

1 **Geo-climatic gradient shapes functional trait variations in *Salix eriocephala* Michx.**

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24 **Running title:** Phenotypic variation of biomass willow

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45 **ABSTRACT**

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47 Intraspecific variations in seasonal phenology and growth physiology reflect adaptation to local  
48 climate. To explore the patterns of local adaptation along latitudinal and longitudinal clines, we  
49 used thirty-four populations of *Salix eriocephala* sourced from its natural ranges across Canada.  
50 The genotypes were examined for 6 phenology and 19 ecophysiology traits over two growing  
51 seasons under common garden condition. Photosynthetic assimilation rate ( $A$ ) increased with  
52 increase in latitude when measured during free growth. In spite, the negative correlation between  
53 stomatal density and stomatal conductance ( $g_s$ ), higher  $A$  is facilitated *via* larger pore length  
54 among genotypes from short growing seasons. In addition, higher  $A$ , was positively associated  
55 with total leaf nitrogen and leaf mass per unit area. No population level differences were  
56 observed for water use-efficiency ( $\Delta^{13}\text{C}$ ), however nitrogen isotope discrimination ( $\delta^{15}\text{N}$ )  
57 displayed latitudinal clines. Growing season phenological traits considered in this study  
58 accounted high heritability ( $H^2 = 0.65-0.94$ ). *Melampsora* rust infestation also displayed a strong  
59 latitudinal cline with high-latitude genotypes being more susceptible. Overall, the results support  
60 the hypothesis that functional trait variations are largely explained by climate of origin and  
61 facilitate selection of parents with superior adaptive traits in the Canadian willow improvement  
62 program for bioenergy and environmental applications.

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## 76 INTRODUCTION

77 The issue of local adaptation is a crucial problem in the view of climate change. Functional trait  
78 variation can provide insights into the physiological processes associated with a species'  
79 persistence across a range of environmental conditions resulting from local adaptation (Aitken  
80 and Whitlock 2013). Largely, local adaptation plays a significant role in maintaining genetic  
81 variation among plant populations (Hodkinson 1999), and to better understand the evolutionary  
82 mechanisms of local adaptation one needs to take into account the environmental factors that  
83 contribute to phenotypic variation in nature (Manzano-Piedras et al. 2014). Such divergent  
84 selection is tested using provenance trial or common garden approaches to better understand trait  
85 trade-off relationships (Stearns 1989). Hence, between-provenance variation probably represents  
86 the most powerful tool for testing hypotheses of climatic adaptation among perennial trees  
87 (Mátyás 1996), whereby environmental gradients have produced genetically based clinal patterns  
88 in phenotype through adaptive evolution.

89  
90 Correlations between trait variation and geo-climatic factors may suggest the adaptive selection  
91 pressure exerted on a trait, thus demonstrating its adaptive significance. A negative latitudinal  
92 cline in tree height growth has been reported for deciduous (McKown et al. 2014a) and conifer  
93 seeds sourced from different provenances (Benomar et al. 2016). Although the height growth of  
94 trees is limited by growing conditions at high latitudes, it is not the case at low latitudes. Hence,  
95 the date of growth cessation is more important in differentiating among provenances height  
96 growth variations (Bridgewater 1990). Such trade-offs results from physiological and/or genetic  
97 links between traits and limit the possibility of evolution of beneficial traits (Weih 2003).

98  
99 Starting with the classical work by Mooney and Billings (1961) on *Oxyra digyna*, studies have  
100 revealed population genetic differentiation in photosynthesis which follows latitudinal and/or  
101 elevational clines (Chapin and Oechel 1983, Robakowski et al. 2012). At the same time, Flood et  
102 al (2011) linked leaf morphological attributes (leaf thickness, stomatal densities, leaf nitrogen)  
103 influencing photosynthetic rates among ecotypes from diverse temperature and moisture  
104 regimes. Stomatal density and pore length determine maximum conductance of CO<sub>2</sub> to the site of  
105 assimilation and also control transpirational water loss from leaves. Over the last 400-million  
106 years the stomatal design features have remained unchanged – i.e., to improve photosynthetic

107 rates and enabling land plants to occupy vast geographic ranges with varying environments thus  
108 contributing to increased genetic diversity (Franks and Beerling 2009). A strong association  
109 among traits suggests that optimization of photosynthesis to local environment along a latitudinal  
110 gradient is one mechanism by which plant fitness is enhanced (Gornall and Guy 2007).

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112 *Populus* species sampled over vast geographic ranges are well studied for their intraspecific  
113 variation in seasonal phenology and ecophysiology related traits (e.g., Soolanayakanahally et al.  
114 2009, McKown et al. 2014a). However, this remains less investigated in native populations of  
115 *Salix* (willow) species adapted to varied growth habitats. For example, willows from varied  
116 habitats provided evidence for their differential expression of water usage strategies when  
117 subjected to drought stress under greenhouse conditions (Savage and Cavender-Bares 2011). The  
118 physiological, anatomical and biochemical processes driving genotypic variation in resource  
119 acquisition and use efficiencies are still elusive in willows.  $\Delta^{13}\text{C}$  is a time integrated proxy  
120 measure of intrinsic water-use efficiency ( $WUE_i$ ) and has been correlated to growth and biomass  
121 accumulation (Farquhar et al. 1989). Considerable genotypic variation in  $\Delta^{13}\text{C}$  has been reported  
122 within the genus *Populus* (Soolanayakanahally et al. 2009, Broeckx et al. 2014, McKown et al.  
123 2014a) and in other tree species (Guy and Holowachuk 2001). These studies suggest that genetic  
124 variation in  $\Delta^{13}\text{C}$  is useful as a selection criterion for improved water-use efficiency. Observed  
125 genotypic differences in stomatal and mesophyll conductance to  $\text{CO}_2$  ( $g_s$  and  $g_m$ , respectively)  
126 were reported to affect  $\Delta^{13}\text{C}$  (Gresset et al. 2014, Barbour et al. 2015). In addition, leaf anatomy  
127 (and its association with LMA), aquaporin activity and enzymatic processes (carbonic anhydrase,  
128 RuBisCO) have recently been shown to influence  $g_m$  (Muir et al. 2014, Flexas et al. 2006).

129

130 In crop plants, the natural abundance of stable N isotopes ( $\delta^{15}\text{N}$ ) appears to be influenced by soil  
131 water availability and together  $\Delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ , has been proposed as an integrative measure for  
132 plant resource use-efficiency (Lopes et al. 2006). Lately, there are growing concerns over  
133 potential effects of soil-derived inorganic N [ammonium ( $\text{NH}_4^+$ ) and nitrate ( $\text{NO}_3^-$ )] affecting  
134 riparian systems and Kohl et al. (1971) established a negative relationship between  $\text{NO}_3^-$  and  
135  $\delta^{15}\text{N}$  to provide an insight into N uptake and assimilation by plants. With growing interest to  
136 establish *Populus* and *Salix* species as bioenergy crops in riparian buffer systems for nutrient  
137 interception and uptake along field edges, it is vital to investigate intraspecific variations in

138 nitrogen isotope discrimination ( $\delta^{15}\text{N}$ ). Except for the study in *P. balsamifera* by Kalcsits and  
139 Guy (2016), no other studies exploited intraspecific variations in  $\delta^{15}\text{N}$ . In addition, one could  
140 improve nitrogen use efficiency (NUE) by understanding plant N uptake, assimilation and  
141 remobilization (between sink and source) during the growing season. These measures of resource  
142 use-efficiencies need careful interpretation, as they are confounded to common garden artefacts  
143 (Soolanayakanahally et al. 2015).

144

145 Seasonal phenologies among deciduous trees in boreal and temperate regions are conditioned by  
146 the environment, especially by photoperiod and temperature. Bud phenology of boreal trees is  
147 characterized by spring bud break (bud flush) and summer growth cessation followed by leaf  
148 senescence in autumn (Fig. 1). The latter two events are cued by photoperiod (Fracheboud et al.  
149 2009) and have an adaptive significance displaying highest heritability (Alberto et al. 2013). As  
150 photoperiod regime is precisely the same from year-to-year, one can calculate critical  
151 photoperiod from observational data given the calendar date and latitude (Withrow, 1959). For  
152 example, Howe et al. (1995) reported that a northern *P. trichocarpa* ecotype (53°N) ceased  
153 height growth and set terminal bud to a critical photoperiod of 15h, whereas for a southern  
154 ecotype (40°N) the critical photoperiod was 9h. Quite similarly, cold hardiness development  
155 displays latitudinal clines during spring and fall in *Tamarix* and *Populus* spp. (Friedman et al.  
156 2011). An earlier bud set among high latitude trees might result in severe infestation of  
157 *Melampsora* leaf rust in a common garden setting due to natural selection trade-offs between  
158 growth phenology and disease resistance (McKown et al. 2014b).

159

160 Given the emergence of willows for biomass, bioenergy and environmental applications, many  
161 genetic resources are currently being made available in North America (Smart and Cameron  
162 2008) and Europe (Lindegaard and Barker 1997) for detailed investigation of phenotypic  
163 variation. One such Canadian genetic resource is the AgCanSalix (Agriculture Canada Salix)  
164 collection comprising both native and hybrid willows. Canada has 76 native willow species  
165 which are adapted to a wide range of environmental conditions (Argus 2010). The diamond or  
166 heart-leaf willow, *S. eriocephala* Michx., spans a broad geographic range coupled with diverse  
167 climatic conditions (Dorn 1970), whereby selective pressure on growth physiology and seasonal  
168 phenology traits is expected to vary extensively.

169  
170 Considerable intraspecific variation in growth phenology and genetic diversity was documented  
171 by earlier studies in *Salix* species that are highly correlated with their latitude of origin and/or  
172 growing season length (Weih et al. 2011, Trybush et al. 2012, Berlin et al. 2014, and Pucholt et  
173 al. 2015). Quantitative Trait Loci (QTLs) associated with growth phenology traits such as bud  
174 burst, elongation growth and leaf abscission were identified in *Salix* spp. (Ghelardini et al. 2014).  
175 Through association mapping analysis significant associations for bud burst, leaf senescence and  
176 biomass traits were reported in *S. viminalis* (Hallingback et al. 2015).

177  
178 In the present study, we investigated the factors that govern local adaptation by making use of a  
179 subset of 34 natural populations of *S. eriocephala*. We hypothesize that the gradient in climatic  
180 conditions (frost free days, mean annual precipitation, mean summer temperature) provide  
181 selection to favor the populations to respond differentially in functional traits (phenology,  
182 photosynthesis, resource use-efficiency) resulting from local genetic adaptation. The specific  
183 questions addressed are:

- 184 (i) Do latitudinal clines exist in photosynthetic assimilation rate ( $A$ ) among genotypes  
185 adapted to varying growing season lengths? If so, to what extent stomatal attributes  
186 (density and pore length) and mesophyll conductance ( $g_m$ ) contributes to higher  $A$ ?
- 187 (ii) Is there variation in water use-efficiency as shown by  $\delta^{13}\text{C}$  and nitrogen discrimination as  
188 shown by  $\delta^{15}\text{N}$ ?
- 189 (iii) Is there a relationship in seasonal phenology and rust infestation among *S. eriocephala*  
190 populations?

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## 193 **MATERIALS AND METHODS**

### 194 **AgCanSalix collection**

195

196 During the winter of 2012 (January to April) dormant stem cuttings of *S. eriocephala* were  
197 collected from within the natural range of the species within Canada. In total, we sampled 34  
198 populations with 15 genotypes per population ( $N = 510$ , Fig. 1). Care was taken to avoid clonal  
199 sampling by selecting distinct genotypes (stools) that were separated by a minimum of 1.0 to 3.0

200 km apart without phenotypic bias. Dormant cuttings (~ 20 cm long) were bagged separately for  
201 each of the 34 populations in re-sealable Ziploc<sup>®</sup> bags and stored at -4°C. Global Information  
202 System (GIS) coordinates and other site information were noted for each genotype (Table 1).  
203 This *in situ* collection of *S. eriocephala* along with other native species such as *S. amygdaloides*  
204 Andersson., *S. bebbiana* Sarg., *S. discolor* Muhl., *S. interior* Rowlee., and *S. petiolaris* Sm. is  
205 commonly referred to as the AgCanSalix collection (Agriculture Canada Salix).

206  
207 In spring (mid-May), 8–10 cm long dormant cuttings were forced to root in Spencer-Lemaire  
208 rootainers (Beaver Plastics, Acheson, Canada) using a mixture of Sunshine No.2 (Sun Gro  
209 Horticulture, Vancouver, Canada) growing mix (60%), peat (30%) and vermiculite (10%) inside  
210 a greenhouse under natural light. Greenhouse conditions were set to day/night temperatures of  
211 23/18 °C, respectively, with relative humidity at 40%. The Agriculture and Agri-Food Canada  
212 (AAFC) greenhouse facility is located at Indian Head, Saskatchewan (50.52°N 103.68°W;  
213 elevation 605 m). Upon bud flush, the plants were regularly watered and fertilized using  
214 Hoagland's solutions at a pH adjusted to 5.8–6.3. After two months of greenhouse growth  
215 (~25–30 cm tall), the plants were transferred to a shade house and allowed to undergo natural  
216 senescence. In late-October, the frozen root plugs were individually bagged and stored at -4°C  
217 till the following spring.

218

### 219 **Common garden establishment**

220 At Indian Head, the site assigned for the establishment of common garden was left fallow during  
221 the 2012 summer. In September, nine soil cores were randomly taken from 0–15 cm depth using  
222 an auger to acquire a representative sample along the length of the common garden (3 acres).  
223 Upon air drying the soil cores were processed separately, bagged and sent for soil testing at  
224 AGVISE laboratories (Northwood, ND, USA). The soil texture was sandy clay loam with an  
225 average pH of 7.9 and with 13.6, 19.7, 242.9 ppm of N, P and K nutrients, respectively. A  
226 detailed soil test report is provided in Supplementary table ST1.

227

228 In spring 2013, the site preparation involved cultivating to a depth of 20 cm or more and disking.  
229 Later, the rows were marked at 3 metre intervals in East-West orientation, with each row running  
230 320 m long. Based on the soil test report, necessary soil amendments were made by drenching

231 the marked rows using micronutrient solutions followed by roto-tilling. Rows were mulched  
232 using black plastic sheet (Crawling Valley Plastics, Bassano, AB, Canada) to avoid intra-row  
233 weed competition. The frozen root plugs were taken out of cold storage and all fifteen genotypes  
234 from each population were planted on mulched rows as a block with 1.0 m spacing. In each of  
235 the three replicates, population blocks were randomized with 3 ramets per genotype planted side-  
236 by-side. In addition, the common garden hosted a gene bank with a single ramet from 34  
237 populations, totalling to 10 ramets for each genotype ( $N = 5100$ ). The site was sprinkler irrigated  
238 as necessary during summer months with mechanical weed control between rows. All trees  
239 survived the first field growing season. A picture narrative of site preparation and planting is  
240 provided in Supplementary Fig. SF1.

241

### 242 **Seasonal growth phenology**

243 Adopting the phenology timetable developed by Saska and Kuzovkina (2010) for *Salix*, bud  
244 flush and leaf emergence were monitored in 2014, while leaf senescence and leaf drop were  
245 monitored in 2013 and 2014. Phenology was monitored by the same personnel walking through  
246 the gene bank every day during spring and twice a week during summer and fall. The  
247 *Melampsora* rust disease onset and severity of infection was scored on all genotypes beginning  
248 mid-August to late-September following the narrative by Mckown et al. (2014b). Here, we report  
249 the *Melampsora* rust scores for the week prior to September 21<sup>st</sup>. Green cover duration (GCD;  
250 days) was calculated as the difference between days to leaf senescence and days to leaf  
251 emergence. Final height and non-coppiced stem dry biomass were recorded in November 2014.  
252 The remaining three replicates were coppiced in November 2013 after initial establishment over  
253 first growing season. In the following years (2014 and 2015), phenology was monitored across  
254 all three replicates and used for estimating broad-sense heritability ( $H^2$ ) along with biomass and  
255 height gain.

256

### 257 **Screening for morpho-physiological traits**

258 Towards gas exchange measurements, we used a subset of 10 randomly chosen genotypes per  
259 population with the exception of Easterville (EAS) where only eight were used ( $N = 338$ ). The  
260 measurements were made between 5<sup>th</sup> July and 31<sup>st</sup> July 2014 during active growth without  
261 water deficit as our common garden was installed with a sprinkler irrigation system. All gas

262 exchange measurements were done on clear, sunny days. Briefly, a Li-COR 6400 XTR (LI-COR  
263 Biosciences, Lincoln, NE, USA) portable infra-red gas exchange system was used for gas  
264 exchange measurements. The gas exchange equipment was switched on by 8:00 am every  
265 morning at the common garden location and allowed to stabilize for 30 min prior to recording.  
266 On any given day, gas exchange measurements were recorded on a single leaf per genotype  
267 between 8:30 and 11:45 am, with the measurement plant randomized among populations and  
268 days of measurement. Inside the leaf chamber, the following conditions were maintained:  
269 reference CO<sub>2</sub> concentration set to 400 ppm using CO<sub>2</sub> cartridges; flow rate 500 μmol s<sup>-1</sup>; block  
270 temperature set at 23 °C; relative humidity of incoming air adjusted to ~50–55%; photosynthetic  
271 active radiation (PAR) 1000 μmol m<sup>-2</sup> s<sup>-1</sup>. Maximum photosynthetic assimilation rate (*A*, μmol  
272 CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>), stomatal conductance (*g<sub>s</sub>*, mol H<sub>2</sub>O m<sup>-2</sup> s<sup>-1</sup>), transpiration rate (*E*, mmol H<sub>2</sub>O m<sup>-2</sup>  
273 s<sup>-1</sup>) and intercellular CO<sub>2</sub> concentration (*C<sub>i</sub>*, ppm) were measured on fully expanded leaves. The  
274 intrinsic water-use efficiency (*WUE<sub>i</sub>*) was determined by calculating *A/g<sub>s</sub>* (μmol CO<sub>2</sub>/mmol  
275 H<sub>2</sub>O). Later, *A-C<sub>i</sub>* curves were constructed on selected populations representing East (NBN,  
276 NBS) and West (CLK, MJW, WAK) using the methodology described by Soolanayakanahally et  
277 al. (2009). The maximum carboxylation rate allowed by Rubisco (*V<sub>cmax</sub>*), rate of photosynthetic  
278 electron transport (*J*), triose phosphate utilization (TPU) and internal conductance (*g<sub>m</sub>*, mol CO<sub>2</sub>  
279 m<sup>-2</sup> s<sup>-1</sup>) were estimated by fitting the *A-C<sub>i</sub>* curve data to the model of Sharkey et al. (2007).

280  
281 Following gas exchange measurements, chlorophyll content index (CCI) was measured on three  
282 fully expanded leaves per plant using an Opti-Sciences CCM-200 meter (Hudson, NH, USA) and  
283 averaged for statistical analyses. Two leaf discs were sampled using a hand held paper punch  
284 exactly from the same leaf used for gas exchange, and oven dried at 50°C for 72 h for recording  
285 leaf mass per unit area (LMA, mg mm<sup>-2</sup>). The stem wood was collected in November 2014 at  
286 15cm above ground after recording non-coppiced biomass. Later, these leaf discs and stem wood  
287 samples were used to analyze leaf and wood carbon (C) and nitrogen (N) content and stable  
288 isotopes ratios (δ<sup>13</sup>C and δ<sup>15</sup>N; ‰) at the UC Davis Stable Isotope Facility (Davis, CA, USA).  
289 All δ<sup>13</sup>C values were converted to Δ<sup>13</sup>C using Farquhar et al. (1989) equation with isotopic  
290 composition of the air to PeeDee Belemnite of -8.3‰. Leaf C to N ratio (C: N; mg/mg) and  
291 photosynthetic nitrogen-use efficiency (PNUE; μmol CO<sub>2</sub> g<sup>-1</sup>N s<sup>-1</sup>) were calculated from these  
292 values. Stomatal density (number of stomata per unit of leaf area, mm<sup>-2</sup>) measurement samples

293 were prepared by applying a thin coat of clear nail polish on the adaxial and abaxial surfaces of  
294 fully expanded leaves (Gornall and Guy 2007). The dried impressions were stripped from leaves  
295 and mounted onto clear microscopic slides for observation. The slides were viewed under Zeiss  
296 phase contrast microscope (Axio Lab A.1, Toronto, ON, Canada) and stomata were counted  
297 under 20× magnification. The final stomatal count was averaged from three randomly selected  
298 field views from one impression. The stomatal pore length ( $\mu\text{m}$ ) was measured on a subset of  
299 populations based on density ranks representing East (high stomatal density: PEI, NSW, QUE,  
300 NBS) and West (low stomatal density: MDN, DRU, KEN, IHD). Five genotypes from each  
301 population ( $N = 40$ ) were randomly chosen to determine pore length on 5 stoma per genotype ( $N$   
302 = 200). Later, maximum stomatal conductance to  $\text{CO}_2$  ( $g_{c(\text{max})}$ ,  $\text{mol m}^{-2} \text{s}^{-1}$ ) was estimated using a  
303 modified version of the Franks et al. (2012) equation.

304

305 The Climate Normals (1981–2010) of closest stations were obtained for all populations from  
306 Environment Canada ([www.climate.weatheroffice.ec.gc.ca/climate\\_normals/index\\_e.html](http://www.climate.weatheroffice.ec.gc.ca/climate_normals/index_e.html)).  
307 Climate variables included frost free days (FFDs, days), mean annual precipitation (MAP, mm),  
308 mean summer temperature beginning from May to September (MST, °C) and degree days above  
309 5°C (DD>5, °C). The FFD was calculated based on the number of days where minimum day  
310 temperature was above 0°C, a proxy for growing season length at each location (Table 1).

311

## 312 **Statistical analysis**

313 All statistical analyses were performed using R studio statistical software (0.99.484 for  
314 Windows). Wherever possible the data from growth phenology traits were calculated from  
315 pooled data across 2013 and 2014. Analysis of variance (ANOVA) and correlation analysis for  
316 traits were performed to estimate the functional trait diversity and relationship among the  
317 populations used in the study. Broad-sense heritability ( $H^2$ ) estimates of traits were calculated as  
318 a ratio of genetic variability ( $\sigma_g^2$ ) to phenotypic variability ( $\sigma_g^2 + \sigma_e^2$ )  $\rightarrow [H^2 = \sigma_g^2 / (\sigma_g^2 + \sigma_e^2)]$ .  
319 Variance components were estimated based on ANOVA, where  $\sigma_e^2 = M_e$  and  $\sigma_g^2 = (M_g - M_e)/b$   
320 [ $M_e$ ; mean square of error,  $M_g$ ; mean square of genotypes and  $b$ ; number of replications].  
321 Pearson's correlation was performed to estimate correlation coefficients ( $r$ ) on all 338 genotypes  
322 among morpho-physiological traits and geo-climatic variables. Significant correlations between  
323 traits were expressed after Bonferroni correction ( $P < 0.001$ ).

324

325

## 326 **RESULTS**

327 The major aim of this study was to evaluate the extent of intraspecific variation in growth  
328 physiology and seasonal phenology of *S. eriocephala*; hence, genotypes were selected to be  
329 representative of a broad range of latitudes and longitudes. The geographical range spanned 15°  
330 in latitude and 52° in longitude, with elevation ranging from 4 to 800 m (Table 1). In general, the  
331 species range for *S. eriocephala* is at higher latitudes in the west, causing similar associations  
332 with longitude and elevation across Canada (Fig. 1). The average number of frost free days (a  
333 proxy for growing season length) ranged from 151 to 210 days with precipitation increasing from  
334 West (316 mm) to East (1709 mm). Growing degree days (DD>5 °C) were 60 units higher for  
335 eastern genotypes. A total of 25 traits related to ecophysiology, phenology and biomass were  
336 measured in 338 genotypes sourced from 34 populations.

337

### 338 **Phenotypic trait variations in *S. eriocephala***

339 All measured traits showed a wide range of variation between genotypes and among populations  
340 (Table 2). Photosynthetic assimilation rates ( $A$ ) ranged from 9.1 to 23.7  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ , with  
341 intrinsic water-use efficiency ( $WUE_i$ ) fluctuating between 19.5–128.7  $\mu\text{mol CO}_2/\text{mmol H}_2\text{O}$ .  
342 Both LMA and stomatal density displayed large variations among genotypes. Overall, leaf N and  
343 leaf C:N ratios were higher than wood, with CCI ranging from 6.5 to 22.2 units. The carbon  
344 isotopic discrimination ( $\Delta^{13}\text{C}$ ) for leaf and wood ranged from 16.8 to 23.1‰. While, the  
345 nitrogen isotopic discrimination ( $\delta^{15}\text{N}$ ) ranged between 3.4 to 21.2‰ in leaf and wood tissues.  
346 Spring bud flush occurred within a week's time, whereas, leaf senescence spanned over 80 days.  
347 At our common garden location, the green cover duration ranged from 98 days to 166 days  
348 leading to variances in overall height gain and biomass accrual.

349

### 350 **Correlation between geo-climatic and phenotypic variables**

351 Pearson correlation coefficients ( $r$ ) between geo-climatic parameters and phenotypic traits for all  
352 338 *S. eriocephala* genotypes are shown in Table 3. It is evident that both  $A$  and  $g_s$  increased  
353 with increase in latitude (LAT), longitude (LON), elevation (ELV) and MSP. Conversely, both  
354 traits were negatively correlated with FFD and MAP.  $WUE_i$  among genotypes increased with

355 increase in FFD and MAP. LMA increased with increase in LAT and LON implying that the  
356 leaves got thicker at high latitudes. While, stomatal density followed opposite trend. Both CCI  
357 and leaf N increased with increase in LAT, LON and ELV and decreased with increase in FFD  
358 and MAP.  $\delta^{15}\text{N}_{\text{leaf}}$  and  $\delta^{15}\text{N}_{\text{wood}}$  paralleled above trends. PNUE, wood N,  $\Delta^{13}\text{C}_{\text{leaf}}$  and  $\Delta^{13}\text{C}_{\text{wood}}$   
359 were not significantly affected by any geo-climatic variables. The relationships between LAT  
360 and resource use-efficiencies [water ( $\Delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ )] are plotted in Fig. 4A and 4B,  
361 respectively. The  $\delta^{15}\text{N}$  values of both leaf and wood increased significantly with increase in LAT  
362 (Fig. 4B). The  $\text{DD}>5^\circ\text{C}$  did not have any significant influence either on gas exchange or  
363 phenology traits.

364

365 Spring phenology (bud flush and leaf emergence) did not correlate with any geo-climatic  
366 variables. However, autumn events (leaf senescence, leaf drop, GCD) were negatively correlated  
367 with LAT, LON and ELV and positively correlated with FFD and MAP. The *Melampsora* rust  
368 incidence among the populations followed the reverse trend. Rust onset and its severity were  
369 positively correlated with LAT, LON, ELV and MST, but were negatively correlated with FFD  
370 and MAP. Genotypes from higher latitudes made significant height gains displaying positive  
371 correlations with LAT and LON. Non-coppiced single stem biomass was negatively correlated  
372 with FFD and MAP in our common garden.

373

### 374 **Correlation among phenotypic variables**

375 Traits related to ecophysiology, phenology and biomass were analyzed for functional  
376 intercorrelations and shown in a heat map (Fig. 3). The ecophysiological traits analyzed among  
377 the populations showed significantly stronger positive or negative correlations among each other.  
378 Photosynthetic assimilation rate ( $A$ ), stomatal conductance ( $g_s$ ), transpiration rate ( $E$ ), PNUE and  
379  $C_i/C_a$  were largely positive correlated to each other. The  $WUE_i$  was negatively correlated with  $A$ ,  
380  $g_s$ ,  $E$  and  $C_i/C_a$ . LMA is negatively correlated with PNUE and positively with Leaf N,  $\delta^{15}\text{N}_{\text{leaf}}$ ,  
381  $\delta^{15}\text{N}_{\text{wood}}$  and height. Stomatal density was positively correlated with autumn phenology and  
382 negatively with  $A$ , rust and height gain. CCI and leaf N are positively correlated with  $A$  and rust.  
383  $\Delta^{13}\text{C}_{\text{leaf}}$  was correlated positively with PNUE and negatively with leaf N.  $\delta^{15}\text{N}_{\text{leaf}}$  and  $\delta^{15}\text{N}_{\text{wood}}$   
384 were positively correlated to each other and also with  $A$ .

385

386 Most of the phenological traits were negatively correlated to ecophysiological traits (Fig.3). Bud  
387 flush was not significantly correlated to either  $g_s$  or PNUE. A similar trend was observed  
388 between leaf unfolding and  $\delta^{15}\text{N}_{\text{leaf}}$  and between rust incidences. Leaf senescence, GCD and leaf  
389 drop were negatively correlated to  $A$ ,  $g_s$ ,  $E$ , CCI, leaf N,  $\delta^{15}\text{N}_{\text{leaf}}$ ,  $\delta^{15}\text{N}_{\text{wood}}$  and rust incidence, and  
390 positively correlated to  $WUE_i$  and stomatal density. The correlation between leaf senescence,  
391 GCD and leaf drop was significantly positive ( $r = 0.75$ ). Height and biomass showed less  
392 correlation with ecophysiological and phenology traits. There was no significant correlation  
393 between biomass and  $\Delta^{13}\text{C}_{\text{wood}}$  and  $\delta^{15}\text{N}_{\text{wood}}$ .

394

### 395 **Stomatal pore length and $g_m$**

396 Based on the stomatal density ranking we measured stomatal pore length in eight populations  
397 representative of either ends of the range. Populations originating from Eastern Canada (low  
398 latitudes) had high stomatal density ( $124 \pm 3.08$  SE) per unit leaf area with a smaller pore length  
399 ( $11.168 \mu\text{m}$ ). In contrast, populations originating from Western Canada (high latitudes) had  
400 fewer stomata ( $68 \pm 2.19$  SE) per unit leaf area but a longer pore length ( $16.864 \mu\text{m}$ ). The  
401 significant differences in stomatal pore length and stomatal density are shown in Fig. 5A–5B ( $P$   
402  $< 0.001$ ). We also observed significant differences in  $g_s$  (Fig. 5C,  $P < 0.001$ ) among eight  
403 populations, however, the maximum diffusive conductance to  $\text{CO}_2$  ( $g_{c(\text{max})}$ ) as determined by  
404 stomatal density and pore length reached physiological optima at either ends of the species range  
405 to achieve maximum carbon gain (Fig. 5D,  $P = 0.09$ ).

406

407 Following gas exchange measurements, a total of ten  $\text{CO}_2$  response curves were constructed and  
408 data analyzed using  $A-C_i$  curve fitting model. The corresponding estimates of  $g_m$ ,  $V_{c\text{max}}$ ,  $J$  and  
409 TPU were plotted in Fig. 6A–6D for three populations from the west (CLK, MJW, WAK) and  
410 two populations from the east (NBN, NBS). The western genotypes had higher  $g_m$  ( $0.288$  vs.  
411  $0.198$ ;  $\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$ ),  $V_{c\text{max}}$  ( $107$  vs.  $93$ ),  $J$  ( $146$  vs.  $114$ ) and TPU ( $10.52$  vs.  $8.48$ ) values than  
412 the eastern genotypes. The variance between their mean values shows the difference in  
413 magnitudes of these values.

414

### 415 **Broad-sense heritability and rust infestation**

416 Heritability estimates ( $H^2$ ) estimates were calculated for seasonal phenology and biomass related  
417 traits. The  $H^2$  estimates ranged from 0.62 to 0.95 among the traits (Table 3). Bud flush, leaf  
418 emergence, leaf senescence and leaf drop had heritability estimates of 0.72, 0.77, 0.62 and 0.78,  
419 respectively. Height gain had higher heritability estimate (0.95) than biomass (0.88). A  
420 latitudinal cline with *Melampsora* rust incidence scores are shown in Fig. 7.

421

422

## 423 **DISCUSSION**

424 This is the first comprehensive study on a substantial number of native populations that  
425 originated from varying latitudes and longitudes in *S. eriocephala*. The above results emphasize  
426 the importance of considering key ecophysiological and phenological traits while studying local  
427 adaptation among willow populations of divergent origin. Besides, these physiological  
428 mechanisms are discussed in the light of convergent evolution among the members of the genus  
429 *Salicaceae* – a sympatric adaptive phenotype to very similar climate and photoperiod.

430

### 431 **Adaptive variations in photosynthesis**

432 Planted into a common environment (indoor greenhouse and/or outdoor common garden),  
433 observed functional traits differences among populations originating along an environmental  
434 gradient can be influenced by past evolutionary history resulting in adaptive genetic variations.  
435 In our study, photosynthetic assimilation rate ( $A$ ) increased with increase in latitude when  
436 measured during free growth in *S. eriocephala* genotypes. This is an agreement with the previous  
437 findings in North American *Populus* species which occupy similar climates (Gornall and Guy  
438 2007, Soolanayakanahally et al. 2009, McKown et al. 2014a, Kaluthota et al. 2015).  
439 Collectively, these studies hypothesised that the observed patterns of higher  $A$  among high  
440 latitude genotypes represent true adaptive variation in response to growing season length.  
441 Whereby, genotypes from shorter growing seasons possess inherently higher  $A$  compared to the  
442 genotypes from longer growing seasons. Again, higher  $A$  in *S. eriocephala* is associated with  
443 higher  $g_s$ , LMA, CCI and Leaf N (Soolanayakanahally et al. 2009, McKown et al. 2014a,  
444 Kaluthota et al. 2015), resulting in greater height gain among high latitude genotypes. A similar  
445 height gain was observed among high latitude *P. balsamifera* populations when photoperiodic  
446 constraints were removed by growing under extended daylength (Soolanayakanahally et al.

447 2009). On the other hand, when daylength was limiting, height rankings reversed leading to  
448 alterations in root:shoot ratios (Soolanayakanahally et al. 2013).

449  
450 Significant increases in  $A$  were found in other deciduous tree species (Benowicz et al. 2000,  
451 Soolanayakanahally et al. 2015) and evergreen conifers (*Picea glauca* [Moench] Voss, Benomar  
452 et al. 2015) sourced along a north-south gradient with varying growing season length. Such  
453 adaptation to growing season length in photosynthetic assimilation rates can be generalized along  
454 elevational gradients as well (Oleksyn et al.1998).

455  
456 Plant species occupying large geographic areas provide cues about adaptation mechanisms to  
457 various environmental conditions (Brosché et al. 2010). Leaf stomata regulate  $\text{CO}_2$  uptake and  
458  $\text{H}_2\text{O}$  use during photosynthesis and transpiration, respectively. Our *S. eriocephala* genotypes  
459 were entirely hypostomatous and stomatal density is negatively correlated with latitude and  
460 longitude (while,  $g_s$  is positively correlated with latitude and longitude). Stomatal density and  
461 pore length were negatively correlated with each other, one compensating for the other. Wang et  
462 al. (2015) studied latitudinal variation in stomatal traits across 760 species to highlight a strong  
463 negative relationship between stomatal density and stomatal length governing physiological  
464 adaptation to the environment. In their study, the plant species at low latitudes had higher  
465 stomatal density and reduced stomatal length than those distributed at high latitudes.

466  
467 Among plant groups, the maximum diffusive conductance to  $\text{CO}_2$  ( $g_{c(\max)}$ ) and water vapor is  
468 ultimately determined by stomatal density and pore length which may serve as a physiological  
469 framework to optimize leaf carbon/water balance (Franks et al. 2012). These long-term  
470 evolutionary scale adjustments in stomatal density and pore length in response to environmental  
471 conditions have facilitated *S. eriocephala* to expand into newer habitats leading to local  
472 adaptation. As the epidermal stomatal design features evolved 400-million years before present,  
473 the observed negative relationship between stomatal density and pore length suggests a  
474 widespread highly conserved genetic basis among vascular plants (Franks and Beerling 2009).  
475 So, we postulate that larger pore length with fewer number of stomata per unit leaf area certainly  
476 contribute to high  $g_s$ , in turn higher  $A$  among high latitude genotypes – a necessary “energy

477 *constraint trade-off* to maximise returns in  $g_s$  and  $A$  for a given investment in stomata  
478 construction costs under global leaf economics spectrum.

479

480 The observed difference in  $A$  is determined by  $g_s$  resulting from a combination of stomatal  
481 density and pore length; however, we must not discount the role of  $g_m$  as well. The physiological  
482 mechanisms involved in higher  $A$  have been the subject of extensive investigation in the recent  
483 past (Muir et al. 2013, Cano et al. 2013, Buckley and Warren 2014, Barbour et al. 2015). Such an  
484 examination of underlying physiological mechanisms in *S. eriocephala* are none and this study  
485 provide a first glimpse into naturally occurring variability in  $g_m$ . Even though our results on a  
486 small subset were not significant for  $A-C_i$  curve fitting estimates, overall the observed trends  
487 points towards higher  $g_m$  at high latitudes. Previously, Soolanayakanahally et al. (2009) reported  
488 an adaptive role of  $g_m$  in enhancing  $CO_2$  uptake efficiency and photosynthetic capacity among *P.*  
489 *balsamifera* trees adapted to short growing season. This enhanced  $g_m$  is linked to increased  
490 palisade surface area exposed to intercellular air space for  $CO_2$  diffusion (Milla-Moreno et al.  
491 2016), accounting for the positive association between LMA and  $g_m$  (Ryan 2015). Such positive  
492 and negative association between LMA (thicker or denser leaf tissue, or both) and  $g_m$  is reported  
493 in other plant species as well. In addition, Th eroux Rancourt et al. (2015) highlighted the  
494 importance of mesophyll-to-stomatal ( $g_m/g_s$ ) ratios while breeding for dry climates within  
495 *Salicaceae* species. Finally, we provide evidences for higher  $A$  among high latitude *S.*  
496 *eriocephala* genotypes ably supported by higher  $g_s$ , larger stomatal pore length and enhanced  $g_m$ .

497

#### 498 **Clines in resource acquisition**

499 Establishing the link between resource acquisition efficiencies and ecophysiological traits among  
500 the populations is essential to further understand their adaptive behaviours. D  az et al. (2016)  
501 mapped global trait spectrum in 46,085 vascular plant species to reflect “acquisitive vs.  
502 conservative” trade-offs between LMA and leaf N in constructing photosynthetic leaf. In their  
503 study, across biomes and plant species, cheaply constructed leaves with short lifespan were  
504 nitrogen-rich with low-LMA (acquisitive leaves), while leaves with long lifespan were nitrogen-  
505 poor with high-LMA (conservative leaves). Conversely, we found opposite patterns in LMA  
506 within *S. eriocephala* collection that occupies temperate-boreal climates. Whereby, shorter  
507 lifespan leaves had higher LMA (nitrogen-rich), while longer lifespan leaves had lower LMA

508 (nitrogen-poor). Similar “within species” patterns in LMA was also observed in *P. balsamifera*  
509 that encompass vast geographic ranges (Soolanayakanahally et al. 2009). Even though no  
510 significant association exists between LMA and ELV in our study, others found that LMA  
511 increases with ELV as well (Poorter et al. 2009).

512

513 Higher leaf N contents are associated with higher *A* as large amounts of inorganic nitrogen  
514 (~75%) are present in the chloroplast (Evans and Seemann 1989). We observed a strong positive  
515 correlation between *A*, leaf N and LMA. At the same time, PNUE is negatively associated with  
516 leaf N and LMA. So possible explanations for high LMA to have lower PNUE could be due to  
517 variation in nitrogen allocation between photosynthetic vs. non-photosynthetic structures, and  
518 also as a result of differential allocation of photosynthetic N between light harvesting complexes,  
519 electron transport and CO<sub>2</sub> fixation (Field and Mooney 1986). It seems that low latitude *S.*  
520 *eriocephala* genotypes invest more N towards foliar structures to withstand biotic and abiotic  
521 stressors, while fast-growing high latitude genotypes allocates more N to photosynthetic  
522 apparatus. Previously, Weih and Rönnerberg-Wastljung (2007) concluded a positive association  
523 between leaf N and photosynthetic capacity in *Salix* genotypes.

524

525  $WUE_i$  decreases as  $C_i/C_a$  increases, suggesting a potential intrinsic trade-off between  $WUE_i$  and  
526 PNUE (Field et al. 1983). Both these resource use-efficiency indicators of gas exchange ( $WUE_i$   
527 and PNUE) mutually depend on  $g_s$ , and are influenced by leaf-to-air temperature, light and  
528 available soil moisture. Unlike H<sub>2</sub>O, CO<sub>2</sub> faces further resistance in diffusion from intercellular  
529 spaces to the site of carbon fixation ( $g_m$ ). But, when *P. balsamifera* was grown without resource  
530 limitation,  $WUE_i$  increased with increase in latitude (Soolanayakanahally et al. 2009). We  
531 recognise the limitation in inferring  $WUE_i$  based on a single common garden. Turner et al. (2010)  
532 were able to differentiate the genetic and plastic responses in  $\Delta^{13}C$  in *Eucalyptus* species by  
533 taking into account the results from two common gardens.

534

535 A negative relationship between  $WUE_i$  and  $\Delta^{13}C$  has been extensively reported in many plants  
536 and is genetically determined (Farquhar et al. 1989).  $\Delta^{13}C$  values reflect on how plant species  
537 adjust their gas exchange metabolism, interplay of CO<sub>2</sub> and H<sub>2</sub>O acquisition and use, and  
538 adaptation patterns to different environments (Dawson et al. 2002). McKown et al. (2014a)

539 reported a 6.6‰ range in  $\Delta^{13}\text{C}$  values among 461 natural accessions of *P. trichocarpa*. Often the  
540 variations in  $WUE_i$  and  $\Delta^{13}\text{C}$  are associated with the variations in photosynthetic capacity of the  
541 populations (Pointeau and Guy 2014). In this study, even though no linkages were observed  
542 between  $\Delta^{13}\text{C}$  and geo-climatic variables, we found trait associations between  $\Delta^{13}\text{C}$ , PNUE and  
543 leaf N. Such variations in  $\Delta^{13}\text{C}$  and their relative role in photosynthetic capacity and adaptation  
544 have been studied in many trees (Anderson et al. 1996; Monclus et al. 2005).

545  
546 Intraspecific variation in nitrogen uptake and assimilation may differ among plant populations  
547 adapted to temperate ( $\text{NO}_3$  dominant soils) and boreal ( $\text{NH}_4$  dominant soils) climates. Soil  
548 derived  $\text{NO}_3$  nitrogen is assimilated by the nitrate reductase (NR) and nitrite reductase (NiR)  
549 pathway, producing  $\text{NH}_4$ . Subsequently, soil derived  $\text{NH}_4$  along with  $\text{NO}_3$  derived  $\text{NH}_4$  is  
550 assimilated via the glutamine synthetase (GS) and ferredoxin glutamate synthase (fd-GOGAT)  
551 pathway resulting in  $\delta^{15}\text{N}$  variations of plant tissues (Lopes and Araus 2006). Hence, natural  
552 abundance of  $\delta^{15}\text{N}$  in a plant provides an insight into the causal relationships between uptake,  
553 assimilation and allocation of nitrogen (Kalcsits and Guy, 2013). If  $\text{NO}_3$  is partially assimilated  
554 in roots than shoots are enriched in  $\delta^{15}\text{N}$  or if wholly assimilated in roots or shoots than shoots  
555 are not enriched in  $\delta^{15}\text{N}$ . We observed between 2 and 4‰ within plant variation (leaf vs. wood),  
556 and this could be due to partial assimilation of source nitrogen (particularly,  $\text{NO}_3$ ) in the roots,  
557 resulting in isotopic differences between tissue types (Evans et al. 1996). The observed  
558 latitudinal clines in  $\delta^{15}\text{N}$  imply that there is an adaptive genetic variation in assimilation of  $\text{NO}_3$   
559 nitrogen between roots and shoots in *S. eriocephala*.

560  
561 **Geographic variation in *Salix* seasonality**  
562 Functional traits that explain ecophysiological capacities are constantly modified during the  
563 growing season as a result of growth cessation and bud set (McKown et al. 2013). For instance,  
564 bud set has high heritability across multiple years ( $H^2 = 0.739$ ), whereas vegetative traits such as,  
565 leaf mass per unit area ( $H^2 = 0.810$  spring;  $H^2 = 0.150$  post bud set) is more plastic within a  
566 given season (McKown et al. 2014a). A number of common garden studies under single  
567 photoperiodic regime suggest daylength sensitivity in bud phenology to have a genetic basis  
568 (Ingvarsson et al. 2006, McKown et al. 2014a). For most deciduous trees, having met the chilling  
569 needs (endo-dormancy), the onset of spring bud flush marks the shift from a dormant, restive

570 stage (eco-dormancy) to an active growth stage upon accumulation of necessary heat sums under  
571 favourable environmental conditions (Worrall 1993).

572

573 Spring leaf emergence has been shown to have advanced over the past century with a steady  
574 lengthening of growing seasons (McMahon et al. 2010) leading to increased carbon fixation by  
575 terrestrial plants (Peñuelas et al. 2009). On the other hand, this increase in carbon sequestration is  
576 partially offset by enhanced rates of respiration (Piao et al. 2008). Temperature driven spring  
577 green-up often displays lower broad-sense heritability ( $H^2 = 0.43$  to  $0.68$ , Tsarouhas et al. 2003)  
578 as temperatures fluctuate a lot from year-to-year. Previous common garden studies have reported  
579 a narrow range for bud flush (~1–3 weeks) among intraspecific populations that display a  
580 latitudinal cline (*Acer saccharum* Marsh., Kriebel 1957; *Betula alleghaniensis*, Clausen and  
581 Garrett 1969; *P. balsamifera* and *P. tremula* L., Soolanayakanahally et al. 2013, 2015; *P.*  
582 *trichocarpa*, McKown et al. 2014a), but higher spring temperature can shorten the duration for  
583 bud flush. So, under common garden environments, trees from low latitudes often display later  
584 bud flush due to higher chilling and heat unit needs than the trees from high latitudes (Hannerz et  
585 al. 2003). Weih (2009) study emphasis spring leaf emergence and leaf abscission events' being  
586 critical for biomass accumulation by *Salix* species and it is important to determine the impacts of  
587 future spring temperature change on the timing of bud flush at a given latitude.

588

589 While *Populus* has been a focus of extensive works in understanding the molecular mechanisms  
590 of autumn phenology primarily cued by photoperiod (Ingvarsson et al. 2006, Keller et al. 2011)  
591 such an understanding is lacking for *Salix* (Hallingbäck et al. 2015). The seasonal variation in  
592 photoperiod is consistent from year-to-year and is a reliable cue for onset of bud set, leaf  
593 senescence and induction of dormancy than temperature which is far less predictable and shows  
594 seasonal fluctuations (Barr et al. 2004). Our observed latitudinal cline in the onset of senescence  
595 and leaf drop is consistent with our *a priori* expectations. As willow plants attain competency to  
596 respond to photoperiod by mid-summer, they would have to wait for the critical daylength to  
597 induce autumn phenological events (Soolanayakanahally et al. 2013), with high latitude  
598 genotypes ceasing growth under longer critical daylength than the trees from low latitude  
599 (Pauley and Perry 1954, Howe et al. 1995).

600

601 At high latitudes, greater susceptibility to insect and disease is largely explained by evolution of  
602 plant defenses which display latitudinal clines (Anstett et al. 2015). As observed in this study,  
603 geographic regions that experience low *Melampsora* rust occurrence, natural resistance could be  
604 negatively selected in the absence of biotic stressors. In addition, larger stomatal pore length  
605 among high-latitude *S. eriocephala* genotypes might provide greater surface area for  
606 *Melampsora* rust spores to penetrate and colonise a given leaf surface area compared to low-  
607 latitude genotypes. Our results find support for the hypothesis “carbon gain and disease  
608 resistance trade-offs” by McKown et al. (2014b), the notion that fast growth might have negative  
609 fitness with disease resistance. In addition, an inherent resistance to *Melampsora* rust is  
610 metabolically costly with substantial increase in certain classes of metabolites, particularly,  
611 tannins.

612

#### 613 **4.4 Adaptive phenotypic trait convergence within Salicaceae**

614 In the Northern Hemisphere the members of *Salicaceae* (*P. balsamifera* and *S. eriocephala*) are  
615 sympatric species with overlapping natural ranges across north temperate-boreal climates (Hosie  
616 RC 1979, Dorn 1970). Both are restricted to moist and nutrient rich sites, exhibit indeterminate  
617 growth, and have a much wider north-south range. In general, parallel evolutionary selection  
618 pressures produce functionally convergent phenotypic traits in related taxa. Broadly, we  
619 hypothesised that the patterns in form and function convergence on a similar adaptive phenotype  
620 among *Populus* and *Salix* species in spite their long divergence (~65 million years).

621

622 In a greenhouse study, without any resource limitations *P. balsamifera* populations displayed  
623 latitudinal gradients in photosynthesis ( $A$ ), whereby higher  $A$  was ably supported by enhanced  $g_m$   
624 and leaf N at high latitudes (Soolanayakanahally et al. 2009). Similar mechanism in *S.*  
625 *eriocephala* was observed in an outdoor common garden, whereby higher  $A$  in genotypes from  
626 high latitudes is partly mediated by higher  $g_m$  and  $g_s$  (mediated by larger stomatal pore length  
627 rather than higher density). These two studies collectively demonstrate a very strong correlation  
628 between  $A$  and latitude of origins and thus suggest that the possibility of a convergent adaptive  
629 phenotypic trait selection to compensate for short growing seasons. In addition, both species  
630 were hypostomatous with stomatal density displaying a strong negative association with latitude  
631 of origin. Unlike greenhouse grown *P. balsamifera*,  $g_s$  was positively correlated with latitude of  
632 origin in *S. eriocephala*. In addition, Leaf N, LMA and CCI were positively associated with

633 each other contributing to greater  $A$  at high latitudes in both species. Four independent common  
634 garden studies along a latitudinal gradient by the same group (Soolanayakanahally et al. 2009,  
635 2015, McKown et al. 2014a, present study) and an additional study by Kaluthota et al. (2015)  
636 showed that genetic divergence in *Salicaceae* members largely explains the variation observed in  
637 functionally important leaf traits –  $A$ , LMA, Leaf N.

638

639 Further, both species display a strong latitudinal cline in autumn phenology (leaf senescence,  
640 green cover period, leaf drop) resulting from photoperiodic adaptation (Soolanayakanahally et al.  
641 2013). Overall, our findings in *S. eriocephala* and *P. balsamifera* lend support to the hypothesis  
642 that natural selective pressures enacted along similar environmental gradients led to phenotypic  
643 trait convergence in sympatric *Salicaceae* members.

644

## 645 **5. CONCLUSION**

646 Our common garden results speak to the paramount role of adaptive trade-offs along latitudinal  
647 gradients, suggesting that certain trait combinations have been favoured by natural selection,  
648 leading to a locally adapted phenotype. First, we found multiple evidences for an enhanced  
649 photosynthetic assimilation rate ( $A$ ) at high latitudes ably supported by stomatal traits (increased  
650  $g_s$ , and larger stomatal pore length) and a greater  $g_m$  which all coevolved along geo-climatic  
651 gradients. In addition, higher  $A$  at high latitudes results from greater LMA with higher leaf N  
652 concentrations. Taken together, our results highlight latitudinal clines in  $A$  as an adaptation to  
653 growing season length. Second, we observed least variations in water use-efficiency as  
654 determined by  $\Delta^{13}\text{C}$  values among genotypes from different latitudes with varying precipitation  
655 patterns. Observed latitudinal cline in  $\delta^{15}\text{N}$  values suggests that  $\text{NO}_3$  nitrogen is partially  
656 assimilated in the roots leading to enrichment of stem wood tissue. Last, a strong photoperiodic  
657 adaption was observed in autumn phenology traits accounting for high heritability that could be  
658 exploited in willow improvement program for biomass and environmental applications. Overall,  
659 an adaptive negative relationship between stomatal density and pore length is optimised to  
660 achieve maximum leaf diffusive conductance to  $\text{CO}_2$  within the physiological framework of  
661 carbon/water balance across a range of a latitudes and climates.

662

663 **AUTHOR CONTRIBUTION**

664 A.S.K.S. participated in data analysis and interpretation and drafted the manuscript. R.Y.S.  
665 conceived the study, performed gas exchange measurements, participated in analysis and  
666 interpretation, and edited the manuscript. R.D.G. interpreted the stable isotope results and  
667 complemented the writing. The authors declare that the research was conducted in the absence of  
668 any commercial or financial relationships that could be construed as a potential conflict of  
669 interest.

670

671

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957 **Table 1.** Geo-climatic information of 34 native *Salix eriocephala* populations used in study. The  
 958 three letters within the brackets correspond to population code.

<b>Population</b>	<b>LAT</b>	<b>LON</b>	<b>ELV</b>	<b>FFD</b>	<b>MAP</b>	<b>MST</b>	<b>DD&gt;5</b>
La Ronge (LAR)	55.00	105.21	390	151	484	11.2	1323
Grand Rapids (GRR)	53.51	99.14	280	167	474	12	1508
Christopher Lake (CLK)	53.30	105.45	531	154	424	12.1	1467
Camrose (CAM)	52.55	112.51	689	165	438	13.6	1431
Easterville (EAS)	52.55	99.10	282	158	443	11.5	1407
Wakaw (WAK)	52.41	105.43	528	161	388	12.3	1517
Stettler (STL)	52.17	113.15	800	159	478	13.6	1446
St. Martin Junction (SMJ)	51.43	98.50	251	159	500	12.6	1588
Watrous (WAT)	51.35	105.27	550	163	435	13	1628
Drumheller (DRU)	51.28	112.43	683	160	338	13.9	1533
Ashern (ASH)	51.07	98.22	264	159	500	12.6	1588
Bow River (BOW)	50.47	112.38	791	169	515	13.6	1490
Indian Head (IHD)	50.30	103.40	609	164	447	13.3	1671
Moose Jaw (MJW)	50.22	105.49	669	176	365	14.1	1845
Portage La Prairie (POR)	49.59	98.16	256	167	515	13.7	1784
Kipling (KIP)	49.55	102.37	751	175	396	15.5	1848
Kenora (KEN)	49.48	94.30	344	182	662	13.6	1759
Taber (TAB)	49.48	112.10	754	170	316	14.9	1717
Fauquier (FAQ)	49.18	82.02	224	151	832	11.2	1370
Deer Lake (DER)	49.10	57.37	12	180	1127	10.9	1298
Morden (MDN)	49.00	98.08	431	183	533	14.6	1955
Fort Frances (FTF)	48.38	93.19	345	180	710	14	1821
Stephenville (STE)	48.33	58.40	8	204	1352	11	1324
Thunder Bay (TBY)	48.31	88.43	231	162	712	11.9	1434
Codroy Valley (COD)	47.51	59.18	4	200	1709	9.4	1023
NB North (NBN)	47.11	67.56	153	181	1134	12.6	1577
Quebec (QUE)	46.98	70.71	15	184	1141	15.4	1707
Batchawana Bay (BAT)	46.54	84.35	169	195	1011	13.4	1718
Field, Sturgeon river (STU)	46.31	80.01	231	183	899	13.3	1705
Prince Edward Island (PEI)	46.26	62.01	21	204	1078	13.1	1722
NB Central (NBC)	46.13	67.42	116	178	1114	13.7	1768
NS Eastern (NSE)	45.52	61.15	24	197	1505	12	1541
NB South (NBS)	45.32	66.03	8	210	1154	14.2	1891
NS West (NSW)	45.21	63.16	21	193	1202	13	1684

959 LAT, latitude (°N); LON, longitude (°W); ELV, elevation (m); FFD, frost-free days (days);  
 960 MAP, mean annual precipitation (mm); MST, mean summer temperature (°C); DD>5, degree  
 961 days above 5°C (°C).

**Table 2.** Mean values for ecophysiology, phenology and biomass traits measured in 338 genotypes of *Salix eriocephala*.

Trait	Mean value ( $\pm$ SD)	Range
<b>Ecophysiology</b>		
<i>A</i>	17.87 $\pm$ 2.74	9.13 – 23.7
<i>g<sub>s</sub></i>	0.48 $\pm$ 0.15	0.07 – 0.9
<i>E</i>	4.59 $\pm$ 1.2	1.13 – 7.59
<i>WUE<sub>i</sub></i>	40.12 $\pm$ 13	19.50 – 128.71
PNUE	15.4 $\pm$ 3.61	7.59 – 33.2
<i>Ci/Ca</i>	0.79 $\pm$ 0.05	0.44 – 0.89
LMA	4.27 $\pm$ 0.64	2.71 – 6.4
SD	91 $\pm$ 26	31 – 174
CCI	12.57 $\pm$ 2.77	6.53 – 22.27
Leaf N	120.16 $\pm$ 26.09	62.93 – 205.13
Leaf C:N	16.19 $\pm$ 2.69	10.26 – 28.98
Wood N	95.06 $\pm$ 19.32	56.77 – 152.82
Wood C:N	7.13 $\pm$ 3.65	0.65 – 19.39
$\Delta^{13}\text{C}_{\text{leaf}}$	20.65 $\pm$ 1.1	16.86 – 23.18
$\Delta^{13}\text{C}_{\text{wood}}$	19.84 $\pm$ 0.76	17.44 – 22.16
$\delta^{15}\text{N}_{\text{leaf}}$	9.85 $\pm$ 2.99	5.67 – 21.21
$\delta^{15}\text{N}_{\text{wood}}$	7.09 $\pm$ 2.57	3.42 – 14.79
<b>Phenology</b>		
Bud flush	140 $\pm$ 1	136 – 143
Leaf emergence	143 $\pm$ 2	140 – 146
Leaf senescence	274 $\pm$ 10	216 – 294
GCD	144 $\pm$ 16	98 – 166
Leaf drop	284 $\pm$ 16	238 – 305
Rust	1 $\pm$ 1	0 – 3
<b>Biomass</b>		
Height gain	215.4 $\pm$ 43.99	78 – 316
Biomass	1.04 $\pm$ 0.56	0.03 – 3.62

965 *A*, photosynthetic assimilation rate ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ); *g<sub>s</sub>*, stomatal conductance ( $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ); *E*, transpiration rate ( $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ); *WUE<sub>i</sub>*, intrinsic water-use efficiency ( $\mu\text{mol CO}_2$   
966  $\text{mmol}^{-1} \text{ H}_2\text{O}$ ); PNUE, photosynthetic nitrogen-use efficiency ( $\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ N s}^{-1}$ ); *C<sub>i</sub>/C<sub>a</sub>*, ratio  
967 of intercellular CO<sub>2</sub> to external CO<sub>2</sub> concentrations; LMA, leaf mass per unit area ( $\text{mg cm}^{-2}$ ); SD,  
968 stomatal density ( $\text{mm}^{-2}$ ); CCI, chlorophyll content index; leaf N, nitrogen content per unit area  
969 ( $\mu\text{mol N cm}^{-2}$ ); C:N, carbon to nitrogen ratio;  $\Delta^{13}\text{C}$ , net carbon discrimination (‰);  $\delta^{15}\text{N}$ , stable  
970 nitrogen isotope ratio (‰); GCD, green cover duration (days); leaf drop (Julian day, DOY);  
971 height gain (cm); biomass (kg).  
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1007 **Table 3.** Pearson correlations coefficients (*r*) between geography, climate and physiological  
 1008 variables for all 338 genotypes. Bold are significant after Bonferroni correction  
 1009 ( $P < 0.001$ ).  
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Trait variables	LAT	LON	ELV	FFD	MAP	MST	DD>5
<i>A</i>	<b>0.57</b>	<b>0.7</b>	<b>0.66</b>	<b>-0.51</b>	<b>-0.65</b>	<b>0.23</b>	0.04
<i>g<sub>s</sub></i>	<b>0.34</b>	<b>0.54</b>	<b>0.53</b>	<b>-0.34</b>	<b>-0.45</b>	<b>0.24</b>	0.03
<i>E</i>	0.19	<b>0.49</b>	<b>0.57</b>	<b>-0.26</b>	<b>-0.36</b>	<b>0.37</b>	0.12
<i>WUE<sub>i</sub></i>	-0.18	<b>-0.36</b>	<b>-0.34</b>	<b>0.24</b>	<b>0.27</b>	-0.18	-0.03
PNUE	0.04	0.16	0.21	-0.06	-0.11	0.17	0.01
<i>C<sub>i</sub>/C<sub>a</sub></i>	0.14	<b>0.31</b>	<b>0.28</b>	-0.2	<b>-0.22</b>	0.16	0.03
LMA	<b>0.26</b>	<b>0.24</b>	0.13	-0.2	<b>-0.27</b>	-0.04	0.12
SD	<b>-0.44</b>	<b>-0.44</b>	<b>-0.32</b>	<b>0.35</b>	<b>0.42</b>	0	0.02
CCI	<b>0.35</b>	<b>0.29</b>	<b>0.32</b>	<b>-0.22</b>	<b>-0.28</b>	-0.07	-0.15
Leaf N	<b>0.39</b>	<b>0.34</b>	<b>0.25</b>	<b>-0.32</b>	<b>-0.37</b>	-0.01	0.03
Leaf C:N	<b>-0.22</b>	-0.15	-0.11	0.19	0.17	0.06	0.09
Wood N	0.13	0.09	-0.01	-0.12	-0.08	0.06	0.02
Wood C:N	0.03	0.16	<b>0.27</b>	-0.04	-0.08	<b>0.24</b>	-0.08
$\Delta^{13}\text{C}_{\text{leaf}}$	0.05	0.1	0.07	-0.06	-0.08	0.03	0.03
$\Delta^{13}\text{C}_{\text{wood}}$	0.06	0.15	0.03	-0.08	-0.17	0.11	0.16
$\delta^{15}\text{N}_{\text{leaf}}$	<b>0.39</b>	<b>0.43</b>	<b>0.35</b>	<b>-0.35</b>	<b>-0.39</b>	0.09	0.08
$\delta^{15}\text{N}_{\text{wood}}$	<b>0.36</b>	<b>0.35</b>	<b>0.24</b>	<b>-0.31</b>	<b>-0.33</b>	-0.01	0.08
Bud flush	-0.02	0.01	0.08	-0.03	-0.04	0.1	0.02
Leaf emergence	0.04	0.12	0.18	-0.09	-0.13	0.18	0.08
Leaf senescence	<b>-0.57</b>	<b>-0.68</b>	<b>-0.61</b>	<b>0.52</b>	<b>0.64</b>	-0.11	-0.05
GCD	<b>-0.74</b>	<b>-0.82</b>	<b>-0.77</b>	<b>0.68</b>	<b>0.79</b>	-0.18	-0.01
Leaf drop	<b>-0.74</b>	<b>-0.83</b>	<b>-0.77</b>	<b>0.69</b>	<b>0.79</b>	-0.18	-0.01
Rust	<b>0.62</b>	<b>0.79</b>	<b>0.83</b>	<b>-0.56</b>	<b>-0.77</b>	<b>0.31</b>	0.1
Height gain	<b>0.25</b>	<b>0.24</b>	0.04	<b>-0.23</b>	<b>-0.31</b>	-0.1	0.14
Biomass	0.18	<b>0.31</b>	<b>0.34</b>	<b>-0.21</b>	<b>-0.28</b>	0.17	0.04

1011 LAT, latitude (°N); LON, longitude (°W); ELV, elevation (m); FFD, frost-free days (days);  
 1012 MAP, mean annual precipitation (mm); MST, mean summer temperature (°C); DD>5, degree  
 1013 days above 5°C (°C); *A*, photosynthetic assimilation rate ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ); *g<sub>s</sub>*, stomatal  
 1014 conductance ( $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ); *E*, transpiration rate ( $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ); *WUE<sub>i</sub>*, intrinsic water-  
 1015 use efficiency ( $\mu\text{mol CO}_2 \text{ mmol}^{-1} \text{ H}_2\text{O}$ ); PNUE, photosynthetic nitrogen-use efficiency ( $\mu\text{mol}$

1016  $\text{CO}_2 \text{ mol}^{-1} \text{ N s}^{-1}$ );  $C_i/C_a$ , ratio of intercellular  $\text{CO}_2$  to external  $\text{CO}_2$  concentrations; LMA, leaf  
1017 mass per unit area ( $\text{mg cm}^{-2}$ ); SD, stomatal density ( $\text{mm}^{-2}$ ); CCI, chlorophyll content index; leaf  
1018 N, nitrogen content per unit area ( $\mu\text{mol N cm}^{-2}$ ); C:N, carbon to nitrogen ratio;  $\Delta^{13}\text{C}$ , net carbon  
1019 discrimination (‰);  $\delta^{15}\text{N}$ , stable nitrogen isotope ratio (‰); GCD, green cover duration (days);  
1020 leaf drop (Julian day, DOY); height gain (cm); biomass (kg).

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1060 **Table 4.** Broad-sense heritability ( $H^2$ ) estimates of phenology and biomass traits in 2015.  
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<b>Traits</b>	<b>Heritability (<math>H^2</math>)</b>
Bud flush	0.72
Leaf emergence	0.77
Leaf senescence	0.62
Leaf drop	0.78
Height	0.95
Biomass	0.88

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1079 Figure legends

1080 **Figure 1A.** Geographical distribution of 34 native populations (blue circle) of *Salix eriocephala*  
1081 from their natural ranges across eastern and western Canada. The green color depicts species  
1082 dominant continuous range while the yellow shaded area is species discontinues range. The  
1083 common garden was established at Indian Head, Saskatchewan, Canada.

1084 **Figure 1B.** Mean temperature (red line) and precipitation (blue line) that prevailed at Indian  
1085 Head common garden during 2013 and 2014. The green band represents the spring bud flush  
1086 period, whereas, the yellow band represents leaf senescence duration among *Salix eriocephala*  
1087 populations.

1088 **Figure 2.** Seasonal phenological timetable of *Salix eriocephala*. Plants become dormant in  
1089 autumn (fall) and remain so until chilling requirements are during winter (white band); during  
1090 spring, heat sum accumulation leads to bud flush and leaf emergence (red band); free growth and  
1091 carbon gain occur after leaf emergence (green band); growth cessation and leaf senescence  
1092 during late summer and early autumn (yellow band).

1093 **Figure 3.** Heat map of Pearson's correlation coefficient for phenotypic traits among 338  
1094 populations of *Salix eriocephala*. The scale bar beneath the heat map denotes the direction/  
1095 magnitude of correlation between the traits, 1 indicated by dark blue being positive and -1  
1096 indicated by dark red being negative.

1097 **Figure 4. A.** Carbon isotope discrimination as determined on leaves ( $\Delta^{13}\text{C}_{\text{leaf}}$ ) ( $\Delta$ ) and wood  
1098 ( $\Delta^{13}\text{C}_{\text{wood}}$ ) ( $\square$ ) and **B.** Nitrogen isotope composition of leaves ( $\delta^{15}\text{N}_{\text{leaf}}$ ) ( $\Delta$ ) and wood ( $\delta^{15}\text{N}_{\text{wood}}$ )  
1099 ( $\square$ ) of *Salix eriocephala* populations plotted against their latitude of origin.

1100 **Figure 5.** The relationship between stomatal dimensions and maximum leaf conductances of  
1101 selected eastern and western populations of *Salix eriocephala*. **A.** Mean stomatal pore length  
1102 (SL,  $\mu\text{m}$ , images above the boxes represent stomatal pore length from respective latitudes viewed  
1103 under 100 $\times$  magnification using phase contrast microscopy), **B.** mean stomatal density (SD), **C.**  
1104 stomatal conductance to carbon dioxide and water vapor ( $g_s$ ,  $\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$ ) and **D.** maximum  
1105 diffusive conductance to carbon dioxide ( $g_{c(\text{max})}$ ,  $\text{mol m}^{-2} \text{s}^{-1}$ ) as determined by stomatal  
1106 dimensions of eastern (NBS, NSW, PEI and QUE) and western (DRU, IHD, KEN and MDN)  
1107 populations.

1108 **Figure 6.** Gas exchange traits of selected eastern and western populations of *Salix eriocephala*.  
1109 **A.** Internal conductance ( $g_m$ ,  $\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$ ), **B.** Maximum carboxylation rate allowed by  
1110 rubisco ( $V_{c\text{max}}$ ,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), **C.** rate of photosynthetic electron transport based on NADPH  
1111 requirement ( $J$ ,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and **D.** Triose phosphate use (TPU,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) of eastern (NBN  
1112 and NBS) and western (CLK, MJW and WAK) populations estimated using Sharkey's  $A-C_i$   
1113 curve fitting model.  $J$  was significantly different ( $P < 0.05$ ) between the eastern and western  
1114 populations.

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1116 **Figure 7.** *Melampsora* rust incidence score of *Salix eriocephala* populations plotted against their  
1117 latitude of origin. The images indicate the rust incidence scoring key, 0 for no rust and 1, 2 and 3  
1118 for minimum, moderate and maximum rust symptoms in the leaves, respectively.

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Fig. 1A

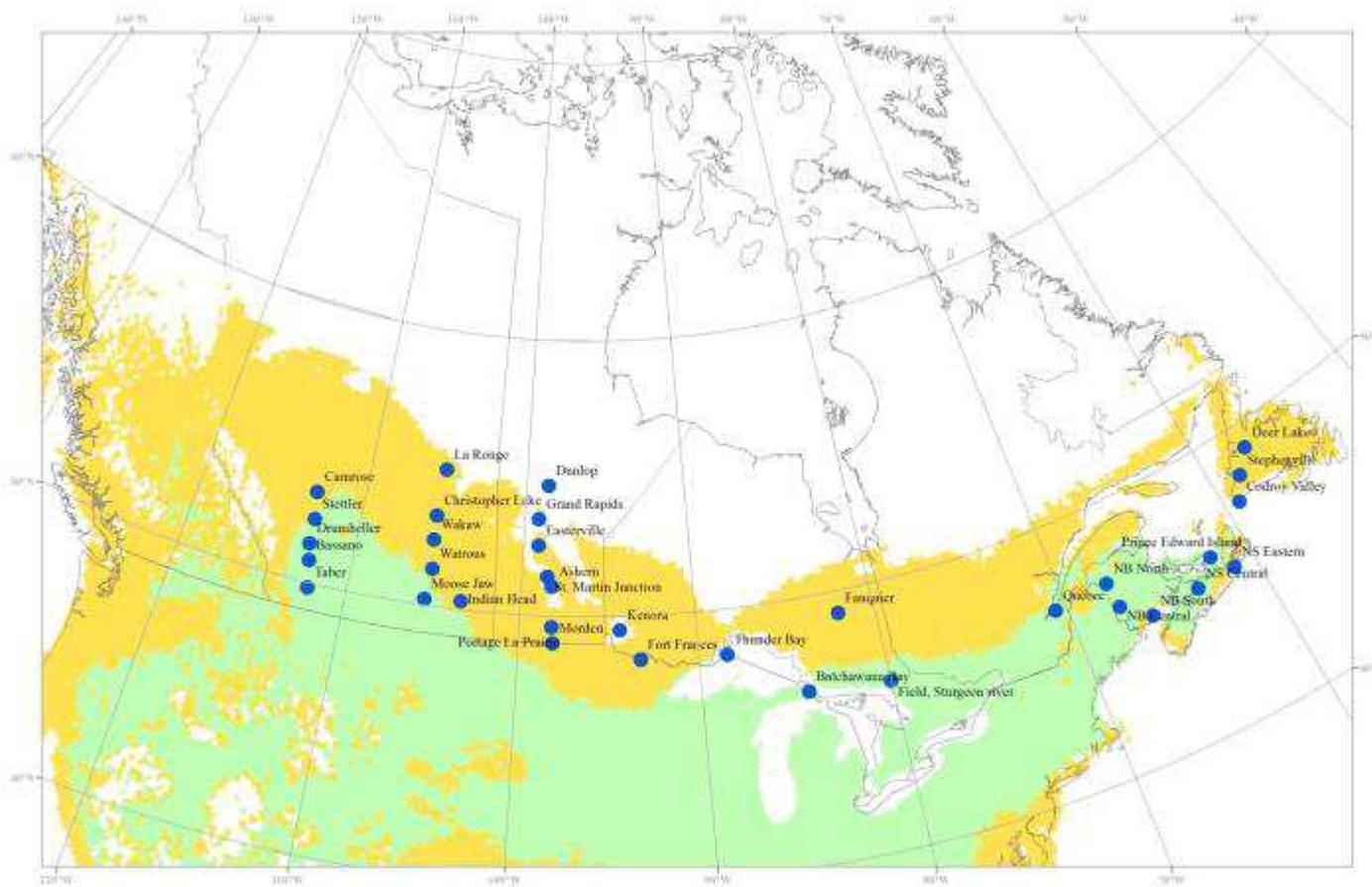


Fig. 1B

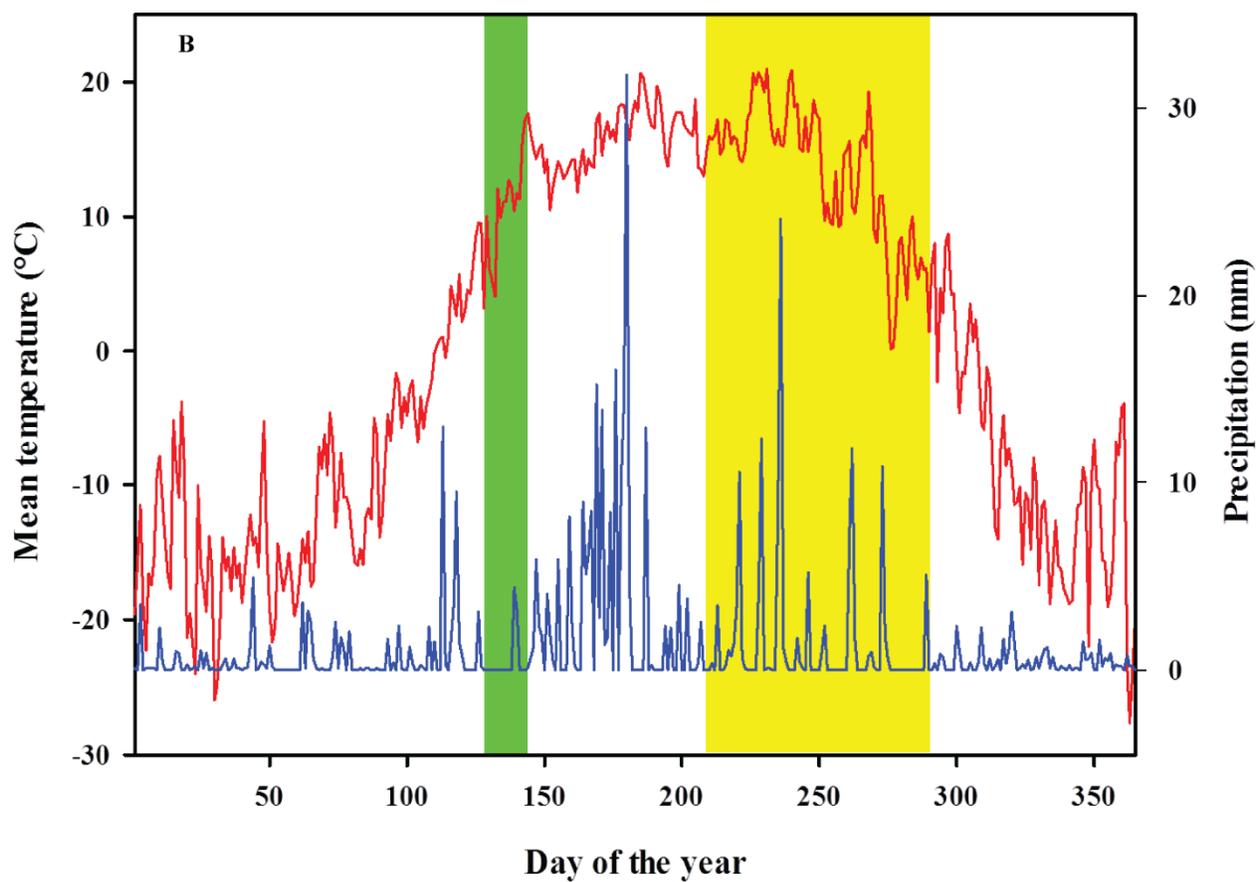


Fig. 2

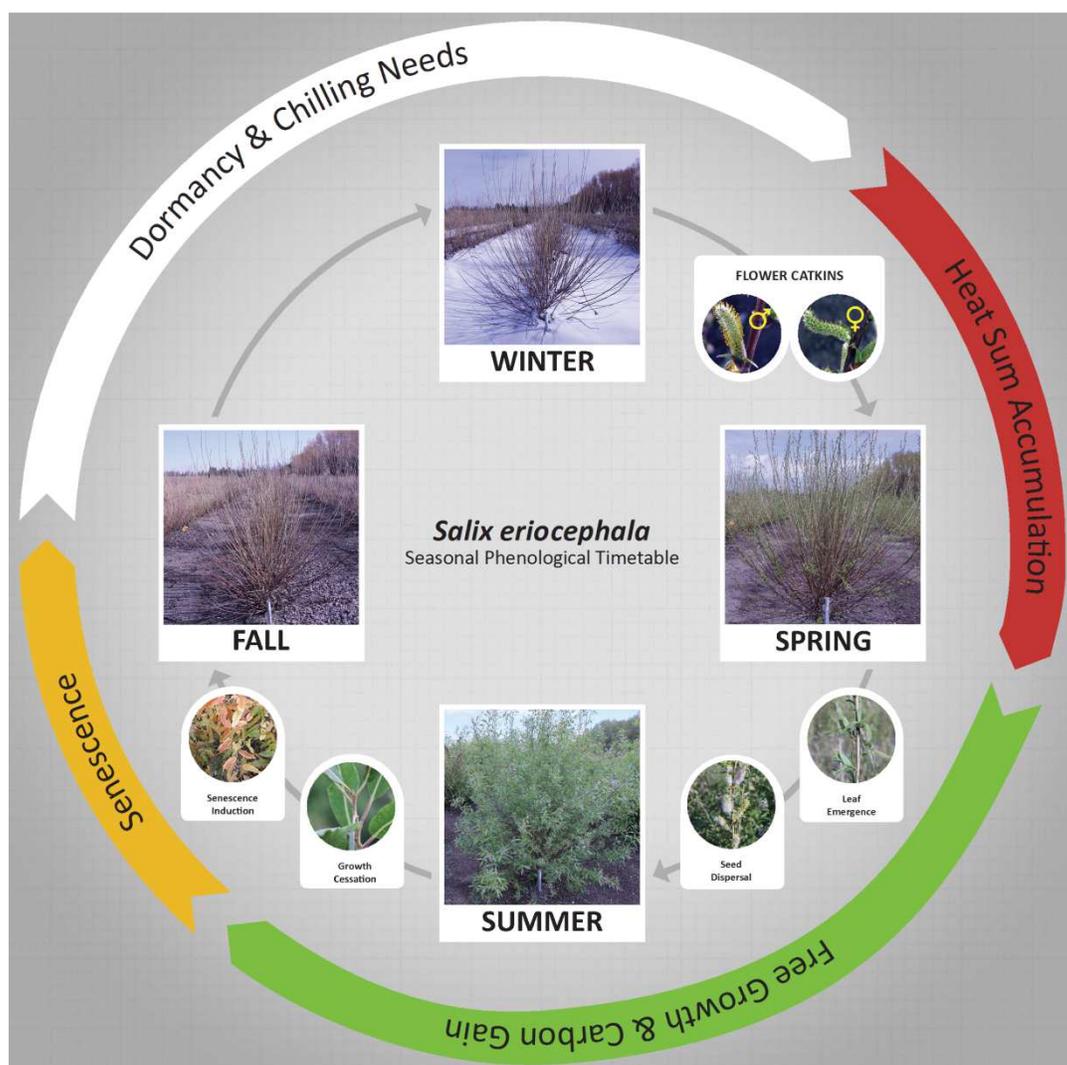


Fig. 3

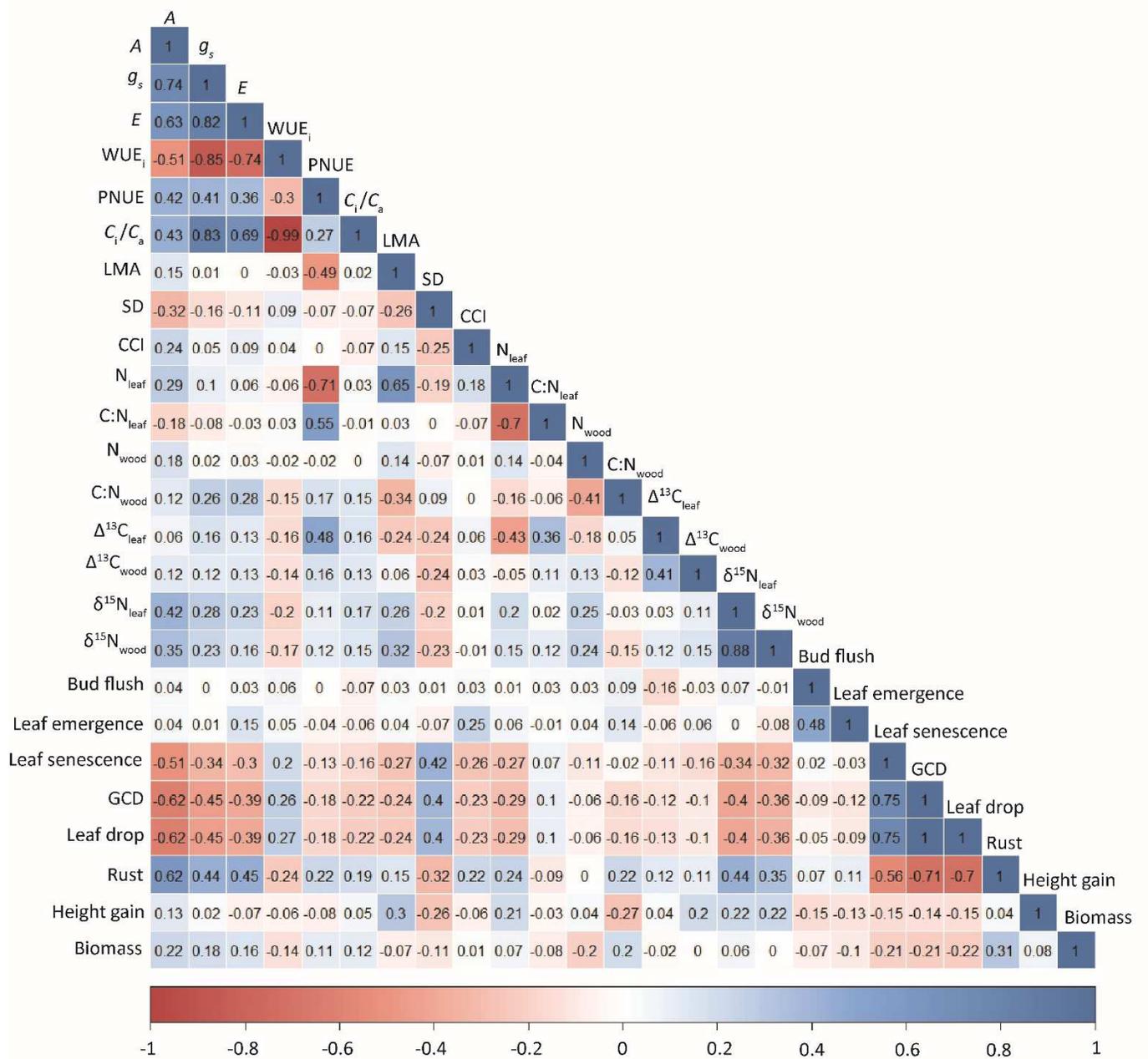


Fig. 4

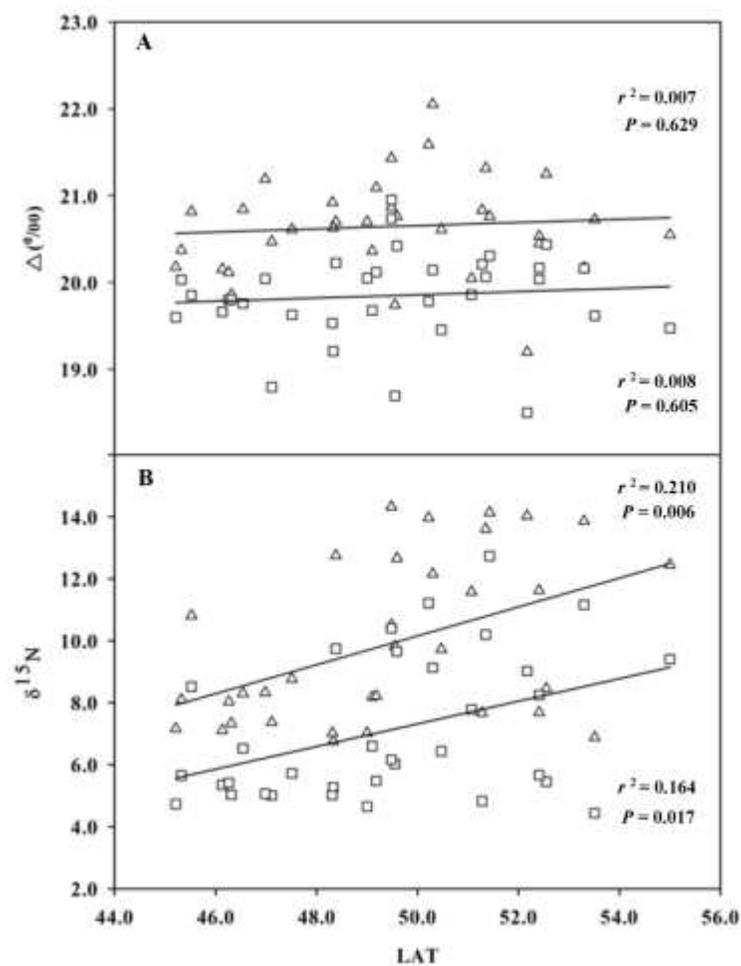


Fig. 5

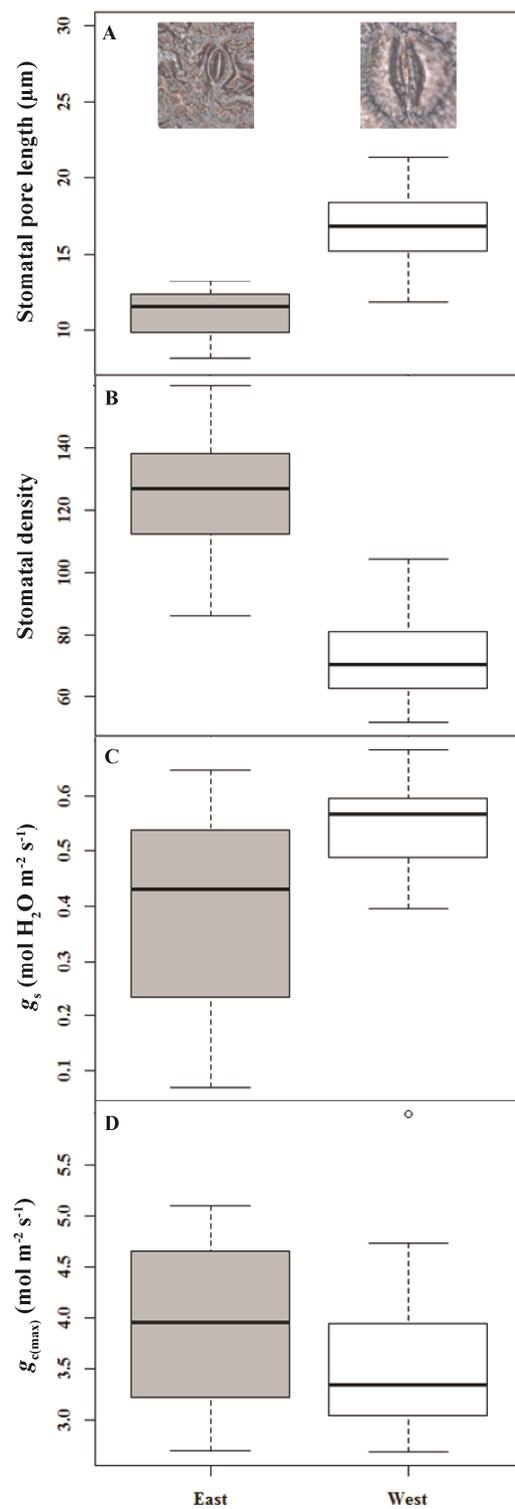


Fig. 6

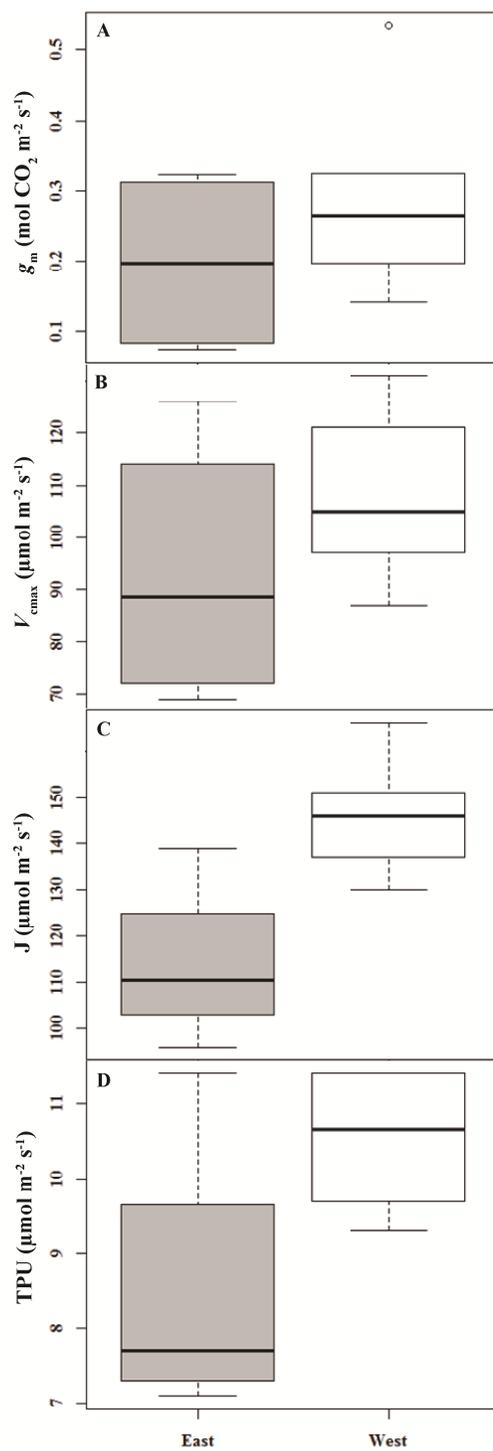
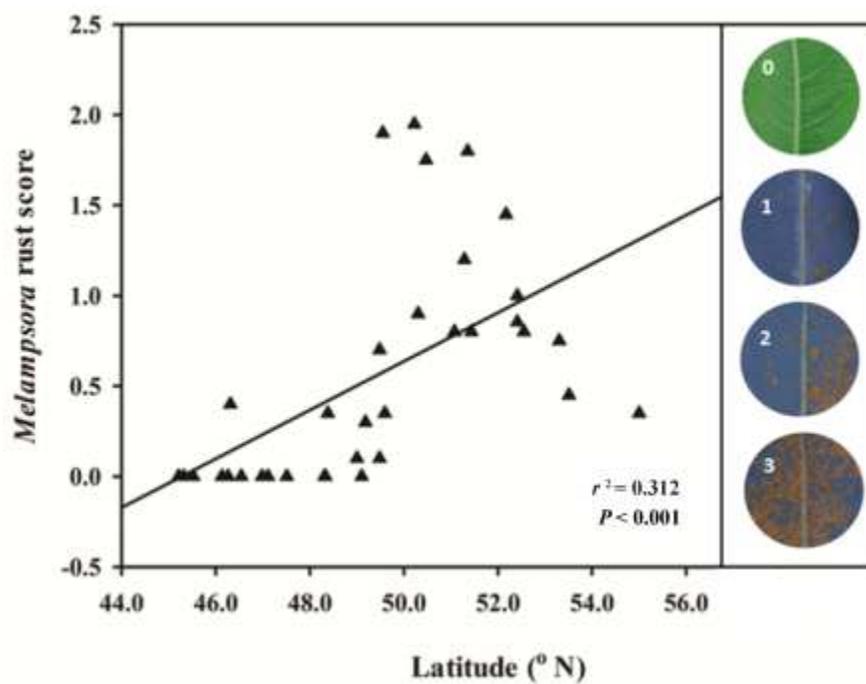


Fig. 7



### Supplementary Table 1 (ST1)

Soil analysis results from 15cm depth at Indian Head common garden, Saskatchewan in 2012. Values shown are means  $\pm$  SD of nine individual samples analyzed from sandy clay loam texture soil.

<b>Soil nutrient composition</b>	<b>Mean <math>\pm</math> SD</b>
pH	7.9 $\pm$ 0.28
Organic matter (%)	2.2 $\pm$ 0.39
Salts (mmhos/cm)	0.3 $\pm$ 0.104
Nitrate nitrogen (N ppm)	13.6 $\pm$ 2.41
Ammonium nitrogen (N ppm)	1.5 $\pm$ 0.26
Olsen phosphorus (P ppm)	19.7 $\pm$ 6.92
Potassium (K ppm)	242.9 $\pm$ 56.55
Calcium (Ca ppm)	3497.4 $\pm$ 950.44
Magnesium (Mg ppm)	534.3 $\pm$ 87.02
Sodium (Na ppm)	20.3 $\pm$ 2.82
Chlorine (Cl ppm)	3.1 $\pm$ 0.84
Cation Exchange Capacity (CEC meq)	22.7 $\pm$ 4.68
Sand (%)	58.3 $\pm$ 6.92
Silt (%)	20.8 $\pm$ 4.05
Clay (%)	20.9 $\pm$ 3.17

**Supplementary Figure 1 (SF1):** Pictorial description of site preparation, planting, management and trait measurements in the common garden at Indian Head research station, Canada.



**SF1 A.** Common garden site preparation prior to willow planting.



**SF1 B.** Nutrient amendments to sandy clay loam soils along planting strips.



**SF1 C.** Laying of plastic mulch to avoid intra row weed competition.



**SF1 D.** Dormant root plugs ready for planting at one metre intervals during the spring of 2012.



**SF1 E.** Uniform establishment of willow common garden during the summer of 2012.



**SF1 F.** Willow growth during the summer of 2014.



**SF1 G.** Gas exchange measurement during active growth in the summer of 2014.