990	<0.01	10	0.506	0.14	10
	3	-0.960	<0.01	8	0.617	0.10	8	-0.869	<0.01	11	0.552	0.08	11	-0.972	<0.01	9	0.678	0.05	9
	4	-0.914	<0.01	8	0.641	0.09	8	-0.973	<0.01	11	0.406	0.22	11	-0.986	<0.01	10	0.592	0.07	10
	5	-0.493	0.22	8	0.832	0.01	8	-0.965	<0.01	11	0.503	0.12	11	-0.952	<0.01	10	0.636	0.05	10
N content per leaf	1	0.974	<0.01	8	-0.330	0.42	8	0.934	<0.01	11	-0.249	0.46	11	0.979	<0.01	10	-0.768	<0.01	10
(mg-N $leaf^{-1}$)	2	0.944	<0.01	8	-0.268	0.52	8	0.909	<0.01	11	-0.363	0.27	11	0.964	<0.01	10	-0.676	0.03	10
	3	0.983	<0.01	8	-0.365	0.37	8	0.903	<0.01	11	-0.233	0.49	11	0.934	<0.01	9	-0.519	0.15	9
	4	0.958	<0.01	8	-0.378	0.36	8	0.963	<0.01	11	-0.362	0.27	11	0.958	<0.01	10	-0.717	0.02	10
	5	0.968	<0.01	8	-0.389	0.34	8	0.981	<0.01	11	-0.355	0.28	11	0.879	<0.01	10	-0.682	0.03	10
$\delta^{15}N$	1	0.005	0.99	8	-0.687	0.06	8	-0.103	0.76	11	0.121	0.72	11	0.353	0.32	10	-0.415	0.23	10
(‰)	2	-0.693	0.06	8	-0.234	0.58	8	-0.500	0.12	11	0.234	0.49	11	-0.694	0.03	10	0.831	<0.01	10
	3	-0.796	0.02	8	0.026	0.95	8	-0.465	0.15	11	0.171	0.62	11	0.153	0.69	9	0.431	0.25	9
	4	-0.410	0.31	8	-0.259	0.54	8	-0.724	0.01	11	0.28	0.41	11	-0.016	0.96	10	0.343	0.33	10
	5	-0.811	0.02	8	0.186	0.66	8	-0.286	0.39	11	0.009	0.98	11	0.208	0.57	10	-0.100	0.78	10
NRA($+NO_3$) per	1	-0.277	0.51	8	0.854	<0.01	8	0.585	0.06	11	-0.088	0.80	11	-0.570	0.09	10	0.225	0.53	10
dry weight	2	-0.137	0.75	8	0.689	0.06	8	0.716	0.01	11	-0.105	0.76	11	-0.340	0.34	10	-0.127	0.73	10
(μmol gdw^{-1} h^{-1})	3	-0.421	0.30	8	0.781	0.02	8	0.700	0.02	10	-0.444	0.20	10	-0.129	0.72	10	0.165	0.65	10
	4	-0.310	0.46	8	0.622	0.10	8	0.647	0.03	11	-0.021	0.95	11	-0.801	<0.01	10	0.751	0.01	10
	5	0.118	0.78	8	0.633	0.09	8	0.792	<0.01	11	-0.345	0.30	11	-0.425	0.22	10	0.242	0.50	10
NRA($+NO_3$) per	1	0.357	0.39	8	0.441	0.27	8	0.761	<0.01	11	-0.350	0.29	11	0.561	0.09	10	-0.574	0.08	10
leaf	2	0.512	0.20	8	0.105	0.80	8	0.840	<0.01	11	-0.401	0.22	11	0.696	0.03	10	-0.655	0.04	10
(μmol $leaf^{-1}$ h^{-1})	3	0.123	0.77	8	0.413	0.31	8	0.717	0.02	10	-0.516	0.13	10	0.509	0.13	10	-0.459	0.18	10
	4	0.341	0.41	8	0.043	0.92	8	0.795	<0.01	11	-0.380	0.25	11	0.678	0.03	10	-0.453	0.19	10
	5	0.519	0.19	8	0.150	0.72	8	0.826	<0.01	11	-0.526	0.10	11	0.480	0.16	10	-0.481	0.16	10

R: Pearson's correlation coefficient. P: p-value. n: number of samples. †: Estimated leaf areas were calculated based on the logistic curves fitted to the number of days after bud break and leaf area for each tree to reduce the effect of sampled leaf sizes. ††: Area growth rate per day was calculated based on the logistic curves to calculate the leaf areas, by subtracting estimated leaf area at a sampling day from the area at the next day.


(a)
(b)
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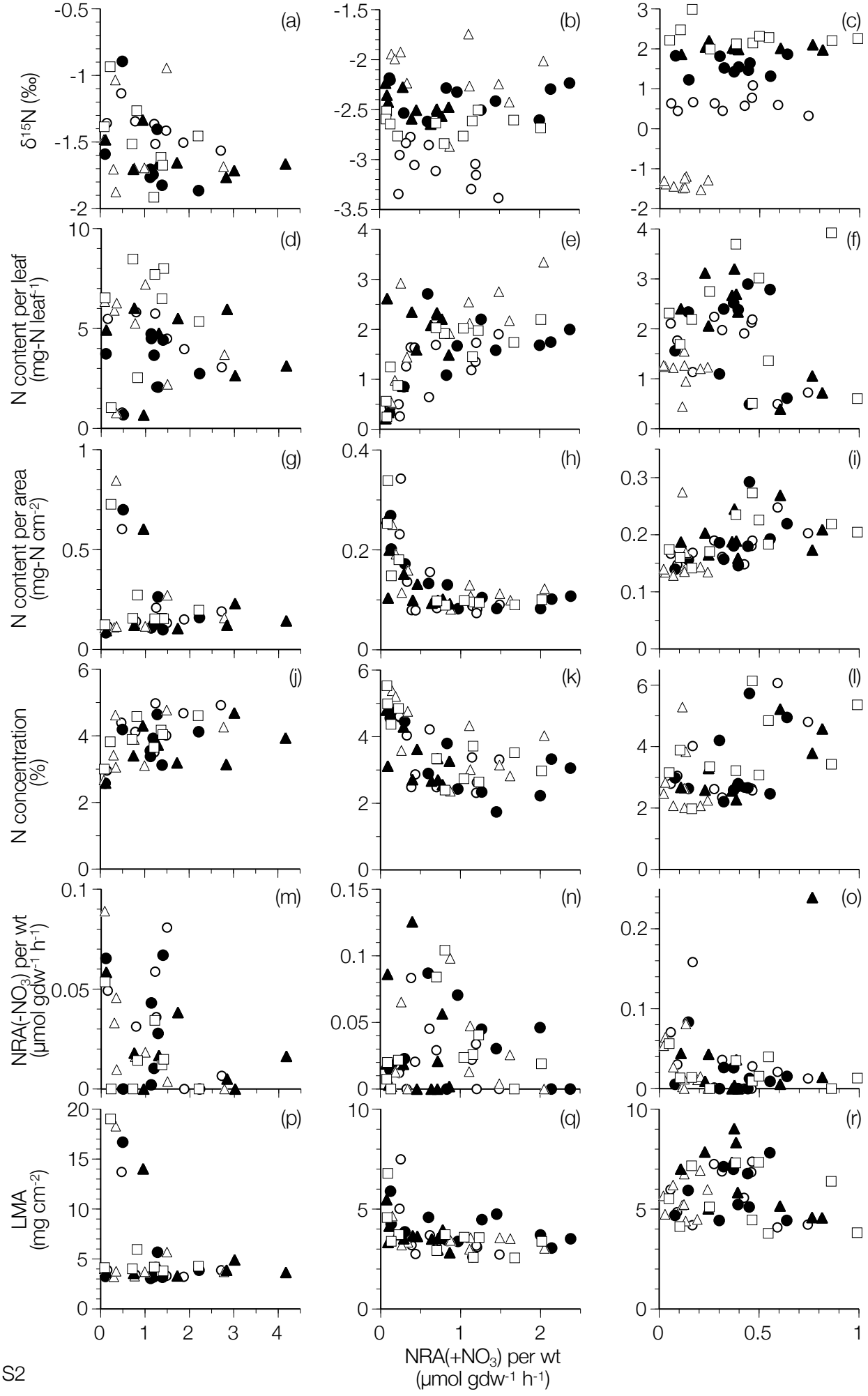
(j)
(k)
(l)
N content per leaf (mg-N leaf-1)
NRA per leaf (μmol leaf-1 h-1)
Leaf expansion rate (cm2 day-1)
LMA (mg cm-2)
Leaf area (cm2)
Leaf growth rate (mg day-1)
Leaf wt (mg)
22 Jul 2009
1 May 2010
8
15
22
29
5 Jun
12
Date of sampling

Fig. S1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(o)
(p)
(q)
(r)
δ15N (‰)
N content per leaf (mg-N leaf-1)
N content per area (mg-N cm-2)
N concentration (%)
NRA(-NO3) per wt (μmol gdw-1 h-1)
LMA (mg cm-2)
NRA(+NO3) per wt (μmol gdw-1 h-1)

Fig. S2

Table S1 Relationship between leaf N traits and leaf mass or leaf mass growth. Pearson's correlation coefficients were calculated between physiological traits regarding N use such as N concentration, N content per leaf, $\delta^{15}N$, NRA($+NO_3$) per dry weight, and per leaf in leaves and leaf growth traits; i.e., estimated leaf mass or its growth rate representing leaf growth stage in view of mass.

	Tree	*A. crispa*						*B. neoalaskana*						*P. tremuloides*					
		Estimated leaf mass (mg)			Mass growth rate (mg day^{-1})			Estimated leaf mass (mg)			Mass growth rate (mg day^{-1})			Estimated leaf mass (mg)			Mass growth rate (mg day^{-1})		
		R	P	n	R	P	n	R	P	n	R	P	n	R	P	n	R	P	n
N concentration	1	-0.869	<0.01	8	0.757	0.03	8	-0.986	<0.01	11	0.584	0.06	11	-0.961	<0.01	10	0.369	0.29	10
(%)	2	-0.874	<0.01	8	0.844	<0.01	8	-0.948	<0.01	11	0.275	0.41	11	-0.951	<0.01	10	0.175	0.63	10
	3	-0.983	<0.01	8	0.577	0.13	8	-0.892	<0.01	11	0.633	0.04	11	-0.993	<0.01	9	0.548	0.13	9
	4	-0.923	<0.01	8	0.726	0.04	8	-0.977	<0.01	11	0.380	0.25	11	-0.949	<0.01	10	0.216	0.55	10
	5	-0.523	0.18	8	0.881	<0.01	8	-0.982	<0.01	11	0.354	0.29	11	-0.933	<0.01	10	0.204	0.57	10
N content per leaf	1	0.948	<0.01	8	-0.167	0.69	8	0.913	<0.01	11	-0.235	0.49	11	0.988	<0.01	10	-0.512	0.13	10
(mg-N leaf^{-1})	2	0.940	<0.01	8	-0.421	0.30	8	0.893	<0.01	11	-0.165	0.63	11	0.977	<0.01	10	-0.375	0.29	10
	3	0.964	<0.01	8	-0.290	0.49	8	0.883	<0.01	11	-0.175	0.61	11	0.880	<0.01	9	-0.255	0.51	9
	4	0.950	<0.01	8	-0.422	0.30	8	0.950	<0.01	11	-0.282	0.40	11	0.977	<0.01	10	-0.379	0.28	10
	5	0.956	<0.01	8	-0.480	0.23	8	0.954	<0.01	11	-0.116	0.73	11	0.884	<0.01	10	-0.249	0.49	10
$\delta^{15}N$	1	0.039	0.93	8	-0.578	0.13	8	-0.036	0.92	11	-0.115	0.74	11	0.424	0.22	10	-0.445	0.20	10
(‰)	2	-0.714	0.05	8	-0.033	0.94	8	-0.627	0.04	11	0.428	0.19	11	-0.783	<0.01	10	0.595	0.07	10
	3	-0.713	0.05	8	-0.097	0.82	8	-0.509	0.11	11	0.319	0.34	11	-0.007	0.99	9	0.547	0.13	9
	4	-0.406	0.32	8	-0.233	0.58	8	-0.736	0.01	11	0.292	0.38	11	-0.134	0.71	10	0.269	0.45	10
	5	-0.801	0.02	8	0.272	0.52	8	-0.369	0.26	11	0.230	0.50	11	0.261	0.47	10	-0.284	0.43	10
NRA($+NO_3$) per	1	-0.348	0.40	8	0.802	0.02	8	0.537	0.09	11	0.003	0.99	11	-0.429	0.22	10	-0.183	0.61	10
dry weight	2	-0.118	0.78	8	0.639	0.09	8	0.551	0.08	11	0.354	0.29	11	-0.174	0.63	10	-0.485	0.16	10
(μmol gdw^{-1} h^{-1})	3	-0.431	0.29	8	0.588	0.13	8	0.686	0.03	10	-0.408	0.24	10	-0.074	0.84	10	-0.088	0.81	10
	4	-0.294	0.48	8	0.567	0.14	8	0.605	0.05	11	0.043	0.90	11	-0.747	0.01	10	0.131	0.72	10
	5	0.075	0.86	8	0.577	0.13	8	0.721	0.01	11	0.007	0.98	11	-0.311	0.38	10	-0.257	0.47	10
NRA($+NO_3$) per	1	0.261	0.53	8	0.589	0.12	8	0.735	0.01	11	-0.283	0.40	11	0.667	0.04	10	-0.634	0.05	10
leaf	2	0.508	0.20	8	0.006	0.99	8	0.760	<0.01	11	0.016	0.96	11	0.794	<0.01	10	-0.602	0.07	10
(μmol leaf^{-1} h^{-1})	3	0.107	0.80	8	0.289	0.49	8	0.711	0.02	10	-0.490	0.15	10	0.574	0.08	10	-0.541	0.11	10
	4	0.351	0.39	8	-0.023	0.96	8	0.797	<0.01	11	-0.345	0.30	11	0.758	0.01	10	-0.465	0.18	10
	5	0.475	0.23	8	0.106	0.80	8	0.817	<0.01	11	-0.248	0.46	11	0.563	0.09	10	-0.408	0.24	10

R: Pearson's correlation coefficient. P: p-value. n: number of samples. †: Estimated leaf mass were calculated based on the logistic curves fitted to the number of days after bud break and leaf weight for each tree to reduce the effect of sampled leaf sizes. ††: Weight growth rate per day was calculated based on the logistic curves to calculate the leaf weights, by subtracting estimated leaf weight at a sampling day from the weight at the next day.

Table S2 Relationship among leaf N traits. Pearson's correlation coefficients were calculated between NRA per unit dry weight or per leaf and N content per unit dry weight or per leaf.

Species	Item	Tree	N concentration (%)			N content per leaf (mg-N leaf^{-1})		
			R	P	n	R	P	n
A. crispa	NRA(+NO_3) per dry wt (μmol gdw^{-1} h^{-1})	1	0.645	0.08	8	-0.125	0.77	8
		2	0.450	0.26	8	0.119	0.78	8
		3	0.506	0.20	8	-0.280	0.50	8
		4	0.451	0.26	8	-0.174	0.68	8
		5	0.681	0.06	8	0.297	0.48	8
	NRA(+NO_3) per leaf (μmol leaf^{-1} h^{-1})	1	0.084	0.84	8	0.473	0.24	8
		2	-0.147	0.73	8	0.694	0.06	8
		3	-0.007	0.99	8	0.281	0.50	8
		4	-0.149	0.73	8	0.438	0.28	8
		5	0.334	0.42	8	0.666	0.07	8
B. neoalaskana	NRA(+NO_3) per dry wt (μmol gdw^{-1} h^{-1})	1	-0.498	0.12	11	0.557	0.08	11
		2	-0.635	0.04	11	0.566	0.07	11
		3	-0.547	0.10	10	0.710	0.02	10
		4	-0.683	0.02	11	0.511	0.11	11
		5	-0.720	0.01	11	0.776	<0.01	11
	NRA(+NO_3) per leaf (μmol leaf^{-1} h^{-1})	1	-0.700	0.02	11	0.748	<0.01	11
		2	-0.823	<0.01	11	0.676	0.02	11
		3	-0.588	0.07	10	0.733	0.02	10
		4	-0.831	<0.01	11	0.680	0.02	11
		5	-0.799	<0.01	11	0.828	<0.01	11
P. tremuloides	NRA(+NO_3) per dry wt (μmol gdw^{-1} h^{-1})	1	0.502	0.14	10	-0.499	0.14	10
		2	0.356	0.31	10	-0.130	0.72	10
		3	-0.105	0.79	9	-0.032	0.94	9
		4	0.726	0.02	10	-0.775	<0.01	10
		5	0.481	0.16	10	-0.089	0.81	10
	NRA(+NO_3) per leaf (μmol leaf^{-1} h^{-1})	1	-0.602	0.07	10	0.611	0.06	10
		2	-0.667	0.04	10	0.836	<0.01	10
		3	-0.554	0.12	9	0.403	0.28	9
		4	-0.734	0.02	10	0.732	0.02	10
		5	-0.332	0.35	10	0.777	<0.01	10

R: Pearson's correlation coefficient. P: p-value. n: number of samples.

Table S3 Relation between $\delta^{15}N$ and other leaf traits regarding N assimilation. Pearson's correlation coefficients were calculated between $\delta^{15}N$ and other traits of leaves such as N content (per unit dry weight or per leaf) or NRA (per unit dry weight or per leaf).

	Tree	*A. crispa*			*B. neoalaskana*			*P. tremuloides*		
		R	P	n	R	P	n	R	P	n
N concentration (%)	1	-0.427	0.29	8	-0.022	0.95	11	-0.305	0.39	10
	2	0.379	0.35	8	0.479	0.14	11	0.624	0.05	10
	3	0.689	0.06	8	0.736	0.01	11	0.045	0.91	9
	4	0.049	0.91	8	0.781	<0.01	11	0.040	0.91	10
	5	0.071	0.87	8	0.328	0.32	11	-0.447	0.20	10
N content per leaf (mg-N $leaf^{-1}$)	1	-0.147	0.73	8	-0.141	0.68	11	0.439	0.20	10
	2	-0.829	0.01	8	-0.569	0.07	11	-0.774	<0.01	10
	3	-0.818	0.01	8	-0.147	0.67	11	0.306	0.42	9
	4	-0.632	0.09	8	-0.635	0.04	11	-0.045	0.90	10
	5	-0.854	<0.01	8	-0.33	0.32	11	-0.117	0.75	10
NRA($+NO_3$) per dry wt (μmol gdw^{-1} h^{-1})	1	-0.765	0.03	8	-0.532	0.09	11	-0.040	0.91	10
	2	-0.560	0.15	8	-0.086	0.80	11	0.055	0.88	10
	3	0.021	0.96	8	-0.196	0.59	10	-0.021	0.96	9
	4	-0.533	0.17	8	-0.677	0.02	11	0.112	0.76	10
	5	-0.515	0.19	8	-0.045	0.90	11	-0.286	0.42	10
NRA($+NO_3$) per leaf (μmol $leaf^{-1}$ h^{-1})	1	-0.522	0.19	8	-0.459	0.16	11	0.553	0.10	10
	2	-0.791	0.02	8	-0.277	0.41	11	-0.640	0.05	10
	3	-0.439	0.28	8	-0.243	0.50	10	-0.071	0.86	9
	4	-0.718	0.05	8	-0.776	<0.01	11	0.125	0.73	10
	5	-0.814	0.01	8	-0.128	0.71	11	-0.154	0.67	10

Table S4 Changes in ten plant traits in three species during the leaf expansion period (mean ± s.d). Repeated measures ANOVA was applied and followed by post hoc Bonferroni test for multiple comparison to determine peak sampling days for each trait in each of three species. Values with the same letters are not significantly different ($p < 0.05$).

	May 3	May6	May 10	May 14	May 17	May 21	May 24	May 27	May 31	Jun 3	Jun 7
A. crispa											
Leaf area				0 ± 0 ^{d}	1.15 ± 0.23 ^{d}	9.38 ± 1.44 ^{d}	21.1 ± 4.52 ^{c}	34.97 ± 6.86 ^{b}	**46.59 ± 8.06** ^{a}	**48.71 ± 6.80** ^{a}	**50.51 ± 7.02** ^{a}
Leaf mass				0 ± 0 ^{e}	18.72 ± 4.81 ^{e}	48.75 ± 6.65 ^{de}	82.15 ± 21.34 ^{cd}	119.27 ± 29.59 ^{bc}	158.53 ± 35.10 ^{ab}	173.81 ± 37.51 ^{ab}	**187.58 ± 36.05** ^{a}
LMA					**16.34 ± 2.42** ^{a}	5.29 ± 0.72 ^{b}	3.88 ± 0.24 ^{b}	3.39 ± 0.24 ^{b}	3.39 ± 0.30 ^{b}	3.54 ± 0.33 ^{b}	3.7 ± 0.38 ^{b}
NRA(+NO_3) per dw					0.5 ± 0.28 ^{b}	1.57 ± 0.85 ^{ab}	**2.82 ± 0.81** ^{a}	1.30 ± 0.40 ^{b}	1.01 ± 0.49 ^{b}	0.94 ± 0.52 ^{b}	1.53 ± 0.74 ^{ab}
NRA(+NO_3) per leaf					0.009 ± 0.004 ^{c}	0.079 ± 0.052 ^{bc}	0.229 ± 0.074 ^{ab}	0.150 ± 0.045 ^{abc}	0.156 ± 0.082 ^{abc}	0.157 ± 0.086 ^{abc}	**0.284 ± 0.144** ^{a}
NRA(-NO_3) per dw					0.01 ± 0.02	0.02 ± 0.02	0.01 ± 0.01	0.01 ± 0.01	0.03 ± 0.03	0.02 ± 0.02	0.04 ± 0.03 ^{ns}
NRA(-NO_3) per leaf					$1.5\times10^{-4}\pm3.2\times10^{-4}$ ^{b}	$7.4\times10^{-4}\pm6.1\times10^{-4}$ ^{b}	$3.4\times10^{-4}\pm5.2\times10^{-4}$ ^{b}	$1.4\times10^{-3}\pm8.6\times10^{-4}$ ^{b}	$4.2\times10^{-3}\pm3.0\times10^{-3}$ ^{ab}	$3.7\times10^{-3}\pm2.5\times10^{-3}$ ^{ab}	**$6.3\times10^{-3}\pm3.5\times10^{-3}$** ^{a}
N concentration					4.27 ± 0.30 ^{ab}	**4.73 ± 0.15** ^{a}	4.37 ± 0.39 ^{ab}	4.08 ± 0.37 ^{abc}	3.69 ± 0.32 ^{bcd}	3.53 ± 0.46 ^{cd}	3.31 ± 0.26 ^{d}
N content per leaf					0.79 ± 0.15 ^{d}	2.3 ± 0.27 ^{cd}	3.59 ± 1.04 ^{bc}	4.83 ± 1.12 ^{ab}	5.83 ± 1.39 ^{ab}	**6.11 ± 1.47** ^{a}	**6.21 ± 1.30** ^{a}
$\delta^{15}N$					**-1.07 ± 0.18** ^{a}	-1.37 ± 0.29 ^{ab}	-1.65 ± 0.15 ^{b}	-1.65 ± 0.09 ^{b}	-1.65 ± 0.14 ^{b}	-1.62 ± 0.20 ^{b}	-1.71 ± 0.21 ^{b}
B. neoalaskana											
Leaf area	0 ± 0 ^{e}	0.85 ± 0.21 ^{e}	2.10 ± 0.23 ^{de}	4.94 ± 0.55 ^{d}	9.42 ± 1.52 ^{c}	16.30 ± 2.30 ^{b}	**21.92 ± 3.47** ^{a}	**22.47 ± 2.58** ^{a}	**22.00 ± 1.51** ^{a}	**20.68 ± 0.82** ^{a}	**20.77 ± 2.23** ^{a}
Leaf mass	0 ± 0 ^{e}	5.72 ± 1.09 ^{e}	9.48 ± 1.47 ^{e}	18.27 ± 1.83 ^{de}	32.50 ± 6.50 ^{cd}	46.18 ± 9.68 ^{bc}	64.97 ± 12.69 ^{ab}	**70.41 ± 12.06** ^{a}	**76.13 ± 7.77** ^{a}	**78.65 ± 11.08** ^{a}	**77.03 ± 12.32** ^{a}
LMA		**6.83 ± 1.22** ^{a}	4.53 ± 0.34 ^{b}	3.69 ± 0.12 ^{bc}	3.43 ± 0.12 ^{bc}	2.81 ± 0.22 ^{c}	2.96 ± 0.36 ^{c}	3.12 ± 0.27 ^{c}	3.45 ± 0.23 ^{bc}	3.79 ± 0.44 ^{bc}	3.72 ± 0.60 ^{bc}
NRA(+NO_3) per dw		0.13 ± 0.08 ^{de}	0.14 ± 0.06 ^{e}	0.32 ± 0.17 ^{de}	0.42 ± 0.26 ^{cde}	1.28 ± 0.49 ^{ab}	**1.66 ± 0.63** ^{a}	0.49 ± 0.35 ^{bcde}	**1.47 ± 0.58** ^{a}	1.02 ± 0.26 ^{abcd}	1.21 ± 0.35 ^{abc}
NRA(+NO_3) per leaf	$7.6\times10^{-4}\pm4.5\times10^{-4}$ ^{c}	$1.4\times10^{-3}\pm6.9\times10^{-4}$ ^{c}	$5.7\times10^{-3}\pm2.3\times10^{-3}$ ^{c}	$1.4\times10^{-2}\pm7.5\times10^{-3}$ ^{c}	$6.0\times10^{-2}\pm2.9\times10^{-2}$ ^{abc}	**$1.1\times10^{-1}\pm5.0\times10^{-2}$** ^{a}	$3.3\times10^{-2}\pm2.1\times10^{-2}$ ^{bc}	**$1.1\times10^{-1}\pm4.4\times10^{-2}$** ^{a}	$8.1\times10^{-2}\pm2.8\times10^{-2}$ ^{ab}	$9.3\times10^{-2}\pm3.5\times10^{-2}$ ^{ab}
NRA(-NO_3) per dw	$1.7\times10^{-2}\pm2.7\times10^{-3}$ ^{bc}	$8.5\times10^{-3}\pm6.8\times10^{-3}$ ^{c}	$2.4\times10^{-2}\pm1.2\times10^{-2}$ ^{bc}	0 ± 0 ^{c}	$1.3\times10^{-2}\pm1.2\times10^{-2}$ ^{c}	0 ± 0 ^{c}	**$6.5\times10^{-2}\pm2.7\times10^{-2}$** ^{a}	$1.4\times10^{-2}\pm2.0\times10^{-2}$ ^{c}	$4.4\times10^{-2}\pm1.0\times10^{-2}$ ^{ab}	$2.7\times10^{-2}\pm5.2\times10^{-3}$ ^{bc}
NRA(-NO_3) per leaf	$9.3\times10^{-5}\pm1.3\times10^{-5}$ ^{c}	$8.3\times10^{-5}\pm6.3\times10^{-5}$ ^{c}	$4.3\times10^{-4}\pm1.6\times10^{-4}$ ^{c}	0 ± 0 ^{c}	$5.3\times10^{-4}\pm4.1\times10^{-4}$ ^{c}	0 ± 0 ^{c}	**$4.7\times10^{-3}\pm2.1\times10^{-3}$** ^{a}	$1.0\times10^{-3}\pm1.4\times10^{-3}$ ^{c}	$3.5\times10^{-3}\pm1.1\times10^{-3}$ ^{ab}	$2.0\times10^{-3}\pm5.0\times10^{-4}$ ^{bc}
N concentration	4.76 ± 0.19 ^{ab}	**5.01 ± 0.41** ^{a}	4.61 ± 0.42 ^{ab}	4.12 ± 0.46 ^{bc}	3.60 ± 0.44 ^{cd}	3.45 ± 0.36 ^{cde}	3.07 ± 0.45 ^{def}	2.73 ± 0.35 ^{ef}	2.61 ± 0.25 ^{ef}	2.46 ± 0.45 ^{f}
N content per leaf	0.27 ± 0.05 ^{e}	0.48 ± 0.09 ^{de}	0.84 ± 0.12 ^{cde}	1.33 ± 0.20 ^{bcd}	1.68 ± 0.52 ^{abc}	**2.26 ± 0.64** ^{a}	2.18 ± 0.58 ^{ab}	2.09 ± 0.43 ^{ab}	2.04 ± 0.22 ^{ab}	1.89 ± 0.40 ^{ab}
$\delta^{15}N$	-2.34 ± 0.43	-2.50 ± 0.53	-2.49 ± 0.35	-2.50 ± 0.25	-2.49 ± 0.56	-2.55 ± 0.52	-2.46 ± 0.42	-2.67 ± 0.32	-2.59 ± 0.32	-2.63 ± 0.27 ^{ns}
P. tremuloides											
Leaf area	0 ± 0 ^{c}	1.74 ± 0.21 ^{c}	3.16 ± 0.33 ^{bc}	6.35 ± 0.72 ^{b}	**11.08 ± 1.26** ^{a}	**14.25 ± 2.17** ^{a}	**12.59 ± 2.18** ^{a}	**13.73 ± 3.41** ^{a}	**13.17 ± 2.65** ^{a}	**12.16 ± 2.38** ^{a}
Leaf mass	0 ± 0 ^{d}	8.19 ± 0.37 ^{d}	13.68 ± 1.87 ^{d}	27.02 ± 1.55 ^{cd}	52.10 ± 8.44 ^{bc}	76.76 ± 11.62 ^{ab}	75.76 ± 15.58 ^{ab}	**92.68 ± 24.82** ^{a}	**96.37 ± 23.24** ^{a}	**94.43 ± 24.12** ^{a}
LMA		4.80 ± 0.50 ^{de}	4.31 ± 0.30 ^{e}	4.28 ± 0.31 ^{e}	4.68 ± 0.33 ^{de}	5.39 ± 0.30 ^{cd}	6.02 ± 0.58 ^{bc}	6.67 ± 0.51 ^{ab}	**7.23 ± 0.42** ^{a}	**7.70 ± 0.81** ^{a}
NRA(+NO_3) per dw		0.45 ± 0.20 ^{ab}	**0.68 ± 0.30** ^{a}	0.38 ± 0.27 ^{ab}	0.11 ± 0.08 ^{b}	0.32 ± 0.13 ^{ab}	0.08 ± 0.05 ^{b}	0.47 ± 0.23 ^{ab}	0.28 ± 0.11 ^{ab}	0.42 ± 0.14 ^{ab}
NRA(+NO_3) per leaf		$3.6\times10^{-3}\pm1.5\times10^{-3}$ ^{b}	$9.2\times10^{-3}\pm4.1\times10^{-3}$ ^{b}	$1.1\times10^{-2}\pm7.0\times10^{-3}$ ^{b}	$6.1\times10^{-3}\pm5.0\times10^{-3}$ ^{b}	$2.5\times10^{-2}\pm1.1\times10^{-2}$ ^{ab}	$6.3\times10^{-3}\pm4.5\times10^{-3}$ ^{b}	**$4.6\times10^{-2}\pm3.1\times10^{-2}$** ^{a}	$2.8\times10^{-2}\pm1.4\times10^{-2}$ ^{ab}	**$4.1\times10^{-2}\pm1.9\times10^{-2}$** ^{a}
NRA(-NO_3) per dw		$1.0\times10^{-2}\pm7.2\times10^{-3}$ ^{b}	$1.3\times10^{-2}\pm1.6\times10^{-3}$ ^{b}	**$1.0\times10^{-1}\pm9.6\times10^{-2}$** ^{a}	$3.1\times10^{-2}\pm2.4\times10^{-2}$ ^{ab}	0 ± 0 ^{b}	$6.2\times10^{-2}\pm1.5\times10^{-2}$ ^{ab}	0 ± 0 ^{b}	$1.7\times10^{-2}\pm1.4\times10^{-2}$ ^{b}	$1.4\times10^{-2}\pm8.9\times10^{-3}$ ^{b}
NRA(-NO_3) per leaf		$8.4\times10^{-5}\pm5.5\times10^{-5}$ ^{bc}	$1.8\times10^{-4}\pm4.1\times10^{-5}$ ^{bc}	$2.9\times10^{-3}\pm2.5\times10^{-3}$ ^{ab}	$1.6\times10^{-3}\pm1.1\times10^{-3}$ ^{bc}	0 ± 0 ^{c}	**$4.7\times10^{-3}\pm1.8\times10^{-3}$** ^{a}	0 ± 0 ^{c}	$1.7\times10^{-3}\pm1.5\times10^{-3}$ ^{bc}	$1.3\times10^{-3}\pm7.1\times10^{-4}$ ^{bc}
N concentration		**5.69 ± 0.43** ^{a}	4.92 ± 0.33 ^{ab}	4.14 ± 0.43 ^{b}	3.21 ± 0.41 ^{c}	2.83 ± 0.29 ^{c}	2.74 ± 0.26 ^{c}	2.71 ± 0.43 ^{c}	2.60 ± 0.43 ^{c}	2.58 ± 0.37 ^{c}
N content per leaf		0.47 ± 0.05 ^{c}	0.67 ± 0.07 ^{c}	1.12 ± 0.15 ^{bc}	1.67 ± 0.30 ^{abc}	2.19 ± 0.46 ^{ab}	2.09 ± 0.47 ^{ab}	**2.57 ± 0.99** ^{a}	**2.57 ± 0.92** ^{a}	**2.48 ± 0.81** ^{a}
$\delta^{15}N$		0.98 ± 1.51	1.61 ± 0.87	1.14 ± 1.45	1.11 ± 1.60	0.97 ± 1.36	0.93 ± 1.39	1.04 ± 1.41	0.95 ± 1.48	1.04 ± 1.52 ^{ns}

Table S5 Results of ANOVA for the comparison among three species at the peak of each plant trait followed by tukey HSD test for multiple comparison. The peak of each trait was determined by repeated measures ANOVA (data shown in bold in Table S4 were used in the analysis), and the peak values of each species were compared by one way ANOVA.

		Results of ANOVA				Results of multiple comparison	
		Df	Sum of Sq	F value	P value	Species combination	P value
Leaf area	species	2	12958	445.9	< 0.01	*A. cri - B. neo*	< 0.01
	Residuals	67	973			*A. cri - P. tre*	< 0.01
						B. neo - P. tre	< 0.01
Leaf mass	species	2	50312	65.1	< 0.01	*A. cri - B. neo*	< 0.01
	Residuals	37	14298			*A. cri - P. tre*	< 0.01
						B. neo - P. tre	0.02
LMA	species	2	311.05	79.78	< 0.01	*A. cri - B. neo*	< 0.01
	Residuals	17	33.14			*A. cri - P. tre*	< 0.01
						B. neo - P. tre	0.69
NRA($+NO_3$) per dw	species	2	11.624	16.56	< 0.01	*A. cri - B. neo*	< 0.01
	Residuals	17	5.968			*A. cri - P. tre*	< 0.01
						B. neo - P. tre	0.04
NRA($+NO_3$) per leaf	species	2	0.1928	19.79	< 0.01	*A. cri - B. neo*	< 0.01
	Residuals	22	0.1072			*A. cri - P. tre*	< 0.01
						B. neo - P. tre	0.11
NRA($-NO_3$) per dw	species	2	0.03718	14.3	< 0.01	*A. cri - B. neo*	0.03
	Residuals	42	0.05457			*A. cri - P. tre*	< 0.01
						B. neo - P. tre	0.22
NRA($-NO_3$) per leaf	species	2	8.36E-06	0.581	0.58		
	Residuals	12	8.64E-05				
N concentration	species	2	2.401	9.598	< 0.01	*A. cri - B. neo*	0.45
	Residuals	12	1.501			*A. cri - P. tre*	< 0.01
						B. neo - P. tre	0.03
N content per leaf	species	2	91.01	45.48	< 0.01	*A. cri - B. neo*	< 0.01
	Residuals	27	27.01			*A. cri - P. tre*	< 0.01
						B. neo - P. tre	0.85
$\delta^{15}N$	species	2	302.52	182	< 0.01	*A. cri - B. neo*	< 0.01
	Residuals	96	79.79			*A. cri - P. tre*	< 0.01
						B. neo - P. tre	< 0.01

Species abbreviation; *A. cri*: *A. crispa*, *B. neo*: *B. neoalaskana*, *P. tre*: *P. tremuloides*

Table S6 Results of ANOVA for the comparison among three species of each plant trait in summer. The data collected during summer on July 22 2009 were analyzed by one way ANOVA followed by tukey HSD test for multiple comparison.

		Results of ANOVA				Results of multiple comparison	
		Df	Sum of Sq	F value	P value	Species combination	P value
Leaf area	species	2	3922	148.5	< 0.01	*A. cri - B. neo*	< 0.01
	Residuals	12	158			*A. cri - P. tre*	< 0.01
						B. neo - P. tre	< 0.01
Leaf mass	species	2	38242	29.47	< 0.01	*A. cri - B. neo*	< 0.01
	Residuals	12	7787			*A. cri - P. tre*	< 0.01
						B. neo - P. tre	0.64
LMA	species	2	38.18	57.9	< 0.01	*A. cri - B. neo*	0.98
	Residuals	12	3.96			*A. cri - P. tre*	< 0.01
						B. neo - P. tre	< 0.01
NRA($+NO_3$) per dw	species	2	0.6775	14.82	< 0.01	*A. cri - B. neo*	< 0.01
	Residuals	12	0.2742			*A. cri - P. tre*	0.33
						B. neo - P. tre	< 0.01
NRA($+NO_3$) per leaf	species	2	0.002496	5.718	0.02	*A. cri - B. neo*	0.02
	Residuals	12	0.002619			*A. cri - P. tre*	0.85
						B. neo - P. tre	0.05
NRA($-NO_3$) per dw	species	2	0.012867	31.73	< 0.01	*A. cri - B. neo*	< 0.01
	Residuals	12	0.002433			*A. cri - P. tre*	< 0.01
						B. neo - P. tre	< 0.01
NRA($-NO_3$) per leaf	species	2	0.000243	12.39	< 0.01	*A. cri - B. neo*	0.11
	Residuals	12	0.0001177			*A. cri - P. tre*	< 0.01
						B. neo - P. tre	0.04
N concentration	species	2	0.8921	11.51	< 0.01	*A. cri - B. neo*	0.32
	Residuals	12	0.4652			*A. cri - P. tre*	< 0.01
						B. neo - P. tre	0.02
N content per leaf	species	2	36.38	30.49	< 0.01	*A. cri - B. neo*	< 0.01
	Residuals	12	7.16			*A. cri - P. tre*	< 0.01
						B. neo - P. tre	1.00
$\delta^{15}N$	species	2	38.58	19.83	< 0.01	*A. cri - B. neo*	0.14
	Residuals	12	11.67			*A. cri - P. tre*	< 0.01
						B. neo - P. tre	< 0.01

Species abbreviation; *A. cri*: *A. crispa*, *B. neo*: *B. neoalaskana*, *P. tre*: *P. tremuloides*