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Bolting and Flowering Response of *Lactuca georgica*, a Wild Lettuce Relative, to Low Temperatures

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Abstract

To learn about the phenological adaptation of *Lactuca georgica* Grossh., a wild relative of domesticated lettuce, we studied seed sampled accessions obtained from individual plants at 19 locations throughout six regions in Armenia, and from two natural populations in Dagestan (Russian Federation) collected as bulk samples. The effects of various vernalization treatments on time to bolting, flowering and seed production time were investigated during four successive years at different growth stages of *L. georgica* plants. We demonstrate that low temperatures play a major role in stimulating the reproduction process of *L. georgica* plants. Our results would suggest that for *L. georgica*: 1) There is an obligatory (or nearly so) vernalization requirement; 2) Plant age, vernalization duration, and genotype of original sample have a role in bolting and flowering regulation; 3) Some plants behaved as typical annuals, responding to vernalization treatment at the seedling stage, but, most did not; 4) Four months of vernalization could be adequate to reach bolting in plants with a developed vegetative rosette, for most—but not all—samples; 5) In order to find the best solution for stimulating the reproductive process of multiple genotypes, it seems that further study should focus on about 4 - 6 months of vernalization at 4˚C applied to plants of about 10 - 22 months old vegetative rosettes, with controlled post-vernalization condition; 6) *L. georgica* germplasm could be used as a source for delayed bolting in breeding of domesticated lettuce varieties.

Keywords

Gene Pools, *Lactuca sativa*, Phenological Adaptation, Plant Age, Vernalization Duration, Wild Lettuce

*AB and BH contributed equally to this publication.

1. Introduction

The genus *Lactuca* L. [Compositae (Asteraceae), tribe Cichorieae, subclade Lactucinae [1] is comprised of 100 [1] to 148 species [2], which are mainly distributed in the Northern Hemisphere ([3], and literature cited therein). The domesticated species in the genus, *Lactuca sativa* L. (lettuce), is one of the most important and widely distributed leafy vegetables around the world. Domestication has resulted in limited genetic variation in the crop making it vulnerable to diseases, pests, and environmental stresses.

In recent years, we have performed extensive studies on the characterization of wild *Lactuca* spp. originating from Southwest Asia, the center of diversity for wild species closely related to domesticated lettuce (Wild *Lactuca* Relatives, WLRs) [4]. Unique new collections of *L. serriola*, *L. aculeata* Boiss., *L. georgica* Grossh., and *L. altaica* Fisch. & C. A. Mey. (four of the seven wild species, according to previous literature [4] [5], in the primary lettuce gene pool, LGP-1), and *L. saligna* (in LPG-2) from Israel and Armenia were studied, as well as a few samples previously collected from Jordan, Turkey and other Mediterranean and European countries. The objectives of our research are related to the identification, collection, characterization, conservation, and sustainable use of these rich genetic sources for lettuce improvement. These studies included eco-geographical distribution [6] [7] [8], genetic, morphological and phenological diversity [9] [10] [11] [12] [13], downy mildew resistance [10] [14] [15] [16] [17] and variation of biologically active sesquiterpene lactone contents [18] [19] [20] [21]. Obtained results strongly support the use of these species specifically and WLRs in general as rich genetic sources for lettuce improvement.

One of the aforementioned species, *L. georgica*, is a diploid ($2n = 2x = 18$) [4] [22], 50 - 300 cm tall, biennial plant according to Gabrielian and Fragman-Sapir ([23], p. 80). Blooming in nature occurs in July-August [23]. *L. georgica*'s distribution is restricted to the Euxinian-Hyrcanian region of southwest Asia (Caucasia, Northeast Anatolia, and North Iran) ([4], and literature cited therein) and in the past, was observed at altitudes ranging from 1500 to 2300 m above sea level [23].

Prior to 2009, in world gene bank collections of wild *Lactuca* spp., *L. georgica* was represented by only a single sample [24] [25], "LAC 327". Consequently, *L. georgica* was not studied by lettuce breeders and crop evolutionists [3]. Thus, we strove to increase the number of available *L. georgica* samples for comparative genetic and physiological studies.

Plants originating from a seed sample of the *L. georgica* "LAC 327" were grown in 2009 and morphologically characterized alongside of wild *Lactuca* spp. samples representing (according to Lebeda *et al.* [5]) the LGP-1 (*L. aculeata*, *L. serriola*, *L. dregeana*, and *L. altaica*), LGP-2 (*L. saligna*), and the section Mulgedium (*Lactuca tatarica* (L.) C. A. Mey.). Field regeneration was conducted at the Institute of Evolution (IOE), Haifa University (HU), Israel and followed all standard seed multiplication protocols, except that seeds were not vernalized. All plants of the *L. georgica* sample did not bolt, even after all plants from the other

species bolted, flowered, and had ripe seeds (data not published). Thus, we hypothesize that plants of *L. georgica* require a cold season for bolting and floral initiation.

Bolting resistance is an important breeding aim of vegetative crops [26]. Therefore, information on the phenology of *L. georgica*, and, in particular, on the environmentally mediated response, is of great importance. It is also important to learn if all *L. georgica* germplasm is indeed biennial, which is a commonly-accepted "fact" that has been cited for over one hundred years in the literature ([23], p. 80), but without any experimental data to supported it. A biennial plant, by definition, completes its life-cycle during two years: in the first year, plants generally make only leaves and other vegetative structures, while in the second year they flower, produce seed and die. The change from vegetative to reproductive growth is a key developmental switch in flowering plants [26]. Environmental conditions such as temperature affect survival, growth, and fitness, particularly during key stages such as seedling growth and reproduction ([27], and literature cited therein). The timing of flowering in both wild and crop plants is a fundamental aspect of adaptation. The control of time to flowering has been studied in numerous species and is quite complex. Under normal circumstances, external cues such as low temperature (vernalization) and light (duration of exposure and intensity) are the prime factors that determine when plants will blossom [28]. The requirement for vernalization has been studied in many wild (and derived domesticated) species with both monocarpic habit (those that flower, set seed and die) and polycarpic species (perennials that can flower repeatedly over many years) showing a requirement for vernalization [29].

In several species, the response to vernalization varies with plant age. The requirement for vernalization usually increases or decreases linearly with respect to time. An increased requirement is found in species with a juvenile phase preceding the inductive phase. A decreased requirement is found in species with a quantitative response to vernalization, since, in these species, floral induction proceeds gradually with age even in the absence of a cold period [30]. In the present study, we examined the effect of low temperatures on the bolting and flowering time of germinating seeds and on different ages of the vegetative rosette of *L. georgica* plants. Experiments were performed during four successive growing seasons. From this, we hoped to gain innovative insights in the fields of phenological adaptation and germplasm exploitation of this WLR, aiming to improve our plant genetic resources (PGR) use efficiency for both basic research and potentially novel sources for breeding programs with domesticated lettuce.

2. Materials and Methods

2.1. Plant Material

A total of 121 wild *L. georgica* samples were used in this study (**Table 1**). The majority of original seed samples were collected from individual plants at 19 unique locations throughout six regions in Armenia (**Figure 1(A)** and **Figure 1(B)**). The remaining two original seed samples were collected as bulk samples

Table 1. List of samples (total 121) representing 19 and two natural locations of wild *Lactuca georgica* in Armenia and Russian Federation, respectively, used for vernalization treatments. (a) Four samples used in experiments 2010, 2011, 2012, 2012-2013-i, and 2012-2013-iv; (b) 120 samples used in experiment 2012-2013-ii; (c) 21 samples used in experiment 2012-2013-iii.

(a)

Pop. no. *	Sample no. *	Curator Institute	Collecting date	Country	Locality	Region	Ln	Lt	El
W6-37141	1	USDA	15/08/2009	Armenia	near Goris	Syunik	46˚19'36"E	39˚30'13"N	1642
W6-37155	1	USDA	17/08/2009	Armenia	Tashtun pass	Syunik	46˚10'25"E	39˚05'28"N	2160
W6-37160	10	USDA	18/08/2009	Armenia	near Shurnuh, Tass pass	Syunik	46˚23'30"E	39˚23'39"N	1555
W6-37168	3	USDA	20/08/2009	Armenia	between Ughedzor pass and Gndevaz	Vayots Dzor	45˚40'33"E	39˚43'11"N	2058

(b)

Pop. no. *	Sample no. *	Curator Institute	Collecting date	Country	Locality	Region	Ln	Lt	El
W6-37138	1, 2	USDA	15/08/2009	Armenia	between Ughedzor pass and Gorhayk	Syunik	45˚44'32"E	39˚40'23"N	2174
W6-37140	1, 2, 3, 4, 5, 6, 7, 8	USDA	15/08/2009	Armenia	vicinity of Gorhayk	Syunik	45˚46'58"E	39˚41'06"N	2085
W6-37141	1, 2, 4, 5, 6	USDA	**						
W6-37145	1, 2, 3, 4, 5, 6	USDA	16/08/2009	Armenia	near Srashen village	Syunik	46˚29'47"E	39˚04'50"N	990
W6-37148	1, 2, 3, 4, 5, 6	USDA	17/08/2009	Armenia	Pass between Tsav and Gyumarants	Syunik	46˚22'02"E	39˚01'06"N	2140
W6-37150	1, 2	USDA	17/08/2009	Armenia	near Gyumarants village	Syunik	46˚22'01"E	38˚59'24"N	1420
W6-37154	1, 2, 3, 4, 5, 6, 7	USDA	17/08/2009	Armenia	between Lichk village and Tashtun pass	Syunik	46˚10'07"E	39˚05'39"N	2040
W6-37155	1, 2, 3, 4, 5, 6, 7, 8, 9, 10	USDA	**						
W6-37159	1, 2, 3	USDA	18/08/2009	Armenia	crossroad to Arachadzor village	Syunik	46˚26'30"E	39˚20'21"N	1320
W6-37160	1, 2, 3, 4, 5, 6, 7, 8, 9, 10	USDA	**						
W6-37167	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	USDA	20/08/2009	Armenia	vicinity of Sisian, near Shaki waterfall	Syunik	45˚59'44"E	39˚32'50"N	1621
W6-37168	1, 2, 3, 4, 5, 6, 7, 8, 9, 10	USDA	**						
433	1, 2, 5, 6, 8	IoE	30/08/2011	Armenia	Geghard I	Ararat	44˚48'51.2"E	40˚08'13.7"N	1722
434	1, 2, 3, 4, 5, 6	IoE	30/08/2011	Armenia	Geghard II	Ararat	44˚49'10.7"E	40˚08'25.7"N	1757
441	1, 3, 4, 5, 6, 7	IoE	01/09/2011	Armenia	Tsovagiugh I	Gegharkunik	44˚58'15.7"E	40˚37'31.1"N	1915
442	2, 3, 4, 5, 7	IoE	01/09/2011	Armenia	Tsovagiugh II	Gegharkunik	44˚58'58.5"E	40˚37'55.1"N	1916
443	2, 3, 4, 5	IoE	01/09/2011	Armenia	Tsovagiugh III	Gegharkunik	45˚00'22.0"E	40˚37'37.5"N	1920
444	1, 2, 3	IoE	01/09/2011	Armenia	Tsovagiugh IV	Gegharkunik	45˚03'25.7"E	40˚36'39.7"N	1915
446	3, 6, 7, 9, 10, 11, 12, 14, 15	IoE	04/09/2011	Armenia	Hamberd	Aragatsotn	44˚16'05.3"E	40˚22'28.7"N	1964
W6-40647	***	USDA	25/07/2010	Russian Federation	Makhachkala	Dagestan	47˚25'26.8"E	42˚52'40.1"N	480
W6-40651	***	USDA	05/09/2010	Russian Federation	Achty	Dagestan	47˚38'47.7"E	41˚23'59.3"N	1255

(c)

Pop. no. *	Sample no. *	Curator Institute	Collecting date	Country	Locality	Region	Ln	Lt	El
433	1, 2, 5, 6, 8, 12	IoE	**						
434	1, 2, 3, 4, 5, 6	IoE	**						
441	1, 3, 4, 6, 7	IoE	**						
446	9, 11, 12, 14	IoE	**						

* Population (Pop.) and sample numbers; Curator Institute: USDA = USDA-ARS, National Plant Germplasm System, Plant Germplasm Introduction and Testing Research Unit, Pullman, WA, USA. IoE = Institute of Evolution, University of Haifa, Israel; Ln = longitude; Lt = latitude; El = elevation (m a. s. l.). ** For details of this population, see above. *** Population was collected as a bulk seed sample.

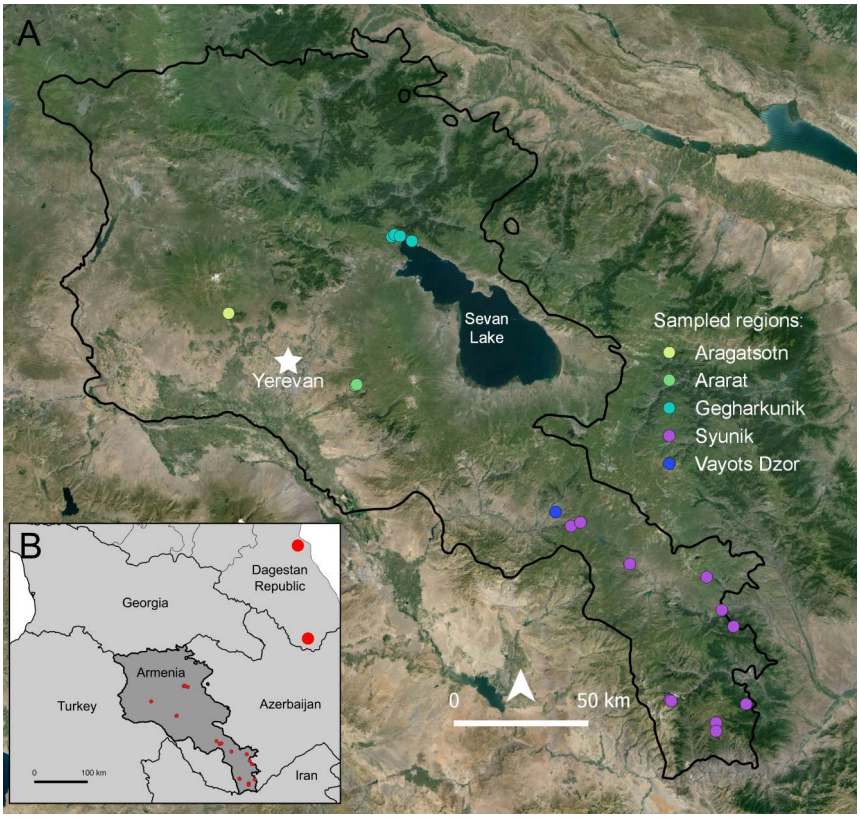

Figure 1. Map of 19 natural populations of wild *L. georgica* collected throughout five regions in Armenia ((A), (B)) and two populations collected in Dagestan Republic (B).

from whole populations at two individual locations in Dagestan, the Russian Federation (**Figure 1(B)**). Plant material was collected during expeditions conducted by: 1) Dr. George Fayvush (Institute of Botany, Armenian Academy of Sciences, Yerevan, Armenia) in 2009; 2) Dr S. Litvinskaya (Kuban State University, Krasnodar, Russian Federation) and Dr. R. Murtazaliev (Mountain Botanical Garden, RAS Dagestan Scientific Center, Makhachkala, Dagestan) in 2010; and 3) Dr. Alex Beharav (IOE, HU, Israel) in 2011. Data on the origin and location of collected samples are summarised in **Table 1**. Note that seeds from some natural populations of *L. georgica* were collected at lower altitudes than the previously reported [23] range of 1500 - 2300 m. In Armenia seeds were collected

from populations starting from 990 m, and in Dagestan a single population was found at 480 m (Table 1).

Various observations supported species identity of the *L. georgica* samples as following: 1) morphological and developmental evaluation, in their native habitats, according to botanical keys and some basic descriptors for wild *Lactuca* spp. [31] [32]; 2) during cultivation and re-determination for the current, as well as for our recent study (see details in Beharav *et al.*, 2018 [9]), also according to morphological and developmental features; and 3) by Target Region Amplification Polymorphism (TRAP) markers, together with samples representing *L serriola*, *L. aculeata*, *L. saligna*, *L. virosa*, and *L. sativa* [9]. A Neighbour-Joining tree clearly clustered the whole set of 238 samples according to their taxonomic determination. Samples from 2009 and 2010 collections were documented and deposited with the USDA-ARS National Plant Germplasm System, Western Regional Plant Introduction Station, Washington State University, Pullman, WA. Seed samples can be requested by US and worldwide researchers via the GRIN-Global database (https://npgsweb.ars-grin.gov/). Samples from 2011 collection were documented and deposited in the seed storage facilities of the IOE's Gene Bank. They are a part of a working collection, and may be shared subject to standard Material Transfer Agreements (MTA).

2.2. Experiments and Growing Conditions

Different vernalization treatments were evaluated at the Western Regional Plant Introduction Station, Pullman WA, USA, during four successive growing seasons, from 2010 until 2013, for a total of seven experiments. The majority of experiments (2010, 2011, 2012, 2012-2013-i, and 2012-2013-iv) included plants from four seed samples from the 2009 collection: W6-37141-1, W6-37155-1, W6-37160-10, and W6-37168-3 (Table 1(a)). Experiments 2012-2013-ii included 120 samples from the 2009, 2010, and 2011 collections (Table 1(b)), while experiments 2012-2013-iii included 21 samples from the 2011 collection (Table 1(c)).

Vernalization treatments were applied to either germinating seed or older plants. The experiment in 2012 on older plants and 2012-2013-iv experiment used plants generated from the 2010 and 2011 experiments, respectively, on germinated seed. The plants used for these two experiments had previous exposure to vernalization as germinated seed. We assumed that these plants did not "remember" the old cold period, thus results can be attributed to the new treatments that included the various vernalization durations as described above. The plants used in the control treatment were the exception to this; they were generated from control treatments in the 2010 and 2011 experiments and had no exposure to vernalization. For the 2012-2013-ii and iii experiments, the plants used had no previous exposure to vernalization.

Treatments in each of the successive years were chosen based on previous results. In all experiments, vernalized plants were moved to growth chambers in order to precisely control the temperature and duration of the vernalization

treatments. Germinating treated seeds were wet and placed at room temperature for between 2 to 10 days (imbibition period), then vernalization treatments were performed. Most experiments of this kind were planned so that all vernalization treatments ended on the same day, allowing the exposure of all plants to the same post-vernalization start date. When the control treatment (no vernalization) was applied, seeds were germinated at room temperature 5 - 7 days before the end of the cold period for the treated plants. After 48 h or when germinated, control seedlings were transplanted to soil. At the end of the vernalization period, depending on the temperature and duration of the cold treatment, seedlings either had not yet emerged or emerged and were at the cotyledonary stage. In other experiments, where vernalization treatments were applied to plants of different ages of the vegetative rosette, we used plants from the previous experiments or other control plants. These kinds of experiments were not optimal because the start and end vernalization date were staggered, however they did provide information if old plants with various ages can be stimulated to bolt.

2.2.1. 2010 Experiment

In a preliminary experiment in 2010, five different combinations of duration and vernalization temperature were applied to just germinating seeds (48 h after imbibition), each included five plants (replicates) from four seed samples representing four populations from the 2009 collection (see Table 1(a)). Treatments were as follows: 1) 27 days at 4˚C; 2) 27 days at 1˚C; 3) 37 days at 4˚C; 4) 37 days at 1˚C; 5) control. To sum up, a total of 25 plants from each sample and a total 100 plants were included in the experiment. All vernalization treatments ended on May 17, 2010. Plants were then moved to a lath house. The temperature from May 17 to May 30 ranged from −0.9 ˚C to 24.7˚C, with an average temp. of 10.7˚C. The high temp. for June was 28.7˚C on June 27. Day-length during this period was above 15 h.

2.2.2. 2011 Experiment

Three vernalization durations were performed at 4˚C using just germinating seeds (48 h) from the same four seed samples that were used in the 2010 experiment (see Table 1(a)): 1) 37 days with four replicates from each sample; 2) 44 days with four replicates; 3) control, *i.e.* without vernalization, with three replicates. A total of 11 plants from each sample, for a total of 44 plants were included in this experiment. All vernalization treatments ended on Aug. 7, 2011, then plants were moved for 14 days of post-vernalization adaptation in a growth room at 15˚C under 18 h-daylength. After 14 days, the temperature was raised to 18˚C under 18 h daylength. The plants were under these conditions for the remainder of the experiment.

2.2.3. 2012 Experiment

A period of 120 days (4 months) of vernalization at 4˚C was applied to a total of 12 plants, three plants (replicates) each from the same four seed samples that were used in the 2010 and 2011 experiments (see Table 1(a)), which had re-

mained in the vegetative rosette stage for 656 days (21.6 months). Due to a limited number of plants, we had no controls (untreated). The plants in this experiment were started from seed placed in germination boxes on Apr. 07, 2010. Seedlings were transplanted to soil after germination and placed in the greenhouse (20˚C - 25˚C under 12 - 18 h-day length). On Jan. 23, 2012, plants were moved to a walk-in growth chamber set at 4˚C under 12 h-day length days until May 22, 2012, *i.e.* for 120 days, when they were moved outside to a lath house until producing seed. Temperatures in the lath house ranged from 1.7˚C to 36.7˚C with an average low of 8.6˚C and an average high of 24.2˚C, with day length between 15.85 to 14.47 hrs.

2.2.4. 2012-2013 Experiments

We tested the effect of various vernalization durations at 4˚C on various plant ages, as following:

1) We applied 73 (2.4 months) and 102 days (3.4 months) of vernalization, as well as no vernalization (controls), to just germinating seeds (eight - ten days after imbibition). Five, six, and eight plants (replicates) from each of the same four seed samples from the 2009 collection (see **Table 1(a)**) that were used in previous experiments were used for the 73 day, 102 day, and control treatments, respectively. A total of 76 plants, 19 plants from each seed sample, were included in this experiment. All vernalization treatments ended on Apr. 08, 2013. Plants were then placed in a growth room set at 18.3˚C under 12 h-day length until June 06, when they were moved outside to a lath house. Temperatures in the lath house ranged from 2.2˚C to 36.1˚C with an average temp of 18.9˚C and day length ranged from 15.8 hrs in June to 11.7 hrs in September.

2) We also applied 168 days (5.5 months) of vernalization to plants 201 - 207 days (6.6 - 6.8 months) old that included between one to 15 plants (replicates) each from a total 120 samples collected in 2009, 2010, and 2011 (see **Table 1(b)**). Seed were started July 25 to July 27, 2012 and transplanted to soil in 32 well planting trays from Aug. 03 to Aug. 09, 2012 and maintained in the greenhouse set at approximately 20˚C with 12 to 18 h light. A total of 217 plants were included in this experiment. Vernalization started on Feb. 26, 2013, ended on Aug. 13. Plants were then placed in a growth room set at 18.3˚C under 12 h-day length until Sept. 03, 2013. They were then transplanted to 2 gal pots and moved outside to a lath house. Ambient temperatures ranged from 35˚C to −6.7˚C with 10 - 13 h day length.

3) In parallel, we applied 55 - 113 days (1.8 - 3.7 months) of vernalization to 126 - 184 (4.1 - 6 month) old plants. Seeds were sown in germination boxes on July 25, 2012. Seedlings were transplanted to soil from Aug. 02 to Aug. 07 and placed in the greenhouse (20˚C - 25˚C under 12 - 18 h-day length). Plants were then separated into two sub-groups, as following: 1) all plants moved into 4˚C on the same date (Nov. 28, 2012, *i.e.* 126 days old plants) and taken out after 58 (Jan. 25, 2013), 85 (Feb. 21, 2013), or 113 days (Mar. 21, 2013); 2) plants were placed in the cold treatment on staggered dates (Nov. 28, 2012; Dec. 27, 2012;

and Jan. 25, 2013, *i.e.* 126, 155, and 184 days old plants, respectively), but all taken out on the same date (Mar. 21, 2013), *i.e.* 113-, 84-, or 55-days of vernalization, respectively. Generally, we used a single, sometimes two plants from each of 21 seed samples representing four populations from the 2011 collection (see Table 1(c)). A total of four-five plants were used for each vernalization duration and each sub-group. In total, 14 plants were included in each of the two sub-groups. In addition, eight plants, two from each of the four populations were used as controls. For all treatments, there were a total of 36 plants included in this experiment. Plants from the 1st sub-group ending vernalization treatment after 58 or 85 days were moved to the greenhouse with temperatures ranging, approximately, from 28.7˚C to 17.5˚C under 12 - 18 h-day length until Mar. 05 when they were moved to a growth room set at 21.1˚C under 18 h-day length. Plants were moved because of the high greenhouse temperatures. On Mar. 21, 2013 all plants from both sub-groups were potted up from flats to 6-inch pots. On Mar. 28, all plants were moved to a walk-in growth chamber set at 18.3˚C days (18 hrs), 12.8˚C nights until June 04, then they were potted to 2 gal pots and moved outside to a lath house. Ambient temperatures ranged from 36.1˚C to −3.9˚C with 10.3 - 15.8 h-day length.

4) Because our previous experiments were not conclusive we continued by applying 36-, 67-, 92-, and 129-days (1.2, 2.2, 3.0, and 4.2 months, respectively) of vernalization—as well as control (without vernalization)—under 12 h-day length to plants with a long vegetative age (between approximately 15 - 20 months, depend on vernalization duration) of the vegetative rosette. We used the plants from the 2011 trial which ended their previous vernalization treatments (37 days, 44 days, or control) on Aug. 07, 2011, but did not respond with bolting. Due to limited number of plants, 67-, 92-, and 129-days vernalization durations included two plants (replicates) from each of the same four seed samples from 2009 collection (see Table 1(a)), that were used in previous experiments, while 36 days of vernalisation treatment and control included only a single plant from each sample. To sum up, a total of eight plants from each sample, (total of 32 plants) were included in this experiment. All vernalization treatments ended on Feb. 21, 2013. The plants were then placed in a growth room set at 21.1˚C under 18 h-day length until Mar. 28, when they were moved outside to the lath house. Ambient temperatures ranged from a high of 30˚C to a low of −2.2˚C from Mar. 28 to June 30 with an average of 11.8˚C. Day length ranged from 12.6 to 15.8 h/day.

3. Results

3.1. Vernalization of Germinating Seeds

3.1.1. 2010 Experiment

A total of 12 out of the 100 plants tested in the 2010 experiment bolted, all except one represented a single seed sample, W6-37160-10. Bolting dates (measured when flower stalk at 5 cm length) ranged from July 20 to Aug. 04, averaging

71 days post-vernalization (dpv). First flower dates ranged from Aug. 11 to Sept. 14, averaged 98.3 dpv. First seed dates ranged from Aug. 25 to Sept. 29, averaged 112 dpv.

All control plants of W6-37160-10 remained strictly vegetative, but plants from all four treatments of this sample bolted, as follows: all five plants (replicates) treated for 37 days, 4˚C bolted; three (out of five) treated for 37 days, 1˚C bolted; two (out of five) treated for 27 days, 4˚C bolted; and a single (out of five) treated for 27 days, 1˚C bolted. Comparing vernalization duration (combined plants from the two treated temperatures), the percentage of bolted plants (eight out of ten = 80%, **Figure 2(A)**) treated for 37 days was significantly higher (chi-square test: df = 1; $\chi^2 = 5.05$; $p = 0.025$) compared to that (three out of ten = 30%) of plants treated for 27 days. However, average bolting date (70.3 dpv) of the three bolted plants treated for 27 days was very similar and not significantly different (un-paired t test: df = 9; t = 0.15; $p = 0.885$) from that (70.9 dpv) of the eight bolted plants treated for 37 days.

Only a single plant (one of ten, **Figure 2(A)**) from W6-37141-1 treated for 37 days, 1˚C bolted, while none of plants from either W6-37155-1 or W6-37168-3 bolted. Note that these two samples were originally collected at higher elevation as compared to the two samples with bolting plants (see **Table 1(a)**).

3.1.2. 2011 Experiment

Only one out of four plants (25%, **Figure 2(B)**) from W6-37160-10 treated for 44 days at 4˚C bolted. Date of bolting was Dec. 12, 127 dpv. All other treated (37 or 44 days at 4˚C) and control plants from all samples remained strictly vegetative.

3.1.3. 2012-2013 Experiment (i)

No plants treated as seedlings for 73 or 102 days of vernalization at 4˚C or any of the control plants bolted.

3.2. Vernalization of Plants with Rosette Leaves (Ordered by the Plant Age at the Time of Vernalization)

3.2.1. 2012-2013 Experiment (iii)

Of the 4.1 - 6 months old plants only a single plant (one out of 8, **Figure 2(D)**), 446-12, from subgroup ii—treated for 55 days—bolted 104 dpv, flowered 143 dpv, and set seed 200 dpv (seed was produced on lateral or secondary stems as the central flower stalk on this plant died). No plants from any of the other two treatments (84 - 85 or 113 days at 4˚C) or the control plants bolted.

3.2.2. 2012-2013 Experiment (ii)

Of the 6.6 - 6.8 months old plants, seven (**Figure 2(E)**) out of the total of 217 plants that were treated with 168 days of vernalization at 4˚C bolted. These were W6-37167-3 (one of two), W6-40647 (one of 15), 441-5 (one of one), 443-3 (two of three), 444-3 (one of one), and 446-15 (one of one). We did not record bolting dates for this set of plants.

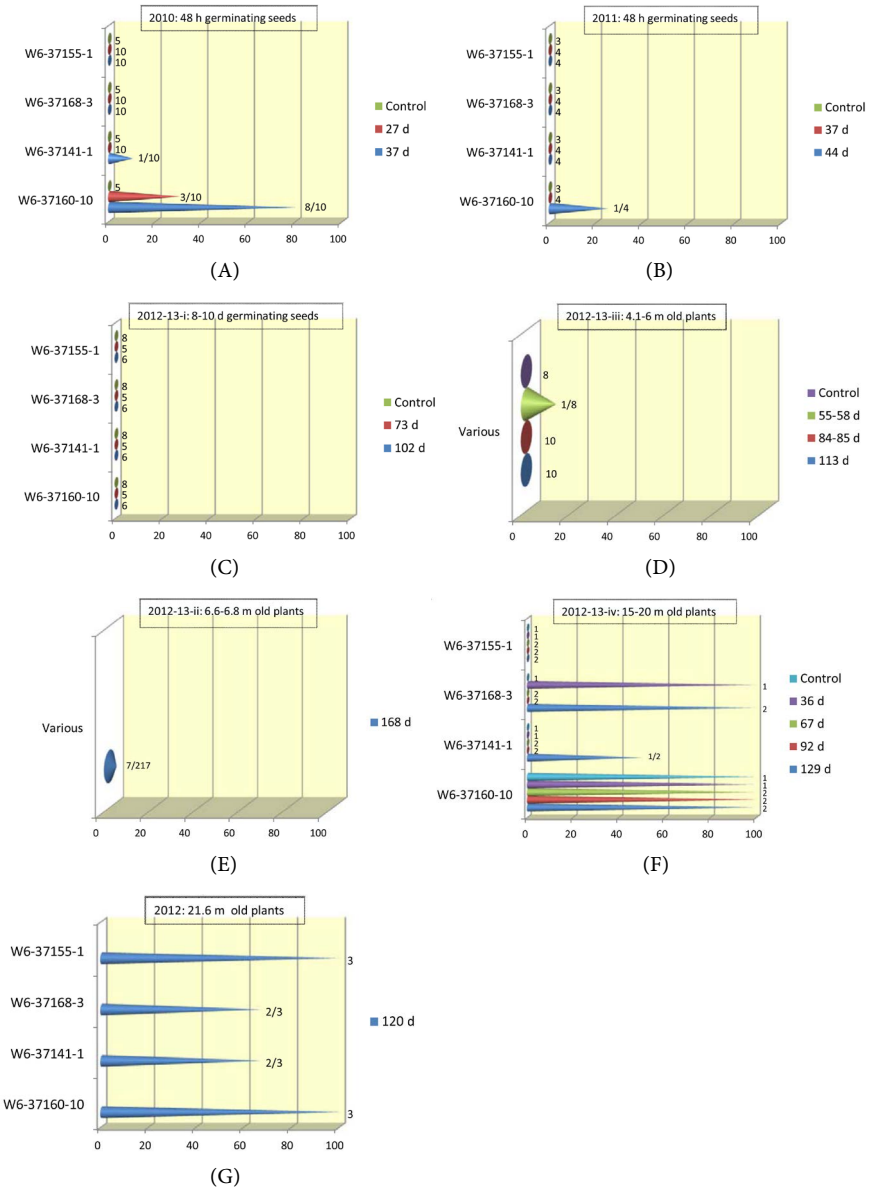

Figure 2. Bolting percentage of progenies of *L. georgica* sampled plants, originally collected throughout seven regions in Armenia and Dagestan Republic, after various vernalization treatments applied to just germinating seeds ((A)-(C)) or different ages of the vegetative rosette ((D)-(G)). Bar number indicates the number of treated plants (where bolting percentage = 0 or 100) or number of bolted out of the treated plants (where bolting percentage > 0 and <100). Vernalization temperature was 4˚C except for A where the results are pooled for the 1˚C and 4˚C treatments.

3.2.3. 2012-2013 Experiment (iv)

Of the 15 - 20 months old plants, a total of 13 out of the 32 plants included in the experiment, bolted. From these, all eight plants (100%, **Figure 2(F)**) that represented sample W6-37160-10 bolted, regardless of vernalization duration (36-, 67-, 92-, and 129-days at 4˚C). The single control plant of W6-37160-10 also bolted on June 30, 2013, 129 days after the end of vernalization for the treated plants. From the seven W6-37160-10 treated plants, a single plant verna-

lized for 67 days bolted 128 dpv, thus we assume that this bolting date was not dependent on vernalization, since it's very similar to the bolting date of the control plant. However, bolting dates for the remaining six W6-37160-10 vernalized plants (for various vernalization durations) ranged from Mar. 03 to Mar. 13, averaging 16.3 dpv. First flower dates of these six plants ranged from Apr. 04 to May 06, averaging 62.8 dpv, while first seed dates ranged from May 21 to May 31, averaging 94.7 dpv. Of the 24 plants representing the other three samples, a single plant from W6-37141-1 (treated for 129 days) bolted 22 dpv, flowered 80 dpv, and seeded 103 dpv; all W6-37155-1 plants remained strictly vegetative; three plants representing W6-37168-3 bolted: two treated for 129 days (one bolted 20 dpv, while the second 100 dpv), and the single plant treated for 36 days (bolted 80 dpv).

3.2.4. 2012 Experiment

Of the 21.6 months old plants, a total ten out of the 12 plants bolted after the four months vernalization at 4˚C. All three plants (100%, **Figure 2(G)**) from samples W6-37155-1 and W6-37160-10 bolted. Two of three plants from samples W6-37168-3 and W6-37141-1 bolted. Bolting dates ranged from June 09 to June 17 with an average of 21.1 dpv. First flower dates ranged from July 08 to Aug. 05, with an average of 60 dpv. First seed production ranged from July 23 to Aug. 9, averaged 74 dpv.

4. Discussion

This study is the first investigation of phenological adaptation of *L. georgica* natural populations and individuals from Armenia and Dagestan, specifically vis-a-vis vernalization requirements. It was shown that low temperatures play a major role in stimulating the reproductive process of *L. georgica* plants. Our results would suggest that: 1) *L. georgica* has an obligate (or nearly so) vernalization requirement since bolting occurred only in a single plant which was not exposed to low temperatures. The single control plant from sample W6-37160-10 that did bolt was a well developed plant, approximately 20 months of vegetative age (2012-2013-iv; **Figure 2(F)**). 2) Plant age, vernalization duration, and genotype of original sample have a role in bolting and flowering regulation of *L. georgica* plants. However, further experiments are required to define the minimal requirements for floral initiation of *L. georgica*. 3) Some samples of *L. georgica* appear to behave as non-obligate typical biennials. Bernier *et al.* [33] indicated that biennial plants with an obligate vernalization requirement normally undergo a juvenile phase during which they are insensitive to low temperatures. However, some of our tested germplasm, in which germinating *L. georgica* seeds did respond to the vernalization treatment, do not fit this scheme (**Figure 2(A)** and **Figure 2(B)**). It seems a vernalization treatment can serve as a substitute to the juvenile phase in some *L. georgica* genotypes. 4) A vernalization period of four months is adequate to stimulate bolting and flowering in plants with a well-developed vegetative rosette, from most—but not all—*L. georgica* samples,

since most plants included in the 2012 (Figure 2(G)) and 2012-2013-iv (Figure 2(F)) experiments bolted after four months of vernalization. However, a longer vernalization duration is needed for some samples, even after attaining a well-developed vegetative stage. 5) To find the best solution for stimulating the reproduction process of *L. georgica* plants from populations that represent different climatic and edaphic environments, it seems that further study should focus on about 4 - 6 months of vernalization at 4˚C applied to plants with about 10 - 22 months of the vegetative rosette, with controlled post-vernalization conditions. Generally, high temperatures and short days may counteract the inductive action of cold temperatures on a plant's bolting and flowering [33]. 6) Due to their vernalization requirement, *L. georgica* germplasm may be used as a source for delayed bolting in breeding domesticated lettuce varieties. High temperature induces early bolting and flowering in lettuce [34]. So, increased temperatures from global climate change pose great challenges for lettuce production. Therefore, it is urgent to study the genetics and molecular mechanism of late bolting and flowering in lettuce by using WLRs to identify novel genes and alleles in known genes that were eliminated following lettuce domestication.

Along with the potential to use *L. georgica* as a source for delayed bolting, the biochemical features [19] [21], and downy mildew resistance [16] point to the uniqueness of this species. Even though recent results indicate that *L. georgica* probably belongs to the LGP-2 [9] [35], we suggest that it should be considered as an attractive germplasm resource for domestic lettuce breeding programs. Clearly, its uniqueness justifies identification and collection of additional samples from multiple locations throughout its geographic distribution.

Due to the limited extent of prior research on *L. georgica*, this study is by necessity exploratory, with the various experiments designed sequentially, and somewhat ad hoc, limited by the availability of seed samples from populations across the species' range, and by the huge amount of work to perform the type of experiments in the present study. It is hard to draw definitive statistically-supported conclusions about the contributions of plant age, vernalization time, and genotype to bolting probability and time to flowering from these results. Nevertheless, a pretty clear basic picture emerges, indicating that for most genotypes vernalization induces bolting with any consistency only in older plants, and there is evidence of genotypic variability in this behaviour. Clearly, more systematic follow-up research on *L. georgica* using germplasm from a wider geographic range is warranted.

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Conflicts of Interest

The authors declare no conflict of interest regarding the publication of this paper.

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