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Seed germination and seedling development ecology in world-wide populations of a circumboreal Tertiary relict

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Abstract

Background and aims	Temperate forests are disjunct in the Northern Hemisphere, having become fragmented from the earlier widespread (Tertiary) boreotropical forest. We asked 'What are the contemporary patterns of population variation in ecological traits of a Tertiary relict in a macroecological context?'. This issue underpins our understanding of variation in populations occurring in the same biome but on different continents.
Methodology	We examined characters associated with root and shoot emergences among populations of *Viburnum opulus* in temperate forests of Asia, North America and Europe. This species has complex seedling emergence extending over several years and requiring various temperature cues.
Principal results	Populations varied in germination responses and clustered into groups that were only partly related to varietal status. Whereas roots (at warm temperatures) and shoots (following a cold period) simultaneously emerged from seeds of all populations when simulated dispersal occurred in winter, they were delayed in some populations when dispersal occurred in summer.
Conclusions	*Viburnum opulus* populations, some separated by 10 300 km, showed high similarity in seedling development and in germination phenology, and we suggest that stabilizing selection has played a key role in maintaining similar dormancy mechanisms. Nevertheless, there was some degree of variation in other germination characters, suggesting local adaptation.

Introduction

The boreotropical forest was a belt of vegetation consisting of tropical and temperate elements concentrated around the Northern Hemisphere during the Eocene epoch (Wen 1999; Xiang and Soltis 2001). Floristic exchanges between North America, Europe and Asia occurred via the North Atlantic Land Bridge and via the Bering Land Bridge (Milne and Abbott 2002). With climatic cooling during the Tertiary (late Miocene and Pliocene) and Quaternary, this forest became fragmented. Today, disjunct populations of these boreotropical elements, commonly called Tertiary relict floras, remain in two or more of the following areas: southeastern Europe, eastern Asia, western Asia, eastern North America and western North America (Xiang and Soltis 2001; Milne and Abbott 2002).

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Stasis of morphological traits has presumably occurred among populations of Tertiary relics given their high degree of morphological similarity (Wen 1999). On the other hand, two schools of thought have emerged regarding ecological traits (Adams *et al.* 2005). The first school believes that ecological tolerances and physiological requirements have remained relatively unchanged over time (Kornas 1972; Axelrod 1983; Ricklefs and Latham 1992; Tiffney and Manchester 2001; Svenning 2003; Qian and Ricklefs 2004). In this view, the main reason for continued survival of taxa was 'climate tracking', i.e. shifts in species' distributions to fit those in climate, while adaptation to a new climate played only a minor role. Researchers in the second school have argued that ecophysiological attributes have changed through time with plant lineages adapting to new environmental conditions (Mason 1947; Wolfe 1969; Dilcher 1973). Ecological studies examining these two viewpoints have resulted in conflicting results. For example, Ricklefs and Latham (1992) found a significant correlation in the geographic ranges of herbaceous species between eastern Asia and eastern North America indicating ecological stasis, but no correlation for woody species. In contrast, Svenning (2003) found strong evolutionary conservatism in climatic requirements among the tree floras of Europe, Asia and North America.

Over the last few years, seed biologists have entered the realm of addressing the stasis/divergence of ecological traits among Tertiary relics by examining their seed dormancy (e.g. Hidayati *et al.* 2000; Walck *et al.* 2002; Adams *et al.* 2005; Vandelook *et al.* 2007; Vandelook and Van Assche 2008). These studies—some finding stasis and others divergence (e.g. Hidayati *et al.* 2010)—have largely focused on species of herbaceous plants usually representing one population on each of two continents. All cases listed above involved seeds that are morphophysiologically dormant (MPD) at maturity, i.e. the embryo is tiny in comparison with the copious endosperm and must grow for the radicle to emerge from the seed, but a physiological block must also be overcome before, during and/or after elongation of the embryo. Thus, levels of MPD, distinguished on the basis of requirements for embryo growth and dormancy break (Baskin and Baskin 2004), have been compared between intercontinental taxa. Yet, differentiation of seed traits is documented best at the population level where fine-scaled adaptations to specific habitat conditions may occur.

Seed traits among populations have been examined from relatively small (e.g. 10^1–10^4 m: Wu *et al.* 1987; Karlsson and Milberg 2007*a*, 2008) to large (e.g. $> 10^5$ m: Schütz and Milberg 1997; Meyer and Allen 1999) geographic scales. Uncommonly, comparisons have been carried out over a continent (Yang *et al.* 1999) and rarely at an intercontinental level. Karlsson and Milberg (2007*b*) reported that germination attributes were relatively similar among populations of the weedy *Conyza bonnariensis* from Mexico, Ethiopia and Morocco. In contrast, Wagner and Simons (2009*a*, *b*, *c*) found divergence in seed, morphology, life-history and phenology traits among North American and European populations of the circumpolar annual *Koenigia islandica* growing in arctic, high-latitude alpine and alpine habitats.

While all these studies have shown population-level variation in seed traits, they may have little bearing on Tertiary relics that are broadly distributed over several continents and that occur in the same biome. Considering that Tertiary relics constitute an important floristic component in the Northern Hemisphere, we wanted to better understand the contemporary pattern of population-level variation for seed and seedling traits in a macroecological context. We selected the woody plant *Viburnum opulus* L., which has a circumboreal distribution (Kollmann and Grubb 2002), for the investigation. Seeds of this species have a special kind of MPD, called epicotyl dormancy, in which temperature requirements differ for root emergence (and prior embryo growth) versus shoot emergence as well as temporally (Giersbach 1937; Knowles and Zalik 1958; Zolobova 1970; Fedec and Knowles 1973).

The 12 populations of *V. opulus* in our study occur in temperate broadleaf and mixed forests (Olson *et al.* 2001) in four countries on three continents, with a gradient of intra- and intercontinental distances (1–10 300 km). We compared embryo and seed sizes, as well as germination (i.e. both root and shoot emergence) responses to a range of temperatures, among *V. opulus* populations from different parts of the world. We were also interested in the developmental timing from seed to seedling and the relationship between the timing of dispersal and that of emergence since seeds of this species have a long dispersal period (Witmer 2001). Overall, we predicted that the seed and seedling traits of *V. opulus* would not differ among populations since they occur in the same (albeit disjunct) biome. Our study is the first to investigate the degree of ecophysiological differentiation in populations of a species over three continents.

Materials and methods

To understand differences in germination responses among circumboreal populations of *V. opulus*, we conducted a series of experiments in a controlled (laboratory) environment as well as in a common garden.

We examined (i) embryo size and seed size and (ii) specific temperature requirements for root and shoot emergences among a subset of 12 populations from Asia, North America and Europe. For all 12 populations, we determined the timing of root and shoot emergences, and of embryo growth, in relation to a sequence of simulated seasonal temperatures. Lastly, emergence phenology of roots and shoots was observed in a common garden in Sweden for 11 of the 12 populations.

Study species

Viburnum opulus occurs from Europe through the Caucasus to Central Asia as var. *opulus*, in East Asia as var. *sargentii* (Koehne) Takeda, and in North America as var. *americanum* Ait. (Hara 1983; United States Department of Agriculture, Agricultural Research Service 2008). In addition, plants of var. *opulus* are naturalized in North America, though they are not always easily distinguishable from var. *americana* (Nellessen 2006). These three taxa are members of the strongly supported *Opulus* clade, which also includes *V. koreanum* and *V. edule* (Moore and Donoghue 2007; Jacobs *et al.* 2008). The circumboreal distribution of this clade is thought to have 'arose quite recently' (Winkworth and Donoghue 2005).

The study species is a deciduous shrub up to 4 m tall, frequently forming adventitious roots and new vertical shoots on branches close to the ground (Kollmann and Grubb 2002). It occurs in a variety of moist to wet habitats: carr, upland deciduous and mixed pine forests, hedgerows, floodplains, alder swamps, and montane and subalpine forests. Fruits mature and are produced in autumn with seeds being consumed and regurgitated (Hernández 2009) mainly during late autumn or winter (Jones and Wheelwright 1987; Englund 1993; Kollmann and Grubb 2002; Drummond 2005) into spring (Witmer 2001).

Seed collections

The fruit is a one-seeded 10- to 15-mm-diameter drupe, with soft pulp and a flattened stone; the fully ripened fruit is red. Fruits were collected from all populations in autumn 2004 (Table 1). Distances between populations were: 1–65 km in Sweden, 175–710 km in Japan, 1000 km in Canada and the USA, 5200–6200 km between Europe and North America, 7600–8200 km between Europe and Asia, and 9600–10 300 km between Asia and North America (Google Inc. 2009). Experiments began 1–22 days after collection and shipment to laboratories. Pulp was removed from the stones (hereafter referred to as seeds) and seeds were dried at room conditions for studies.

General laboratory procedures

Laboratory work on Asian and North American seeds was performed at Middle Tennessee State University in Tennessee, USA (MTSU), and that on European seeds at Linköping University in Sweden (LiU). A preliminary study showed that the timing of emergence and the percentages of emergence for roots and shoots from seeds collected in Tokarp (Sweden) and in Sapporo (Japan) did not differ between laboratories.

Incubators (MTSU: I-36LL, Percival Scientific, Perry, IA, USA; LiU: Rumed Cooled 3122, Rubarth Apparatebau,

Table 1 Collection locations and dates for seeds from three varieties of *V. opulus*.

Variety	Location collected[a]	Latitude	Longitude	Status at collection location	Date collected
sargentii	Japan, Hokkiado Prefecture, Mombetsu	44°21′N	143°21′E	Native	19 Sep 2004
sargentii	Japan, Hokkaido Prefecture, Sapporo	43°00′N	141°21′E	Native	8 Oct 2004
sargentii	Japan, Hokkaido Prefecture, Kaminokuni	41°47′N	140°07′E	Native	4 Oct 2004
sargentii	Japan, Miyagi Prefecture, Sendai	38°16′N	140°52′E	Native	7 Dec 2004
americanum	Canada, New Brunswick, Moncton	46°06′N	64°47′W	Native	6 Oct 2004
opulus	USA, New York, Ithaca	42°25′N	76°32′W	Non-native	11 Sep 2004
opulus	Sweden, Östergötland County, Motala	58°32′N	15°05′E	Native	26 Sep 2004
opulus	Sweden, Östergötland County, East Stocklycke	58°18′N	14°38′E	Native	25 Sep 2004
opulus	Sweden, Östergötland County, West Stocklycke	58°18′N	14°37′E	Native	25 Sep 2004
opulus	Sweden, Östergötland County, Örnslid	58°19′N	14°38′E	Native	25 Sep 2004
opulus	Sweden, Östergötland County, Rimforsa	58°08′N	15°36′E	Native	25 Sep 2004
opulus	Sweden, Östergötland County, Tokarp	58°25′N	15°30′E	Native	7 Oct 2004

[a]Collection sites are named by the nearest town or by place names occurring on detailed maps.

Laatzen, Germany) were set at alternating 12/12 h (day/night) temperatures of 15/5, 20/10, 25/15 and 30/15 (or 30/20) °C. A constant temperature of 5 °C was achieved in another incubator (MTSU) or in a cold room (LiU; room: Ki-PANEL, Huurre Sweden AB, Sweden; temperature control: ADU 200, Styrprojektering AB, Sweden). We used the same set of temperature regimes among all populations to simulate seasons: 5 °C, winter; 15/5 °C, early spring and late autumn; 20/10 °C, late spring and early autumn; and 25/15 and 30/15 (or 30/20) °C, summer. With the exception of photoperiod, light conditions were similar in incubators and the cold room [described by Walck and Hidayati (2007) for MTSU and by Karlsson *et al.* (2005) for LiU]. The photoperiod was 14 h (MTSU) or 12 h (LiU), which apparently had no effect given that root and shoot emergences were similar between laboratories in the preliminary study (see above).

Since germination in *V. opulus* has two distinct phases, temporally separated, we use the term 'rootling' for seedlings in the first phase of germination (i.e. a seed with only an emerged radicle and no shoot) to distinguish it from seedlings in the second phase (i.e. a seed with a root and shoot). To examine root emergence, seeds were placed on two filter papers moistened with distilled water in 10-cm-diameter Petri dishes and incubated in light. Dishes were wrapped with plastic film and placed in polyethylene plastic bags to reduce water loss during the experiment, and additional water was added if needed. Roots were determined to have emerged when the radicle protruded ~1 mm beyond the seed. To study shoot emergence, a rootling was removed from the dish and planted in a 7-cm-deep plastic container, filled with 2–3 cm of moist sand, and covered with plastic film; only the root and lower portion of the seed were covered with sand. Shoots were judged to have emerged when at least one cotyledon was completely exposed from the seed coat. Seeds/rootlings were either placed in one dish/container or divided equally among two to three dishes/containers.

Lengths of 20–25 embryos were obtained each time by excising them from seeds using a razor blade and measuring them with a micrometer under a dissecting microscope. At the start of the study, seed and embryo lengths were determined for fresh seeds 24 h after they were placed on moist filter paper at room temperature (~22 °C). Measurements for fresh seeds and embryos were obtained for Mombetsu, Sapporo, Sendai (Japan), Moncton (Canada), Ithaca (USA) and Tokarp (Sweden) populations; the embryo : seed (E : S) ratios were calculated from these measurements.

Root and shoot emergences were evaluated fortnightly. Percentages of root emergence are based on number of viable seeds, and those of shoot emergence on the number of seeds in which a radicle had emerged. Seeds from which radicles did not protrude were checked to determine whether the embryos were white and firm, indicating that they were viable, or if the embryos were brown and soft, indicating that they were nonviable. Throughout all laboratory studies, 70–97 % of seeds maintained their viability. Survivorship of rootlings until the end of all experiments varied from 62 to 93 % among populations, except for 22 and 39 % in the Kaminokuni and Sendai (Japan) populations, respectively.

Specific requirements for root emergence

Twenty-six Mombetsu (Japan), 150 Moncton (Canada) and 50 Tokarp (Sweden) seeds were used for each treatment. Dishes were placed at 5, 15/5, 20/10, 25/15 °C and either 30/15 (MTSU) or 30/20 °C (LiU) for 12 weeks of stratification and were then transferred to (or remained at) each of these temperature regimes for 12 weeks of incubation, e.g. seeds stratified at 5 °C were incubated at 5, 15/5, 20/10, 25/15 °C and either 30/15 or 30/20 °C. Seeds were checked for an emerged radicle fortnightly, and if present, counted and removed from the dish. At the end of the experiment, seeds from which radicles did not protrude were checked for viability.

Specific requirements for shoot emergence

Several hundred seeds from the Moncton (Canada), E Stocklycke and Tokarp (Sweden) populations were placed at 25/15 °C to allow radicle emergence. After many seeds had radicles, the experiment was set up using 15, 33 or 40 seeds, respectively, per treatment. They were cold stratified at 5 °C for 0, 12 and 24 weeks (Canadian population) or 0, 6, 12 and 24 weeks (Swedish populations). After each cold period, seeds were incubated at 15/5, 20/10 and 25/15 °C for 12 weeks. Seeds in which the shoot emerged during stratification were removed from the containers before incubation and subtracted from the total tested. Control seeds were kept at 5 °C for 36 weeks. Rootlings were checked for an emerged shoot fortnightly and, if present, were counted and removed from the dish.

Temperature sequence for root and shoot emergences and for embryo growth

Seeds from all populations were exposed to two conditions. (i) In continuous temperature regimes, seeds were incubated at 5, 15/5, 20/10 and 25/15 °C for 80 weeks. (ii) In annual temperature cycles, which simulated a natural sequence of seasonal temperatures, seeds were given a winter (5 °C) regime progressing to a summer (25/15 °C) regime (cold (C) temperatures → warm (W) temperatures) or a summer regime

Table 2 Characters, and their acronyms, used in the Mantel test and PCA.

Characters
Incubated at continuous temperature regimes (see Fig. 4)
Root emergence at 5 (*R5*), 15/5 (*R15*), 20/10 (*R20*) and 25/15 °C (*R25*)
Shoot emergence at 15/5 (<u>S15</u>), 20/10 (<u>S20</u>) and 25/15 °C (<u>S25</u>)
Incubated at annual temperature cycles (see Figs 5 and 6)
Final root emergence in C → W during 1st cycle (RC1) and 2nd cycle (RC2)
Final root emergence in W → C during 1st cycle (**RW1**) and 2nd cycle (**RW2**)
Final shoot emergence in C → W during 2nd cycle (<u>SC2</u>)
Final shoot emergence in W → C during 1st cycle (<u>**SW1**</u>) and 2nd cycle (<u>**SW2**</u>)
Number of days until the start of root emergence in C → W (dsRC) and W → C (**dsRW**)
Number of days until the start of shoot emergence in C → W (<u>dsSC</u>) and W → C (<u>**dsSW**</u>)
Number of days until 50 % of maximum root emergence in C → W during 1st cycle (d50RC) and W → C during 1st cycle (**d50RW**)[a]
Number of days until 50 % of maximum shoot emergence in C → W during 2nd cycle (<u>d50SC</u>) and W → C during 2nd cycle (<u>**d50SW**</u>)[a]
Number of days until maximum root emergence in C → W (dmRC) and W → C (**dmRW**)
Number of days until maximum shoot emergence in C → W (<u>dmSC</u>) and W → C (<u>**dmSW**</u>)

[a]Emergence speed, measured as the number of days to reach 50 % of final emergence (T50), was calculated using a formula taken from Amjad and Anjum (2002).

Fig. 1 Mean lengths for fresh embryos (E) and seeds (S), and their ratio, of *V. opulus* from five native populations in Asia (A), North America (NA) and Europe (E), and from a non-native population in North America of the European variety (E/NA). Confidence intervals ± 95 % are shown.

incubation for the Canadian and Swedish populations. In the annual temperature cycles, embryos from these four populations were measured each time dishes or containers were moved to a new temperature regime (for up to 60 weeks). Embryo length was measured only in ungerminated seeds.

Phenology of root and shoot emergences in a common garden

With the exception of Mombetsu (Japan), all populations were used in this study. Japanese and North American seeds were sent to LiU 1–2 days after arriving at MTSU. Fifty seeds were sown on top of soil on 13 November 2004 (except for Sendai, sown on 1 January 2005) in each of 2–5 (2 L) pots per population. The soil used was found locally (Ledberg) and sterilized for 20 min at 120 °C before being placed in pots containing a non-woven glass-fibre sheet in the bottom. The pots were placed at Ledberg, Sweden (58°26′N, 15°28′E), close to the Tokarp population. A 0.15-m-deep hole was dug in the ground and filled with 2- to 6-mm-diameter ceramic clay pellets (AB Svenska, Leca, Sweden) before pots were added. The pots were buried to a depth such that the surface of the soil was at the same level as the surface of the surroundings. They were protected from direct wind, but otherwise subjected to natural weather conditions. Animals were excluded by a 20-mm-mesh net. Temperature (without protection from sunshine) was measured every hour (TinytagPlus, Intab, Sweden) at the soil surface in an additional pot and

progressing to a winter regime (W → C) for two annual cycles equalling 80 weeks. The number of seeds used in this study was: 45 for Sapporo (Japan); 132 for Kaminokuni (Japan); 100 for Sweden (except for Tokarp); 150 for North America, Mombetsu and Sendai (Japan), and Tokarp (Sweden). Rootlings were removed from dishes, planted in containers, and the cycle continued.

Embryo growth was examined for Mombetsu, Sapporo (Japan), Moncton (Canada) and Tokarp (Sweden) populations. In each of the continuous temperature regimes, embryos were measured following 12 and 36 weeks of incubation at 5, 15/5, 20/10 and 25/15 °C for Japanese populations, and following 6, 12, 24 and 36 weeks of
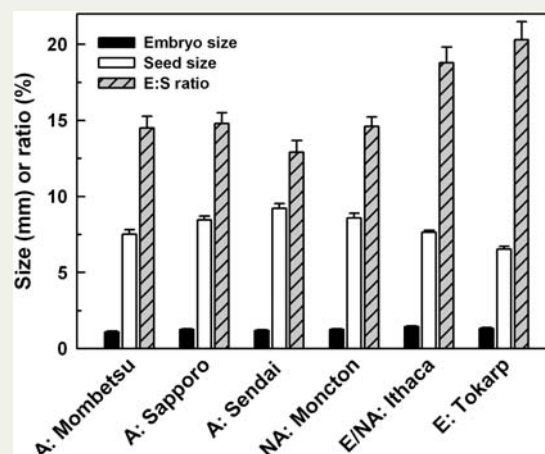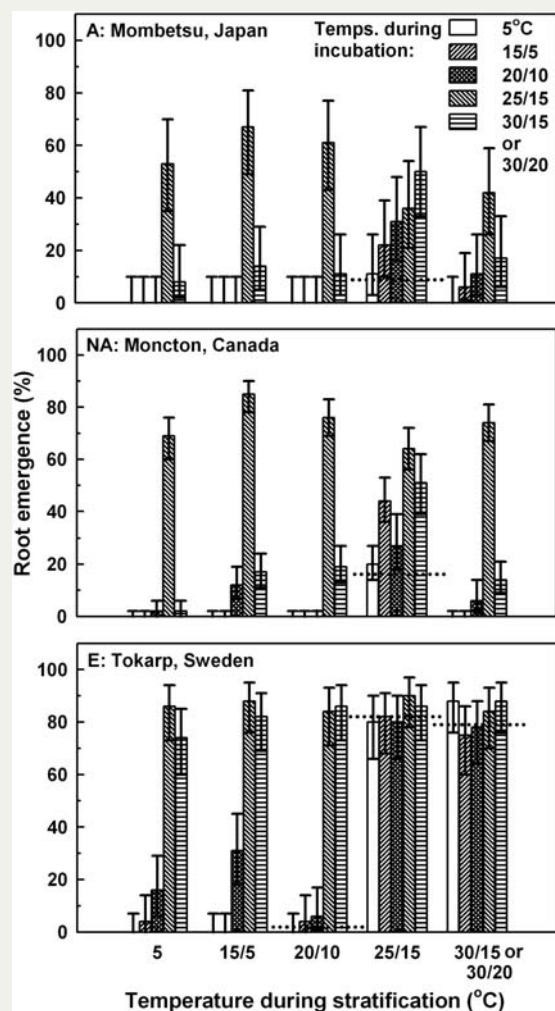
Fig. 2 **Root emergence for seeds of *V. opulus* from three populations representing Asia (A), North America (NA) and Europe (E).** Seeds were incubated for 12 weeks at 5, 15/5, 20/10, 25/15 and 30/15 (or 30/20) °C following 12 weeks of stratification at each of these temperatures. The dotted line represents emergence that occurred during stratification. Confidence intervals (95 %), based on statistics for binomial distribution, are shown.

Fig. 3 **Effects of cold stratification on shoot emergence from rootlings of *V. opulus* populations representing North America (NA) and Europe (E).** Control seeds were maintained at 5 °C for 36 weeks. Treated European seeds were cold stratified at 5 °C for 0, 6, 12 and 24 weeks, and North American seeds for 0, 12 and 24 weeks, before incubation at 15/5, 20/10 and 25/15 °C for 12 weeks. Confidence intervals (95 %), based on statistics for binomial distribution, are shown.

Statistical analyses

For embryo/seed size data and root/shoot emergence data, we determined 95 % confidence intervals. This approach, as opposed to testing specific null hypotheses, allows the presentation of the magnitude of effect size as well as the precision of the estimate for this magnitude (Cumming and Finch 2005; Nakagawa and Cuthill 2007). Confidence intervals for emergence data were calculated using the exact method for binomial parameters. A principal components analysis (PCA) was conducted to summarize the complex data for 26 characters (Table 2) per studied population from the seasonal sequence experiment. Principal components analysis was applied with the purpose of displaying the relative similarity in characters among the populations (software CANOCO 4.5, ter Braak and Smilauer 2002). The 26

precipitation was measured daily, ~15 m away from the pots. On 7 October 2005, 1–2 pots from each population were examined for rootlings, and after recording their number they were replanted. Shoot emergence was examined fortnightly throughout the study, except when the pots were covered with snow. At the end of the study (31 August 2007), the number of remaining seeds (with and without a radicle) that had not produced a shoot during the study were counted. Percentages of root and shoot emergences were calculated based on the number of seeds sown at the start of the study.
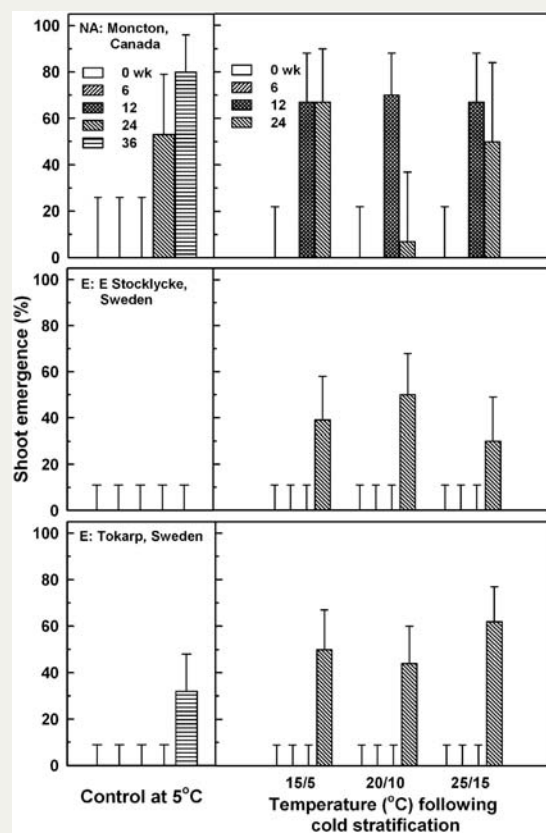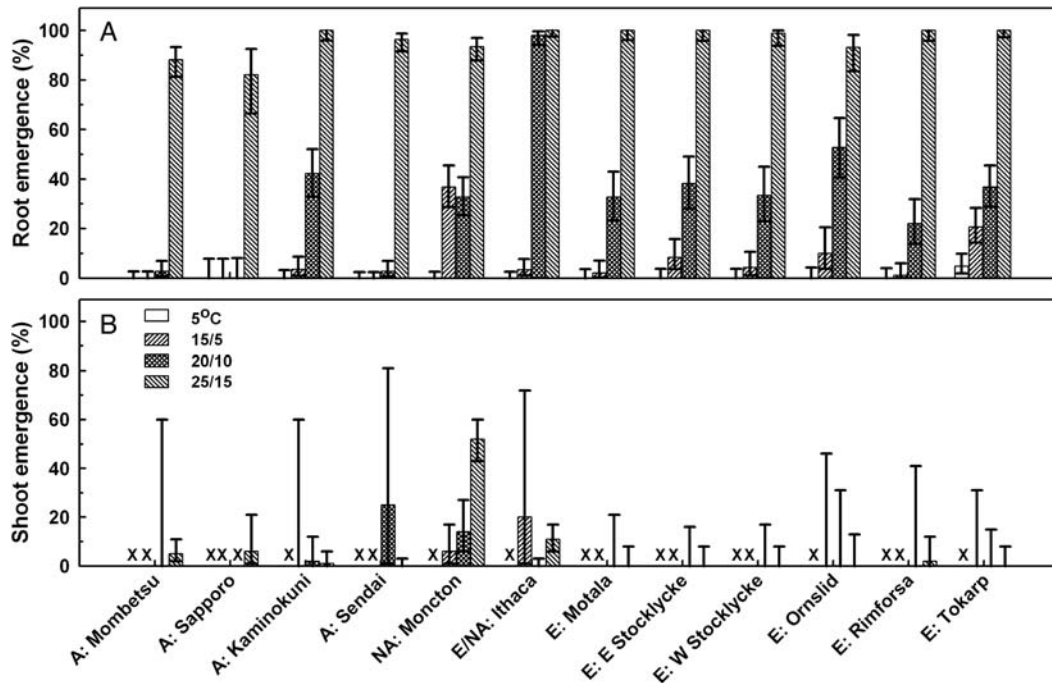
Fig. 4 Root emergence from seeds (A) and shoot emergence from rootlings (B) of *V. opulus* from 11 native populations in Asia (A), North America (NA) and Europe (E), and from one non-native population in North America of the European variety (E/NA). Seeds were incubated continuously for 80 weeks at four temperature regimes (shown in (B)). An X in (B) indicates that no rootlings were available to follow shoot emergence. Confidence intervals (95 %), based on statistics for binomial distribution, are shown.

characters were normalized before the PCA. The length of an arrow indicates relative importance to the solution and the angle of one arrow relative to another shows the relative correlation between them. The same data set was also used to calculate Euclidean distances between populations, i.e. their relative similarity in the seasonal sequence experiment. This Euclidean distance matrix was then compared with a corresponding one on geographic distances by a Mantel test. Pair-wise geographic distances were $\log(x + 1)$ transformed.

Results

Seed and embryo sizes

Seed size varied among populations, but embryo size did not (Fig. 1). Embryos were 13–20 % the length of seeds. Embryo : seed ratios were similar among three Asian and the Moncton (Canada) populations, but they were smaller than those in the Ithaca (USA) and Tokarp (Sweden) populations. Both of these latter populations are var. *opulus*.

Specific requirements for root emergence

During 12 weeks of stratification, roots emerged from ≤16 % of the seeds from Mombetsu (Japan) and Moncton (Canada) populations at 25/15 °C (Fig. 2). During incubation, the highest root emergence (42–85 %) from both populations was generally at 25/15 °C, regardless of stratification temperature. The broadest range of temperatures for root emergence during incubation occurred if seeds of these populations were stratified at 25/15 °C. Root emergence occurred in 2 and 79–82 % of seeds from the Tokarp (Sweden) population during stratification at 20/10 and 25/15–30/20 °C, respectively. The highest root emergence (74–88 %) from this population was when seeds were exposed to 25/15 or 30/20 °C.

Specific requirements for shoot emergence

Although 12 weeks of cold stratification were adequate for high shoot emergence from the Moncton (Canada) population (≥ 67 %), 24 weeks were needed for Swedish populations (30–62 %) (Fig. 3). Up to 80 % of shoots emerged from the Moncton population during
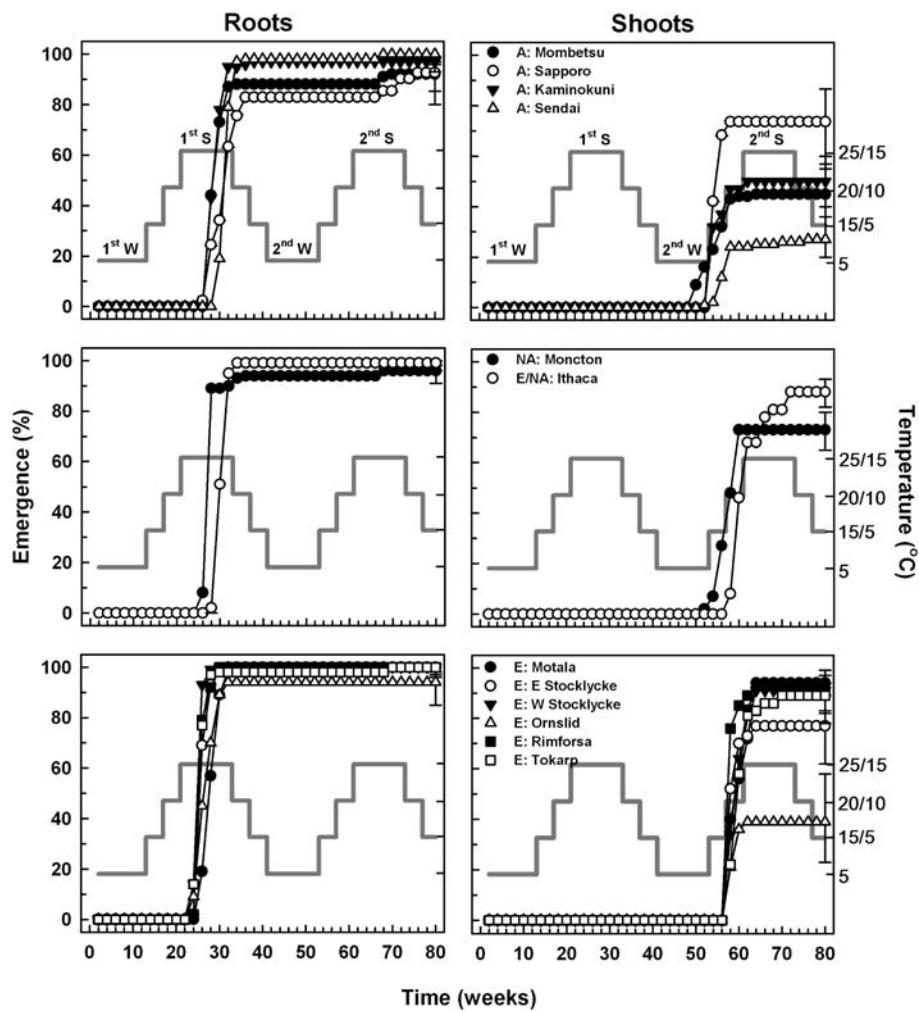
Fig. 5 Cumulative root emergence from seeds and shoot emergence from rootlings of *V. opulus* from 12 populations (as explained in Fig. 4). Population symbols for root emergence are shown in the corresponding panel for shoot emergence. Seeds were subjected to an annual temperature cycle (thick grey lines) starting at a winter (W) regime and progressing to a summer (S) regime. Confidence intervals (95 %), based on statistics for binomial distribution, are shown only for the last data point.

36 weeks of incubation at 5 °C, but $\leq$32 % from the Swedish populations.

Temperature sequence for root and shoot emergences and for embryo growth

The most suitable continuous temperature regime for root emergence ($\geq$82 %) was 25/15 °C for all populations and additionally 20/10 °C for the Ithaca (USA) population (Fig. 4A). For rootlings, shoot emergence from populations was $\leq$25 % regardless of temperature, with the exception of 52 % at 25/15 °C for the Moncton (Canada) population (Fig. 4B). Embryo growth in Mombetsu, Sapporo (Japan), Moncton and Tokarp

(Sweden) occurred only at 25/15 °C during 36 weeks of incubation (data not shown).

For the C $\rightarrow$ W treatment, root emergence ($\geq$83 %) for all populations occurred during the first summer at 25/15 °C (Fig. 5) concomitantly with embryo growth (data not shown). Roots from European populations emerged an average of 4 days earlier than those from Asian and North American ones. Shoot emergence for all populations took place starting late in the second winter (at 5 °C) through the second spring (at 15/5 and 20/10 °C) (Fig. 5). Shoot emergence varied between 27 and 94 % among populations. Shoots began to emerge ~6 days earlier (at 5 and 15/5 °C) in Asian and the
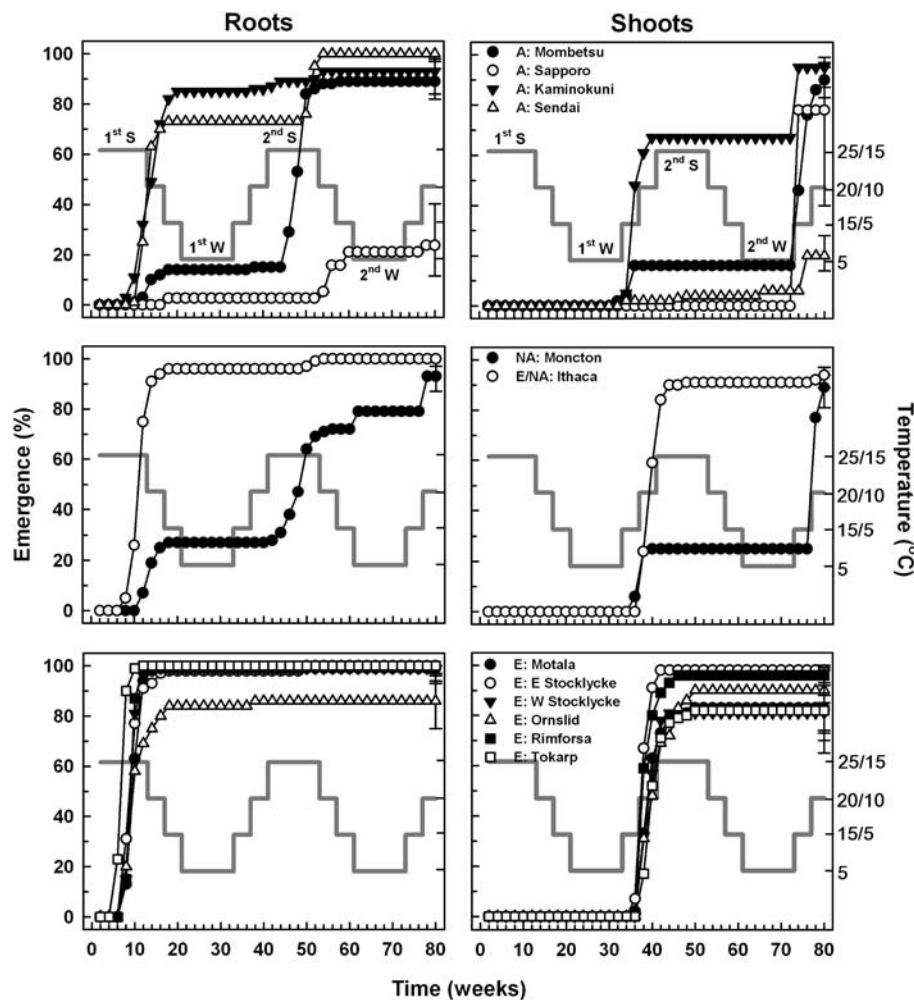
Fig. 6 Cumulative root emergence from seeds and shoot emergence from rootlings of *V. opulus* from 12 populations (as explained in Fig. 4). Population symbols for root emergence are shown in the corresponding panel for shoot emergence. Seeds were subjected to an annual temperature cycle (thick grey lines) starting at a summer (S) regime and progressing to a winter (W) regime. Confidence intervals (95 %), based on statistics for binomial distribution, are shown only for the last data point.

Canadian populations than in the Ithaca (USA) and European populations (at 20/10 °C).

Root and shoot emergences among populations varied more in the W → C treatment than in the C → W treatment. High percentages (≥70 %) of roots emerged from all European populations, the Ithaca (USA) population and two Japanese (Kaminokuni, Sendai) populations during the first summer and first autumn (25/15 to 20/10 °C) (Fig. 6). Root emergence (21–89 %) in the other Japanese (Mombetsu, Sapporo) populations and the Moncton (Canada) population occurred mostly in the second summer and second autumn (25/15 to 15/5 °C). Embryo growth in Mombetsu, Sapporo (Japan), Moncton (Canada) and Tokarp

(Sweden) populations took place from the first summer through the first autumn followed by a delay until the second summer (data not shown). The highest shoot emergence (≥79 %) was in the first spring and second summer (20/10 to 25/15 °C) following the first winter for all European populations and the Ithaca population, whereas the highest shoot emergence (20–95 %) occurred in the second spring (15/5–20/10 °C) following the second winter for all Asian populations and the Moncton population (Fig. 6).

Geographic distance was strongly correlated with difference in characters (Mantel test, $R = 0.5739$, $P = 0.0021$ in a test with 9999 permutations), indicating that the further apart the populations, the more
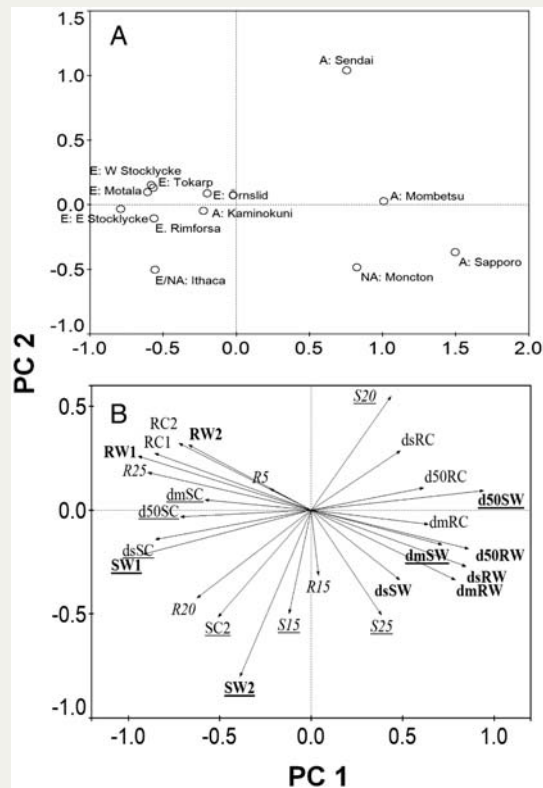
Fig. 7 Principal components analysis results displaying the 12 populations (A) and 26 characters (B). See Fig. 4 for population information and Table 2 for acronyms.

dissimilar they are in germination characters. The percentage of variance explained for the first, second, third and fourth principal components was 57.3, 14.8, 11.1 and 5.9, respectively. All European populations, the non-native population in North America and one Asian population formed a tight cluster (EIK group), whereas two other Asian populations and the native North American population formed a second cluster (MMS group; Fig. 7A). One Asian population was a distinct outlier (S group). The characters that best identified the EIK group were **SW1** and **RW1**, and for the MMS group were **d50SW**, **d50RW**, **dsRW** and **dmRW** (Fig. 7B). Roots and shoots emerged during the first cycle in the W → C treatment in the EIK group, and during the second cycle in the MMS group. The S group had the highest shoot emergence at constant 20/10 °C (*S20*).

Phenology of root and shoot emergences in a common garden

On 7 October 2005, 2–10 % of the seeds sown in November 2004 (or January 2005 for Sendai) had an emerged radicle (up to 10 mm long) (Fig. 8). During summer (June–August)

2005 preceding root emergence, temperatures averaged 17.5 °C. Shoot emergence occurred between 26 April 2006 and 27 May 2006 (0–5 % among populations), and between 2 May 2007 and 27 May 2007 (additional 1–26 %). Temperatures averaged −0.9 and −0.1 °C during winters (December–February) 2005–06 and 2006–07, respectively, and 11.6 and 13.2 °C during the shoot emergence periods of 2006 and 2007, respectively (Fig. 8). Root and shoot emergence was independent of precipitation events (Fig. 8), which is not surprising as soils in Sweden rarely dry out in spring or autumn. During the ~3-year phenology study, 1–26 % of the seeds produced roots and shoots, and 3–10 % died (Fig. 9). At the end of the study (31 August 2007), 13–46 % of seeds had roots (slightly branched and 30–50 mm long) and 30–63 % were ungerminated but viable.

Discussion

Seed size, but not embryo size, varied among the *V. opulus* populations. In contrast to our work, other studies have reported trends in embryo size. For *Osmorhiza aristata*, embryos in a southern population were longer than those in a northern population in Japan, but seed length did not differ (Walck *et al.* 2002). Nikolaeva (1999) reported decreased embryo lengths in *Fraxinus excelsior* with increasing latitude in Europe.

Root emergence (and also prior embryo growth) for nearly all seeds from each of the *V. opulus* populations occurred at 25/15 °C. In addition, roots from seeds in the Ithaca (USA) and Tokarp (Sweden) populations also emerged at 20/10 and 30/20 °C. Very low shoot emergence occurred at any temperature unless seeds received cold stratification: either ≥ 12 weeks for the Canadian population or 24 weeks for the Swedish populations. Thus, embryo growth and root emergence occurred in warm temperatures without a requirement for pre-treatment at any other temperature; in contrast, a cold period was required before shoot emergence took place. Seeds in the 12 populations would be classified as having 'deep simple epicotyl morphophysiological dormancy' (Baskin *et al.* 2009).

In nature, root emergence from seeds of 11 populations from Asia, North America and Europe sown in a common garden in Sweden occurred in the first autumn following the autumn–winter sowing. Although none to a few shoots emerged from these populations during the second spring following sowing, larger percentages did so in the third spring. In contrast, germination (presumably root emergence) of *V. opulus* seeds sown outdoors in England was observed during the first spring after fruiting (Lee *et al.* 1991). Kollmann and Grubb (2002) suggested that the first spring would
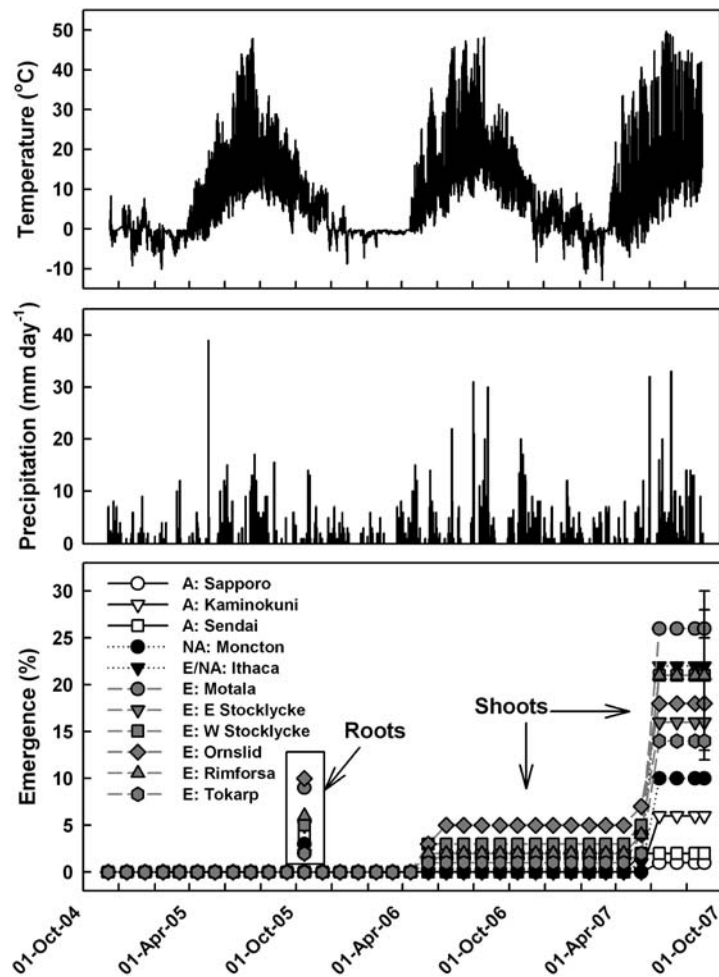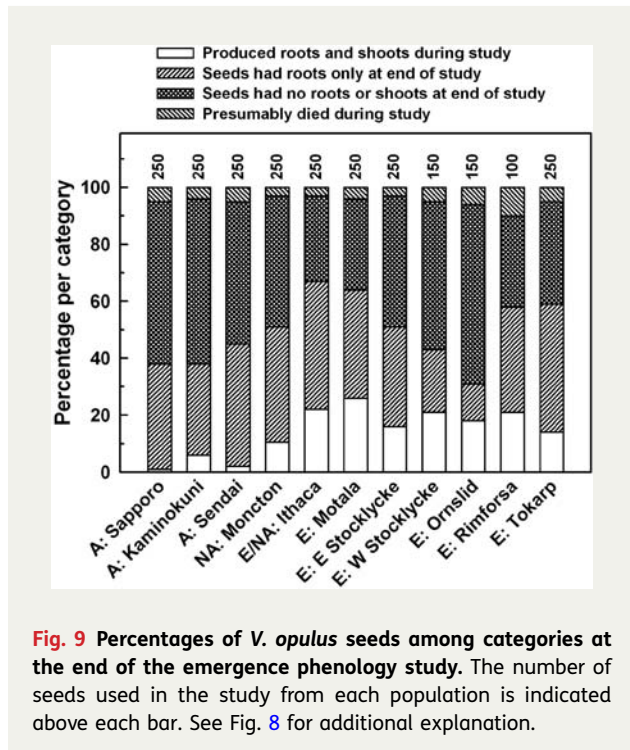
Fig. 8 Temperature, precipitation, root emergence and cumulative shoot emergence for *V. opulus* seeds from 11 populations. Populations were as explained in Fig. 4 without Mombetsu (Japan) and were sown on 13 November 2004 (or 1 January 2005 for Sendai) in a common garden in Ledberg, Sweden. Root emergence (single data points) was examined only on 7 October 2005, and shoot emergence throughout the study until 31 August 2007. Confidence intervals (95 %), based on statistics for binomial distribution, are shown only for the last data point of shoot emergence if both upper and lower intervals are ≥ 6 % (i.e. West Stocklycke, Örnslid and Rimforsa populations).

be the normal germination season of the species and that studies reporting delayed germination until years 2 or 3 may have been influenced by storage conditions of the seeds. However, our results for *V. opulus*, along with those for other temperate species of *Viburnum* with epicotyl dormancy (Giersbach 1937; Hidayati *et al.* 2005), show that delayed germination is normal. Thus, germination of *V. opulus* in the spring (after the previous autumn dispersal) is highly unlikely.

At the end of our common garden experiment (~3 years following sowing), low to moderate percentages of remaining seeds were viable and had only roots (and no shoots yet) or had not yet produced roots (or shoots). These results show that *V. opulus* has the capability of forming a persistent soil seed bank (*sensu* Walck *et al.* 2005) and a rootling bank. In contrast, transient soil seed banks (i.e. seeds persist in soil for <1 year) have been mostly reported for *V. opulus* (Thompson *et al.* 1997). Animal exclusion in our study might have affected the likelihood of seeds remaining in the soil. This idea is supported by the observation that 66 % of 150 *V. opulus* fruits placed on the ground disappeared after 16 days, primarily due to rodents (Jones and Wheelwright 1987).

The 12 populations in our study clustered into three distinct groups based on the germination characters.

Fig. 9 **Percentages of *V. opulus* seeds among categories at the end of the emergence phenology study.** The number of seeds used in the study from each population is indicated above each bar. See Fig. 8 for additional explanation.

The groups did not correspond to the (morphological) varietal status assigned to the species. Instead, populations from Asia were clustered with those from North America or from Europe; one Asian population did not group with any other population. The similarity of European populations is not too surprising as they are in close proximity. The Asian populations were geographically widely separated from each other, and as such, the amount of variation in germination was relatively broad given their wide dispersion in the PCA. The clustering of populations was mainly driven by the W → C treatment. Whereas roots and shoots from all populations in the C → W treatment emerged simultaneously, they were delayed in some populations in the W → C treatment. The different germination responses between these treatments suggest that the timing of dispersal for *V. opulus* seeds in relation to ambient temperatures may have important ecological consequences of spreading germination over time.

Viburnum opulus collected in Ithaca (USA) was determined to be a naturalized population of the European var. *opulus* based on morphology. In Witmer's (2001) study, only one of more than 50 shrubs surveyed in the Ithaca area was morphologically identified as var. *americanum*. Likewise, the germination characters of the Ithaca population clustered within a group comprising all the European populations and its E : S ratio more closely matched that recorded for the Tokarp

(Sweden) var. *opulus* population. Thus, not only have the naturalized plants in North America retained their morphological resemblance to their counterparts in Europe, but their seed ecophysiology also closely resembles them. Our results are in contrast to other studies on shrubs (Erfmeier and Bruelheide 2005) and herbs (Kudoh *et al.* 2007) that documented differences in germination between native and non-native ranges of species.

Conclusions and forward look

The degree of population divergence in our study varied among the seed and seedling traits that we examined. First, seedling development for winter (peak)-dispersed seeds (C → W treatment) and the phenology of germination were remarkably similar among populations of *V. opulus*. Comparable to morphological (Milne and Abbott 2002) or ecological (Ricklefs and Latham 1992; Svenning 2003) stasis in other Tertiary relicts, we suggest that stabilizing selection has maintained similar dormancy mechanisms, allowing the near simultaneous seedling emergence among *V. opulus* populations. There are selective advantages for root emergence in summer, allowing root system development in autumn without exposing the shoot to winter conditions, and then for shoot emergence at the beginning of a growing season (Kondo *et al.* 2011). These traits apparently have been maintained in these populations since their separation. Apart from being a long time period, a substantial part of the current geographic distribution of *V. opulus* has been glaciated or under periglacial conditions during the Pleistocene. Hence, there have been substantial range shifts, likely fluctuations in population size and possible small refugia during glacial maxima (Habel *et al.* 2010; Qiu *et al.* 2011) and, thus, ample opportunity for differentiation.

Second, at a finer scale, populations varied in seed lengths and in the level and duration of temperatures required for root and shoot emergences. In addition, seedling development for late-dispersed seeds (W → C treatment) was delayed in some populations. Seed and seedling traits may adapt to environmental conditions and differentiate populations, if the variation is genetically based. To what extent variation in *V. opulus* is genetically controlled remains to be determined. However, an interesting trend can be observed. The northern-most populations (Sweden) in our study had the broadest temperature range for root emergence and the longest cold stratification requirement for shoot emergence. In a climate where summer temperatures average 21/13 °C, it is advantageous to have root emergence occur at slightly lower temperatures as compared with southern populations. Moreover, the cold stratification

period coincides with the relatively long winter, possibly preventing precocious seedling emergence during unusual winter thaws. Altogether, these responses suggest that adaptation to the local environment has occurred.

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Contributions by the authors

The order of authorship corresponds to decreasing levels of contribution in conceiving the idea, leading the research, collecting and analysing the data, and writing the manuscript.

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Conflict of interest statement

None declared.

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