

Refugial persistence and postglacial recolonization of North America by the cold-tolerant herbaceous plant *Orthilia secunda*

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Abstract

Previous phylogeographical and palaeontological studies on the biota of northern North America have revealed a complex scenario of glacial survival in multiple refugia and differing patterns of postglacial recolonization. Many putative refugial regions have been proposed both north and south of the ice sheets for species during the Last Glacial Maximum, but the locations of many of these refugia remain a topic of great debate. In this study, we used a phylogeographical approach to elucidate the refugial and recolonization history of the herbaceous plant species *Orthilia secunda* in North America, which is found in disjunct areas in the west and east of the continent, most of which were either glaciated or lay close to the limits of the ice sheets. Analysis of 596 bp of the chloroplast *trnS-trnG* intergenic spacer and five microsatellite loci in 84 populations spanning the species' range in North America suggests that *O. secunda* persisted through the Last Glacial Maximum (LGM) in western refugia, even though palaeodistribution modelling indicated a suitable climate envelope across the entire south of the continent. The present distribution of the species has resulted from recolonization from refugia north and south of the ice sheets, most likely in Beringia or coastal regions of Alaska and British Columbia, the Washington/Oregon region in the northwest USA, and possibly from the region associated with the putative 'ice-free corridor' between the Laurentide and Cordilleran ice sheets. Our findings also highlight the importance of the Pacific Northwest as an important centre of intraspecific genetic diversity, owing to a combination of refugial persistence in the area and recolonization from other refugia.

Keywords: climate change, glaciations, last glacial maximum, Laurentide/Cordilleran ice sheets, phylogeography

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Introduction

Climate change has occurred throughout the history of the Earth. The Pleistocene epoch (1.8–0.01 MYA) in particular was an age of constant change, when numerous climatic fluctuations between glacial and interglacial periods continuously altered the landscape. During the Last Glacial Maximum (LGM; ca. 18–21 kya), large parts of northern Europe and northern North America were covered by ice sheets

and sea levels dropped by as much as 120 m, exposing extended coastline in certain areas (Clark & Mix 2002). The LGM was the last major force to shape the distribution ranges of species before the current period of anthropogenically induced climate change, which has also resulted in observed range shifts in various plants and animals (Parmesan & Yohe 2003; Root *et al.* 2003; Perry *et al.* 2005; Hickling *et al.* 2006; Parmesan 2006; Kelly & Goulden 2008). Consequently, knowledge of how organisms have responded to previous periods of climate change may allow insights into the possible impacts of present day global warming on species' ranges.

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Over the last two decades, phylogeographical studies have shown that the majority of species persisted through the LGM and previous glacial maxima in climatically suitable refugial areas, usually south of the ice sheets and permafrost. The original phylogeographical studies on the biota of Europe identified the three main southern peninsulas of Iberia, Italy and the Balkans as key refugia for temperate species (reviewed in Taberlet *et al.* 1998; Hewitt 1999; Schmitt 2007), with extant populations outside these regions being founded by northwards recolonization during the current interglacial. A growing number of studies, however, have highlighted the existence of 'cryptic' refugia at higher latitudes, particularly for cold-adapted species (reviewed in Provan & Bennett 2008; Bennett & Provan 2008; Stewart *et al.* 2010). The main implications of these cryptic refugia for species' distributions are that recolonization patterns may involve more than a simple range expansion from low to high latitudes, with a subsequent uncertainty surrounding the rate of migration during postglacial warming periods.

Phylogeographical studies in North America have produced an even more complex picture of postglacial recolonization, with a growing body of evidence for the existence of a number of putative refugia across the continent (summarised in Fig. 1; also Pielou 1991). Difficulties in identifying the locations of these refugia and

patterns of recolonization have been exacerbated by uncertainties about the exact extents of the Laurentide and the Cordilleran ice sheets during the LGM. Modelling approaches and geological and palaeontological analyses have often provided inconsistent information concerning the limits of the ice sheets and even whether the two coalesced at the LGM (Pielou 1991; Dyke *et al.* 2002; Charbit *et al.* 2007).

Cold-tolerant plant species with limited dispersal capabilities represent potentially good candidates to gain phylogeographical insights into the extent of ice sheets at the LGM, as they are generally more likely to have persisted in northern refugia than temperate species. *Orthilia secunda* (syn. *Pyrola secunda*—one-sided or serrated wintergreen) is a perennial, herbaceous plant with hermaphroditic flowers belonging to the Pyrolaceae (in some taxonomic treatments this family is subsumed into the Ericaceae) which exhibits a circum-boreal distribution in Eurasia and North America. The main areas of the species' current range in North America correspond to largely disjunct areas in the west and east of the continent that were either glaciated or which lay close to the limits of the ice sheets, although it is sporadically found further south, usually in mountainous areas in the west. Because the species is mostly restricted to dry to mesic forest habitats, it is rare or absent from central northern US states such as North

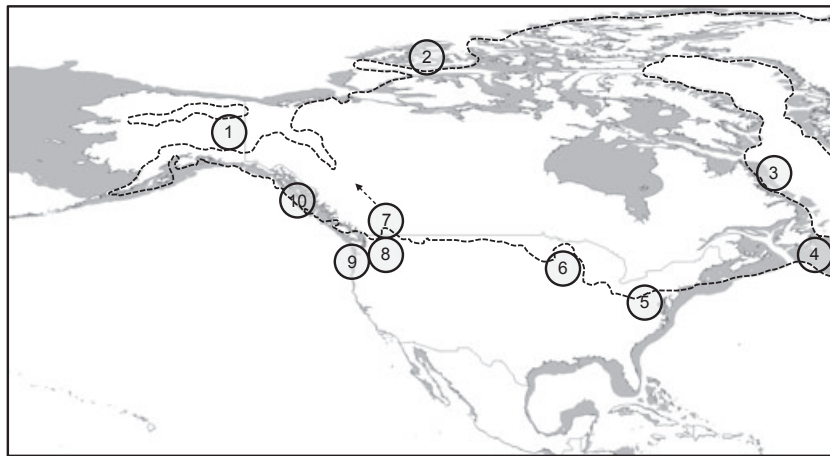


Fig. 1 Locations of proposed glacial refugia for terrestrial plants and animals in northern North America based on evidence from previous phylogeographical studies. Clockwise from top left: 1—Beringia (Holder *et al.* 1999; Tremblay & Schoen 1999; Fedorov & Stenseth 2002; Fleming & Cook 2002; Anderson *et al.* 2006; Loehr *et al.* 2006; Eidesen *et al.* 2007a,b; Maroja *et al.* 2007; Godbout *et al.* 2008; Marr *et al.* 2008; Aubry *et al.* 2009); 2—Canadian High Arctic (Holder *et al.* 1999; Tremblay & Schoen 1999; Fedorov & Stenseth 2002; Eidesen *et al.* 2007a); 3—Eastern Arctic Canada (Tremblay & Schoen 1999; Jaramillo-Correa *et al.* 2004; Godbout *et al.* 2005); 4—Grand Banks (Holder *et al.* 1999); 5—Northeastern USA (Tremblay & Schoen 1999; Austin *et al.* 2002; Jaramillo-Correa *et al.* 2004; Godbout *et al.* 2005; Eidesen *et al.* 2007a,b; Lee-Yaw *et al.* 2008); 6—'Driftless Area' (Jaramillo-Correa *et al.* 2004; Rowe *et al.* 2004; Godbout *et al.* 2005; Lee-Yaw *et al.* 2008); 7—'Ice-free Corridor' (dashed arrow indicates uncertainty concerning the extent of the ice-free corridor at the Last Glacial Maximum (LGM)—Golden & Bain 2000; Loehr *et al.* 2006); 8—Clearwater Refugium (Brunsfield & Sullivan 2005; Brunsfield *et al.* 2007); 9—Northwestern USA (Jaramillo-Correa *et al.* 2004; Loehr *et al.* 2006; Maroja *et al.* 2007; Godbout *et al.* 2008; Aubry *et al.* 2009; Latch *et al.* 2009; Tomomatsu *et al.* 2009); 10—Coastal Pacific Northwest/Haida Gwaii (Holder *et al.* 1999; Soltis *et al.* 1997; Conroy & Cook 2000; Clarke *et al.* 2001; Cook *et al.* 2001; Fleming & Cook 2002; Godbout *et al.* 2008). The heavy dashed line shows the approximate extent of the ice sheets at the Last Glacial Maximum (after Dyke *et al.* 2002).

and South Dakota and eastern Montana, and eastern Alberta and western Saskatchewan in Canada. In this study, we used a phylogeographical approach to elucidate the refugial and recolonization history of *O. secunda* in North America, and in particular to determine (i) whether this cold-adapted species may have persisted in northern refugia during the LGM, and (ii) whether its current disjunct distribution is because of survival in separate western and eastern refugia.

Materials and Methods

Sampling and DNA extraction

Samples, including material from herbarium specimens, were obtained from 84 locations spanning the entire range of *O. secunda* in North America plus one population from Greenland and three individuals from a population from Siberia (Appendix 1). Two of the North American populations (ALDM and ALSP) had been identified in herbarium records as '*O. secunda* subsp. *obtusata*'. As there is some uncertainty surrounding the taxonomic status of subsp. *obtusata* (Dorr & Barrie 1993), these populations were included in this study. DNA was extracted using the Qiagen DNeasy kit. In total, 458 individuals were sequenced for the chloroplast *trnS-trnG* intergenic spacer and 553 individuals were genotyped for five nuclear microsatellite loci.

Palaeodistribution modelling

Ecological niche modelling (ENM) was carried out to determine suitable climate envelopes for *O. secunda* in North America for the current period and at the last glacial maximum (LGM, ca. 18 kya) using the maximum entropy approach implemented in the MAXENT software package (V3.2.1; Phillips *et al.* 2006). Present-day species occurrence data were downloaded from the Global Biodiversity Information Facility data portal (<http://www.gbif.org>). A distribution model based on the 19 BIOCLIM variables in the WorldClim data set (Hijmans *et al.* 2005) was generated at 2.5 min resolution using MAXENT with the default parameters for convergence threshold (10^{-5}) and number of iterations (500), and projected onto reconstructed LGM data [Community Climate System Model (CCSM); Palaeoclimate Modelling Intercomparison Project Phase II: <http://pmip2.lscce.ipsl.fr>] to identify potential refugial areas. Duplicate records from the same locality were removed to reduce the effects of spatial autocorrelation. A presence threshold was determined using the sensitivity-specificity sum maximization approach (Cantor *et al.* 1999), and the performance of the model was tested using 25% of the occurrence data points to determine

the area under the receiver operating characteristic (ROC) curve (AUC).

Chloroplast *trnS-trnG* sequencing

Four hundred and fifty-eight samples were sequenced for the chloroplast *trnS-trnG* intergenic spacer. A product was initially amplified using the *trnS-trnG* primers of Zhang *et al.* (2005) and sequenced, and the resulting sequence was used to design species-specific primers OS-*trnS* 5'-AGATATGACTATCGTTACATTTTCATG-3' and OS-*trnG* 5'-GTATAGTAATGGATCTTTTGTCACAA-AC-3'. PCR was carried out on a MWG Primus thermal cycler using the following parameters: initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 2 min and a final extension at 72 °C for 5 min. PCR was carried out in a total volume of 20 µL containing 200 ng genomic DNA, 20 pmol of each primer, 1× PCR reaction buffer, 200 µM each dNTP, 2.5 mM MgCl₂ and 0.5 U GoTaq Flexi DNA polymerase (Promega). Five-microlitre PCR product were resolved on 1.5% agarose gels and visualised by ethidium bromide staining, and the remaining 15 µL was sequenced commercially (Macrogen, Korea).

Microsatellite genotyping

Five hundred and fifty-three individuals were genotyped for five *O. secunda* microsatellite loci previously described in Beatty *et al.* (2008). Forward primers were modified by the addition of a 19-bp M13 tail (5'-CAC-GACGTTGTAAAACGAC-3'), and reverse primers were modified by the addition of a 7 bp tail (5'-GTGTCTT-3'). PCR was carried out in a total volume of 10 µL containing 100 ng genomic DNA, 10 pmol of dye-labelled M13 primer (6-FAM or HEX), 1 pmol of tailed forward primer, 10 pmol reverse primer, 1× PCR reaction buffer, 200 µM each dNTP, 2.5 mM MgCl₂ and 0.25 U GoTaq Flexi DNA polymerase (Promega). PCR was carried out on a MWG Primus thermal cycler using the conditions described in Beatty *et al.* (2008), and genotyping was carried out on an AB3730xl capillary genotyping system. Allele sizes were scored in GENEMAPPER V4.1 using ROX-500 size standards and were checked by comparison with previously sized control samples.

Data analysis

DNA sequences for the *trnS-trnG* intergenic spacer were aligned in BIOEDIT (V7.0.9.0; Hall 1999). Three mononucleotide repeat regions were removed from the alignment, as well as four indels that appeared to have arisen via duplication. Given that the mechanisms that

generate such duplications may be similar to those that generate microsatellite length variation, it was decided to remove them from the alignment prior to further analysis. The *trnS-trnG* sequence alignment was used to construct a median-joining network using the Network software package (V4.5.1.6; <http://www.fluxus-engineering.com>).

Tests for linkage disequilibrium between pairs of microsatellite loci in each population were carried out in the program *FSTAT* (Goudet 2001). Levels of genetic diversity and F_{IS} were calculated for populations with a sample size of $N \geq 5$. Gene diversity (H) based on chloroplast *trnS-trnG* haplotype frequencies and observed and expected heterozygosity (H_O and H_E) based on nuclear microsatellite allele frequencies were calculated using the *ARLEQUIN* software package (V3.01; Excoffier *et al.* 2005). To visualise range-wide gradients in genetic diversity for both chloroplast and nuclear markers based on H and H_E , respectively, data interpolation was carried out using kriging in ArcGIS (V3.2; ESRI, Redlands, CA, USA). Levels of allelic richness (A_R) and F_{IS} were calculated using *FSTAT*.

The software package *BAPS* (V3.2; Corander *et al.* 2003) was used to identify clusters of genetically similar populations from the nuclear microsatellite data using a Bayesian approach. Ten replicates were run for all possible values of the maximum number of clusters (K) up to $K = 40$, with a burn-in period of 10 000 iterations followed by 50 000 iterations. As the program identified $K = 40$ as the most likely number of clusters, the analysis was re-run multiple times for values up to $K = 10$, $K = 20$ and $K = 30$. In each case, the program identified the maximum value of K as the most likely. It has been highlighted previously that *BAPS* tends to overestimate the true number of clusters (Latch *et al.* 2006) so because of this, and because we primarily wanted to test for congruence between the chloroplast sequence data and the nuclear microsatellite data, we ran the final analysis for $K = 3$, the number of groups identified by the chloroplast data, although the analysis was also carried out for $K = 2$ and $K = 4$ for comparison. An analysis of molecular variance (AMOVA) was carried out for both the chloroplast *trnS-trnG* data and the nuclear microsatellite data based on the three clusters identified by the *BAPS* analysis using *ARLEQUIN*.

Results

Palaeodistribution modelling of O. secunda at the LGM

The ENM based on current climate data was a largely accurate description of the present-day distribution of *O. secunda* in North America (Fig. 2). Modelling based

on reconstructed climate at the LGM identified suitable regions for the persistence of *O. secunda* both south and north of the limits of the ice sheets. South of the ice sheets, the suitable climate envelope encompassed areas such as western Washington and Oregon, where the species is currently common, as well as southern US states such as California, Arizona and New Mexico, where the species is now rare, and extensive areas in the southeast well beyond the current range of *O. secunda*. To the north, suitable areas corresponded to unglaciated regions in Alaska and Yukon, as well as large parts of the Pacific coast of British Columbia that may also have remained unglaciated at the LGM.

Chloroplast trnS-trnG sequencing

Removal of mononucleotide repeats and duplicated indels from the *trnS-trnG* alignment of the 458 samples resulted in an overall alignment length of 596 bp. These chloroplast microsatellite regions and indels did contain some degree of phylogenetic information, as they were usually correlated with other synapomorphies in the alignment, but were removed from the final analysis as they introduced homoplasious reticulations into the network, most likely due to their high levels of bidirectional mutation (Provan *et al.* 2001). The network constructed from the alignment revealed a total of 16 haplotypes (GenBank accession numbers HQ162684—HQ162699; Fig. 3). Two major groups of haplotypes exhibited a primarily northwestern vs. southern/eastern distribution, but with some occurrences of haplotypes from one group in the region occupied primarily by the other group as well. Haplotypes related to and including the common blue haplotype, hereafter referred to as the 'blue clade', were by far the most frequent in populations from Alaska, Yukon and British Columbia. One of the haplotypes in this group was found in the two populations from Alaska originally designated as '*O. secunda* subsp. *obtusata*' and the three Siberian samples. Conversely, the group of haplotypes related to the common red haplotype (the 'red clade') were relatively rare in these areas but common in southern and eastern populations. Haplotypes from a third group (the 'yellow clade') were found only in populations from Oregon, Washington and western British Columbia.

Gene diversity (H) values calculated for populations with a sample size of $N \geq 5$ ranged from 0.000 (many populations) to 0.788 [WAOF (Washington); Table S1 Supplementary Information]. Interpolation of gene diversity values across the range of *O. secunda* identified the area spanning Washington, Oregon and northern Idaho, through southwestern Alberta and southern British Columbia to the Alexander Archipelago as the main centre of diversity (Fig. 4a). Gene diversity

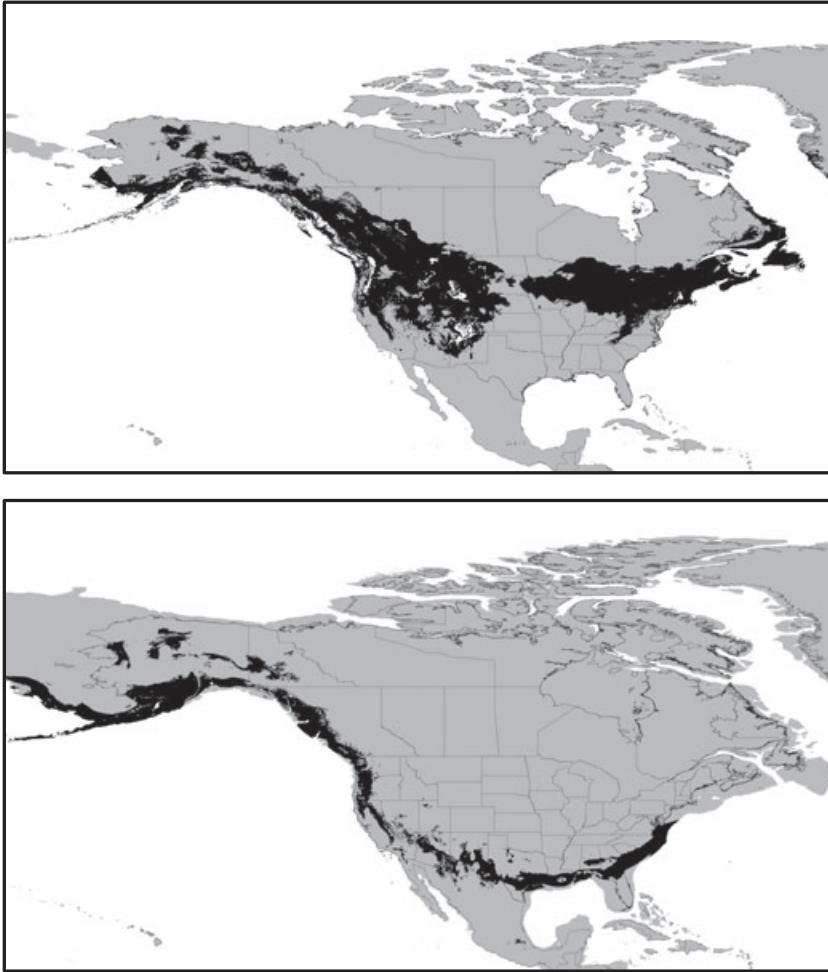


Fig. 2 Ecological niche modelling of suitable climate envelopes for *O. secunda* based on current climate (top) and reconstructed climate at the Last Glacial Maximum (LGM) (bottom).

decreased with longitude towards the east, with all populations east of *ca.* 110 °W fixed for a single haplotype, the exception being the WICC (Wisconsin) population (Fig. 3).

The AMOVA based on the three BAPS clusters indicated that 34.50% of the total genetic variation was partitioned between groups, 36.27% between populations within groups, and the remaining 29.23% within populations (Table 1a).

Nuclear microsatellite genotyping

No significant linkage disequilibrium was detected between pairs of loci after sequential Bonferroni correction. Between 13 and 33 alleles were detected at the five microsatellite loci studied (mean = 24.6) and levels of observed and expected heterozygosity (H_O and H_E) calculated for populations with a sample size of $N \geq 5$ ranged from 0.218 [COLH (Colorado)] to 0.675 [NSBU (Nova Scotia)] and from 0.455 [NSUT (Nova Scotia)] to 0.840 [ALLS (Alaska)], respectively (Table S1 Supplementary Information). Levels of allelic richness (A_R)

ranged from 2.354 [MEBM (Maine)] to 4.938 [MNCF (Minnesota); Table S1 Supplementary Information]. Interpolation of expected heterozygosity values across the range of *O. secunda* identified the area around the northwest coast of British Columbia and the Alexander Archipelago, southwest Yukon and southern Alaska as the main centre of diversity (Fig. 4b). The area of lowest diversity was in the east, around New England and the eastern provinces of Canada. Levels of inbreeding were generally high, with a mean F_{IS} of 0.321 (Table S1 Supplementary Information). Summary statistics for all microsatellite loci are given in Table S2 (Supplementary Information).

The Bayesian assignment of populations to one of $K = 3$ clusters based on microsatellite allele frequencies gave results that were highly congruent with those from the chloroplast *trnS-trnG* sequence data (Fig. 5). Most strikingly, the five populations that contained chloroplast haplotypes solely or predominantly from the yellow clade [ORHR (Oregon), WAOF (Washington), V188686, V179192 and V150749 (all British Columbia)] formed a cluster with the WAMA (Washington)

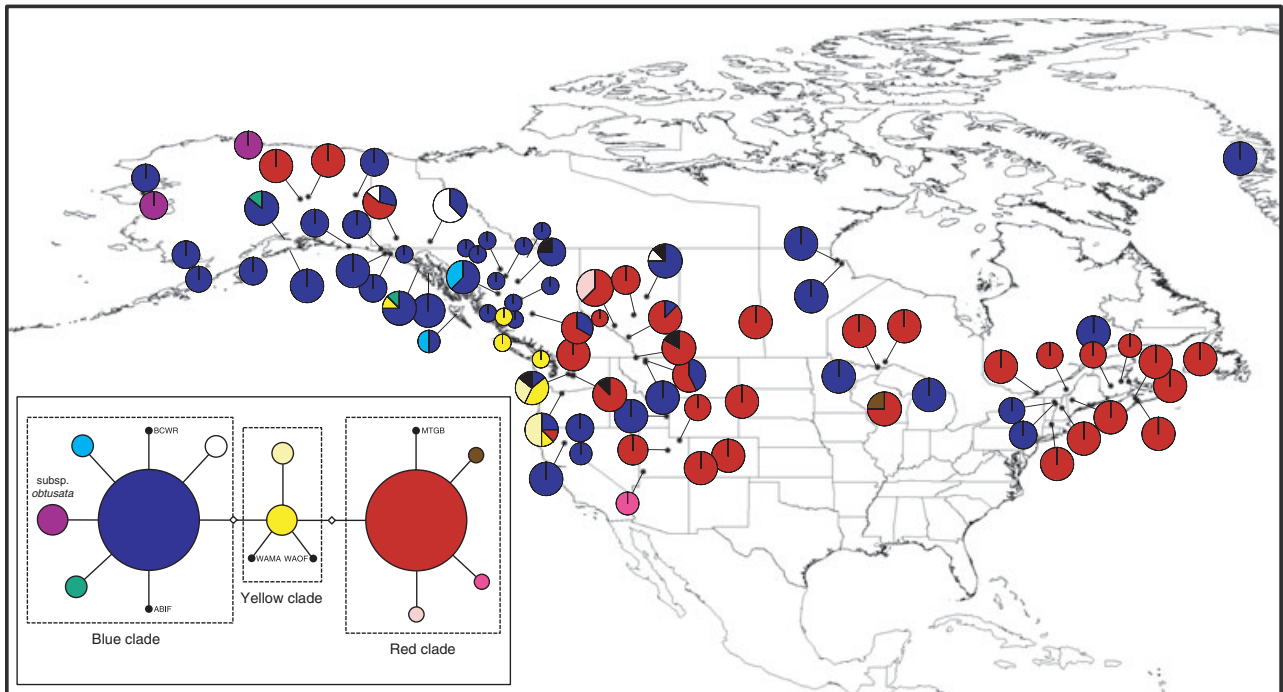


Fig. 3 Distribution of chloroplast *trnS-trnG* haplotypes. Pie chart sizes are approximately proportional to sample size, with the smallest circles representing $N = 1$ and the largest representing $N = 8$. Inset shows the phylogenetic relationships between the 16 haplotypes. Open diamonds represent missing haplotypes, and small black circles represent unique haplotypes i.e. those found in a single individual. The population of origin of each unique haplotype is indicated.

population, which contained a unique chloroplast haplotype that also belonged to the yellow clade. With a few exceptions, the remaining populations formed two clusters that corresponded to the red and blue chloroplast clades. The BAPS analysis based on $K = 2$ grouped the populations from the yellow cluster with the blue cluster populations. Analysis based on $K = 4$ separated the most northeasterly populations [NHVW (New Hampshire), MEBM (Maine), NBTY, NBBG (New Brunswick), NSWN, NSBU, NSUT (Nova Scotia) and NLMI (Newfoundland)] from the remaining populations in the red cluster. Finally, the AMOVA based on the three BAPS clusters indicated that 9.72% of the total genetic variation was partitioned between groups, 18.34% between populations within groups and the remaining 71.94% within populations (Table 1b).

Discussion

Postglacial recolonization by O. secunda from western refugia

The findings of the present study indicate that the major strongholds for *O. secunda* in North America during the glacial episodes associated with previous periods of climate change were confined to western

areas of the continent. The main centre of diversity based on a combination of chloroplast and nuclear markers is the area stretching from Oregon through Washington and British Columbia to southern Alaska. The chloroplast data indicate particularly high genetic diversity west of the Rocky Mountains in Washington and Oregon, an area just south of the limit of the Cordilleran ice sheet at the LGM that would have been a suitable refugium for the species at the time according to the ENM. An early comparative phylogeographical study of plants in the Pacific Northwest by Soltis *et al.* (1997) also highlighted this region as an area of high diversity, but this was because of contact between divergent lineages from the north and the south. This is not solely the case for *O. secunda* because the occurrence of several endemic haplotypes is characteristic of refugial persistence rather than admixture (Provan & Bennett 2008), although some admixture may have occurred here. The presence of a refugium in this area is further confirmed by the geographical localisation of populations belonging to the yellow cluster in the BAPS analysis in the region. Populations from this cluster are not found elsewhere, suggesting *in-situ* persistence throughout the LGM. Furthermore, this region has previously been identified as a putative refugium for the plant species *Camassia quamash* (Tomimatsu *et al.* 2009).

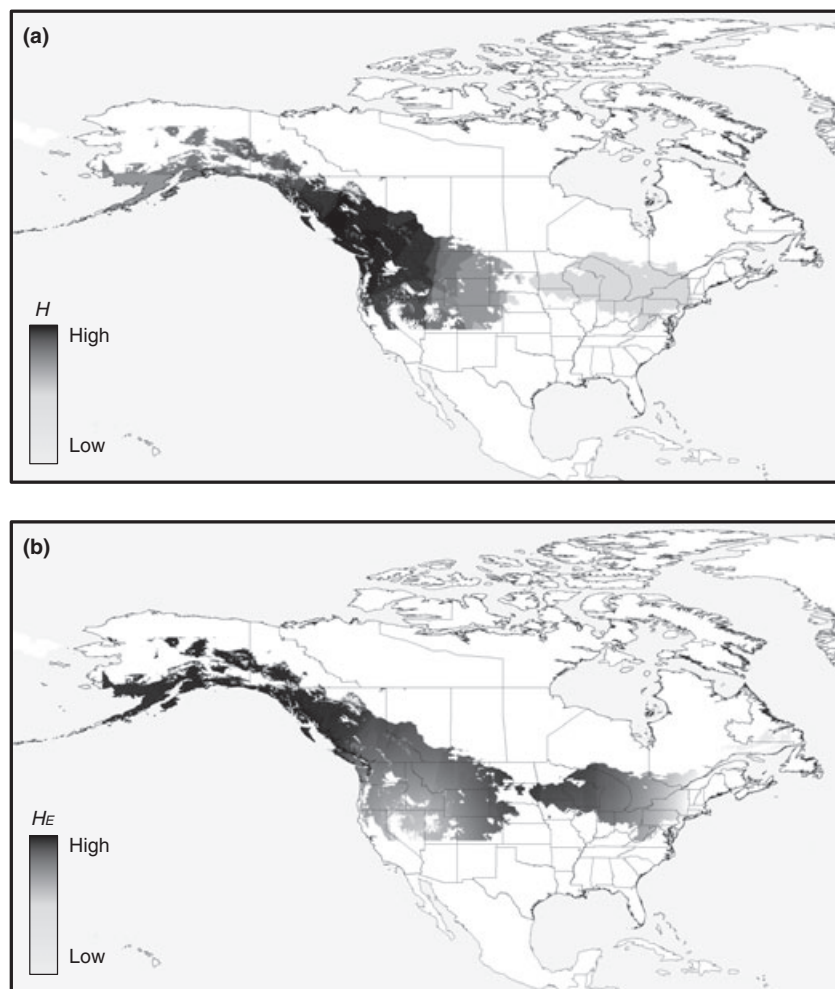


Fig. 4 Geographical gradients in genetic diversity based on (a) chloroplast haplotype gene diversity (H) and (b) nuclear microsatellite expected heterozygosity (H_E). Note that the lower part of the western distribution range is not included in the kriging, because all populations from the south had sample sizes less than 5, and thus, diversity values were not calculated for these populations.

Table 1 Analysis of molecular variance based on (a) chloroplast *trnS-trnG* haplotype frequencies, and (b) nuclear microsatellite allele frequencies. Groups were defined based on the results of the BAPS clustering ($K = 3$)

Source of variation	Sum of squares	Variance	% variation
(a)			
Among groups	87.302	0.191	34.50
Among populations within groups	277.243	0.201	36.27
Within populations	304.868	0.162	29.23
(b)			
Among groups	129.080	0.218	9.72
Among populations within groups	523.380	0.411	18.34
Within populations	1474.621	1.613	71.94

The predominance of both the blue clade haplotypes and populations assigned to the blue cluster in the BAPS analysis in northwestern populations suggests the existence of a further refugium, possibly in Beringia or

in the coastal British Columbia/Alexander Archipelago region. Both these regions have previously been highlighted as putative refugial areas based on molecular, fossil and biogeographical evidence (reviewed in Cook *et al.* 2001; Abbott & Brochmann 2003) and would have been climatically suitable for *O. secunda* based on the ENM. There is a possibility that the dominance of the blue clade in the north may reflect a pattern of 'leading edge' recolonisation from the Oregon/Washington refugium (Cwynar & MacDonald 1987; Soltis *et al.* 1997), but the occurrence of several haplotypes in northern populations (e.g. in Haida Gwaii and the Alexander Archipelago) that are absent from areas south of the limit of the ice sheets would suggest that persistence in one or more northern refugia is the more likely scenario.

Phylogeographical studies have indicated Siberian Beringia as a refugium for several plant species (Eidensen *et al.* 2007a,b; Schönswetter *et al.* 2007; Ehrich *et al.* 2008). Thus, there is the possibility that *O. secunda* recolonized North America from a Siberian refugium.

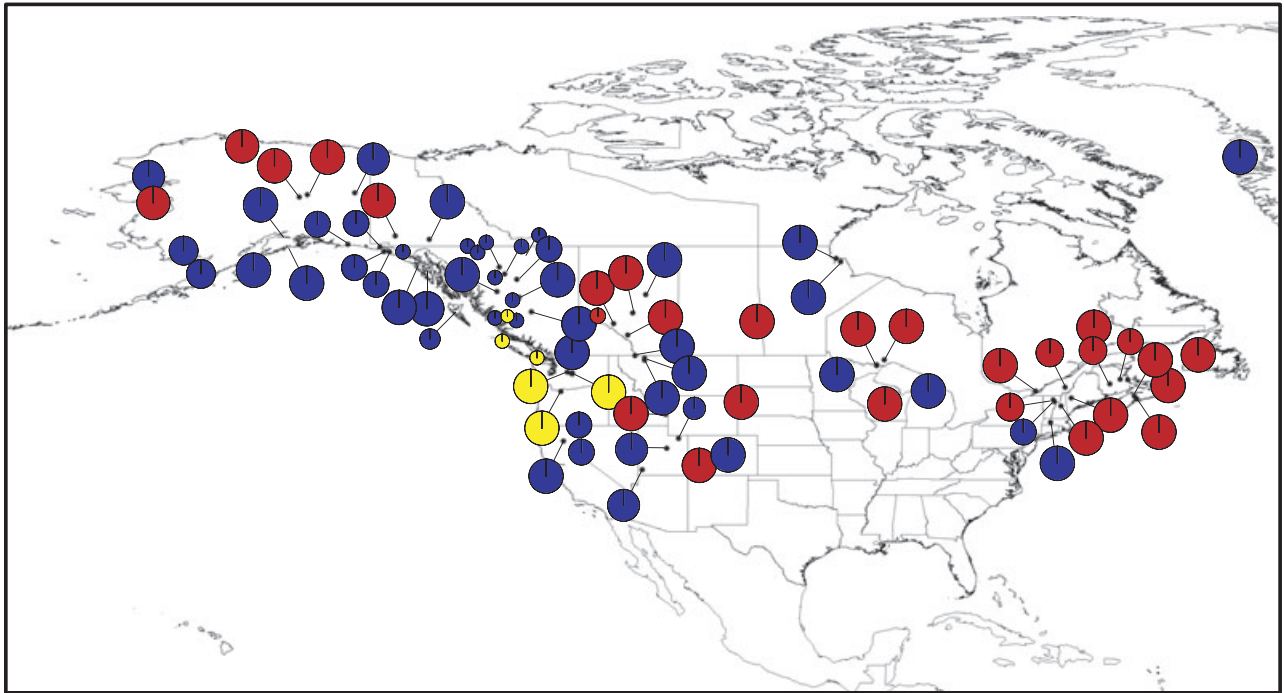


Fig. 5 Assignment of populations to $K = 3$ clusters based on BAPS analysis of the nuclear microsatellite data. Each cluster is represented by a different colour.

Although only a limited number of samples from Siberia were analysed in the present study, as the species is rare and most of the region is difficult to access, they shared a haplotype with the two *O. secunda* subsp. *obtusata* populations analysed which came from north-western Alaska. The taxonomic status of subsp. *obtusata* is a matter of some debate, but the fact that the unique chloroplast haplotype representative of these three populations was not found elsewhere in the populations analysed suggests at best a limited role of Siberian *O. secunda* populations in any colonization of North America. This could be further clarified by more extensive sampling in Eurasia.

The relative absence of haplotypes from the red clade and low levels of population assignment to the red microsatellite cluster in more northerly populations suggests that the refugium associated with these haplotypes was situated south of the ice sheets. The occurrence of private haplotypes from this clade in two of the populations along the Alberta/British Columbia border (MTGB and ABMS) coupled with the relatively high levels of genetic diversity in the surrounding area might represent further evidence for a refugium associated with the proposed 'ice-free corridor' between the Cordilleran and Laurentide ice sheets at the LGM. The existence of such a region, and its possible extent, is a matter of ongoing debate (Pielou 1991; Mandryk *et al.* 2001; Dyke *et al.* 2002). Modelling studies (Charbit *et al.* 2007) based on

several atmospheric general circulation models have indicated a range of possible scenarios, from complete coalescence of the ice sheets to the existence of continuous unglaciated regions between the two ice sheets. Geological and palaeontological evidence (Catto *et al.* 1996) suggests that a lack of synchrony between the advances of the Cordilleran and Laurentide glaciers would have led to a 'fluctuating' ice-free zone between Alberta and British Columbia that was habitable by plants and small animals. Phylogeographical studies have also indicated the existence of putative cryptic glacial refugia at the southern end of the ice-free corridor (Golden & Bain 2000), as well as in regions further north that were generally believed to be situated well within the limits of the ice sheets (Loehr *et al.* 2006; Marr *et al.* 2008). The apparently limited gene flow between populations north and south of the ice sheets in this study, characterized by a paucity of haplotypes from the red clade north of ca. 54°N latitude suggests that the corridor was not completely open during the LGM. Populations of the cold-tolerant *O. secunda* were more likely confined to small areas of suitable habitat near the glaciers or to nunataks, and any gene flow through the corridor would have followed the relatively rapid deglaciation that commenced ca. 14 KA (Kleman *et al.* 2010).

An alternative location for a red clade refugium could be the 'Driftless Region', an apparently unglaciated area

around southwestern Wisconsin, southeastern Minnesota and northeastern Iowa. Phylogeographical (Jaramillo-Correa *et al.* 2004; Rowe *et al.* 2004; Godbout *et al.* 2005; Lee-Yaw *et al.* 2008) and palaeontological (Jackson *et al.* 2000) evidence suggest that this area was a refugium for several plant and animal species during the LGM. The occurrence of a unique haplotype in the WICC (Wisconsin) population and the fact that this area possessed the highest levels of nuclear genetic diversity in the eastern part of the range of *O. secunda* also indicate a possible refugium. Alternatively, it may be that *O. secunda* is now absent from the area that was the source of the red clade and cluster, after the change from forest to grassland since the LGM.

Although *O. secunda* is also relatively common in northeastern North America, we found no evidence for an eastern refugium. Phylogeographical signatures of refugial areas include high levels of genetic diversity and the presence of endemic haplotypes (reviewed in Provan & Bennett 2008), but levels of genetic diversity for both the chloroplast and nuclear markers were lowest in the east. This is somewhat surprising, given that previous rangewide phylogeographical studies of plant species with a largely continental distribution in northern North America have generally indicated the occurrence of separate western and eastern refugia (Jaramillo-Correa *et al.* 2004; Alsos *et al.* 2005; Godbout *et al.* 2005; Keller *et al.* 2010; de Lafontaine *et al.* 2010). A comparison of the current and past modelled distributions of *O. secunda* shows that whilst there are areas of overlap in the west, which correspond to the putative refugial regions described previously, this is not the case in the east, where possible refugial areas lie well south of the current distribution of the species. This would suggest that *O. secunda* did not track changes in available habitat during the last glacial period to any great degree, and that populations in climatically unsuitable areas were extirpated. Subsequent recolonization from separate western refugia would have given rise to the pattern of chloroplast haplotype diversity observed today, with central and eastern populations comprised solely of either the most common red or blue haplotypes. Although *O. secunda* currently exhibits a disjunct distribution in North America, pollen and macrofossil evidence indicates the existence of transcontinental boreal forests immediately south of the Laurentide ice sheet at both the LGM and throughout the deglaciation (Strong & Hills 2005; Yansa 2006). The presence of extensive suitable habitat would have facilitated the eastward spread of *O. secunda* from a southern refugium (red haplotype) followed by recolonization from the northern refugium (blue haplotype).

The importance of the Pacific Northwest: a hotspot and a melting pot

The Pacific Northwest has previously been identified in phylogeographical and biogeographical studies as a key centre of diversity and endemism for a range of plant and animal species (Hultén 1968; Rogers *et al.* 1991; Soltis *et al.* 1997; Brunfield *et al.* 2001; Cook *et al.* 2001). Phylogeographical studies on various plant (Godbout *et al.* 2008; Tomomatsu *et al.* 2009) and animal (Fleming & Cook 2002; Stone *et al.* 2002; Burg *et al.* 2005; Latch *et al.* 2009) species have indicated high levels of genetic variation and unique genotypes in the area, the 'classic' signatures of glacial refugia (Provan & Bennett 2008). This is also the case for the distribution of intraspecific genetic diversity in *O. secunda*. A previous comparative phylogeographical study on European tree and shrub species (Petit *et al.* 2003) demonstrated that whilst refugial areas contained unique genetic variation ('hotspots'), the areas with the highest levels of genetic diversity were those further north, where haplotypes from distinct refugial lineages had become admixed ('melting pots'). The high diversity observed in the Washington and Oregon populations in the present study would appear to be the result of both of these processes. In addition to being the likely refugium for the yellow clade, based on both chloroplast and nuclear data, these areas were also probably recolonized from the northern (blue) refugium and the refugium for the red clade to the east. Thus, the area is both a 'hotspot' and a 'melting pot' for genetic diversity in *O. secunda*, a factor that should be borne in mind should the necessity to formulate conservation strategies for the species arise.

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Gemma Beatty is studying for a PhD under the supervision of Jim Provan, comparing how postglacial recolonization and range-edge effects have shaped the genetic diversity of several northern hemisphere Monotropeae species. This PhD represents a continuation of her Masters research into range-edge effects in *Orthilia secunda*. Jim Provan is a Senior Lecturer in Evolutionary Genetics at Queen's University Belfast. His research interests focus on examining the effects of threats to biodiversity such as climate change and habitat loss on populations at the genetic level.

Supporting information

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

Table S1 Summary statistics by population. (NC—not calculated owing to small sample size). H —gene diversity; H_O —observed heterozygosity; H_E —expected heterozygosity; A_R —allelic richness standardised to $N = 5$; F_{IS} —inbreeding coefficient

Table S2 Summary statistics by locus averaged over populations (calculated for populations with $N \geq 5$). A —number of alleles; H_O —observed heterozygosity; H_E —expected heterozygosity; F_{IT} , F_{ST} , F_{IS} —Weir and Cockerham's F -statistics

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Appendix 1

Samples analysed in the present study. N_{nuc} —number of samples analysed for nuclear microsatellites; N_{cp} —number of samples sequenced for the chloroplast *trns-trnG* intergenic spacer

Country	State/Province	Location	Code	Lat/Long (N/W)	N_{nuc}	N_{cp}	Collector	
Canada	Alberta	Mount Stelfox	ABMS	52.174/116.491	8	8	Dorothy Fabijan	
		Coyote Lake	ABCL	53.272/114.543	8	6	Dorothy Fabijan	
		Ilka Fen	ABIF	55.097/113.260	8	6	Dorothy Fabijan	
	British Columbia	Calgary	ABCA	51.033/115.050	8	8	Lawrence Harder	
		Manning Park	BCMP	49.064/120.782	8	8	Ron Long	
		Wrede Range	BCWR	56.632/126.206	5	4	Ken Marr	
		Brothers Lake	BCBR	57.200/127.417	4	3	Ken Marr	
		Anemine Mountain	BCAM	57.867/127.967	3	1	Ken Marr	
		Prince George	BCPG	53.400/124.750	8	6	Bruce Rogers	
		Babine Lake Road	BCBL	54.771/126.843	4	4	Karen McKeown	
		Babine Mountains	BCBM	54.854/125.991	8	8	Karen McKeown	
		Chapman Lake Road	BCCL	54.865/126.646	4	4	Karen McKeown	
		Kitwanga	BCKI	55.407/128.214	8	8	Karen McKeown	
		Vancouver Island	V179192	48.733/124.283	1	1	Royal BC Museum Herbarium	
		Vancouver Island	V188686	50.466/127.800	1	1	Royal BC Museum Herbarium	
		Mount Robson Park	V080791	52.833/118.500	1	1	Royal BC Museum Herbarium	
		Kimsquit River	V167706	52.950/127.133	1	1	Royal BC Museum Herbarium	
		Kitlope River	V150749	53.217/127.850	1	1	Royal BC Museum Herbarium	
		Queen Charlotte Islands	V171416	53.267/132.233	1	1	Royal BC Museum Herbarium	
		Princess Royal Island	V193787	53.275/129.057	1	1	Royal BC Museum Herbarium	
		Queen Charlotte Islands	V189596	53.559/132.344	1	1	Royal BC Museum Herbarium	
		N/A	V194659	56.952/128.594	1	1	Royal BC Museum Herbarium	
		Muncho Lake Park	V200496	58.829/125.519	1	1	Royal BC Museum Herbarium	
		N/A	V192022	59.437/130.247	1	1	Royal BC Museum Herbarium	
		Tatshenshini River	V079085	59.483/137.733	1	1	Royal BC Museum Herbarium	
		N/A	V079476	59.917/131.300	1	1	Royal BC Museum Herbarium	
	Manitoba	Wapusk National Park	MBWP	58.433/93.644	8	6	Bruce Bennett	
		Churchill	MBCH	58.745/94.115	8	7	Peter Kevan	
	Newfoundl and	Mount Ignoble	NLMI	48.999/57.752	8	7	Maria Voitk	
		Taymouth	NBTY	46.183/66.600	6	6	Mike MacDonald	
	Canada	New Brunswick	Rogersville	NBRG	46.639/65.564	4	4	Mike MacDonald
			Upper Tantallon	NSUT	44/700/63.883	8	8	Richard Lapaix
	Nova Scotia	Windsor	NSWN	44.983/64.133	8	8	Richard Lapaix	

Appendix 1 (Continued)

Country	State/Province	Location	Code	Lat/Long (N/W)	N_{nuc}	N_{cp}	Collector
		Baddeck	NSBD	46.100/60.750	8	8	Richard Lapaix
	Ontario	Thunder Bay	ONTB	48.550/89.283	8	8	Erika North
	Quebec	Parc Nacional d'Oka	QCPO	45.467/74.052	8	8	Stephane Bailleul
		Parc Frontenac	QCPF	45.853/71.097	5	5	Andre Lapointe
		Mont Manic	QMMM	51.601/68.266	8	8	Andre Lapointe
	Saskatchewan	Hudson Bay	SKHB	52.850/102.400	8	8	Glen Lee
	Yukon	Whitehorse	YKWH	60.690/135.006	8	8	Bruce Bennett
		Kluane Lake	YKKL	61.029/138.408	8	7	Jennie McLean
		Boutellier Summit	YKBS	61.033/138.367	8	7	Jennie McLean
USA	Alaska	Mount Parish	ALMP	57.828/135.145	8	6	Brad Kriekhaus
		Neka River	ALNR	58.072/135.811	8	8	Brad Kriekhaus
		Harlequin Lake	ALHL	59.416/139.013	5	5	Ellen Anderson
		Lower Situk River	ALLS	59.459/139.581	5	5	Ellen Anderson
		Fairbanks Quad	ALFQ	64.936/148.087	8	8	Carolyn Parker
		Chatanika River	ALCR	65.192/147.259	8	8	Allan Batten
		Mud Creek	UAFM132987	57.150/158.080	5	4	University of Alaska at Fairbanks
		Kodiak Island	UAFM157544	57.668/153.258	8	4	University of Alaska at Fairbanks
		Togiak Lake	UAFM113283	59.530/159.700	5	5	University of Alaska at Fairbanks
		Malaspina Forelands	UAFM153489	59.902/139.951	5	2	University of Alaska at Fairbanks
		Weeping Peat	UAFM136860	60.200/143.200	4	4	University of Alaska at Fairbanks
		Yukon River Valley	UAFM147332	65.380/142.510	6	4	University of Alaska at Fairbanks
		Seward Peninsula	UAFM112148	65.550/163.860	5	5	University of Alaska at Fairbanks
		Kakagrak Hills	UAFM144319	67.270/163.670	6	5	University of Alaska at Fairbanks
		De Long Mountains	UAFM134760	68.270/159.910	6	6	University of Alaska at Fairbanks
		Tonsina Point	ALTP	60.051/149.435	8	7	Matt Carlson
		Kenai Peninsula	ALKP	60.870/149.630	8	7	Matt Carlson
USA	California	Lassen National Forest	CALF	40.376/121.333	8	8	Kirsten Bovee/Chris Ivey
		Fort Bidwell	NYBG762091	41.861/120.161	4	4	New York Botanical Garden
	Colorado	Lizard Head	COLH	37.867/108.050	8	8	Al Schneider
		Dome Rock	CODR	38.840/105.190	8	7	Tass Kelso/George Maentz
	Idaho	Anderson Creek	NYBG737389	43.113/111.271	8	7	New York Botanical Garden
	Maine	Blueberry Mountain	MEBM	44.768/70.480	8	8	Andy Cutko
	Massachusetts	Elmer Brook	MAEB	42.298/72.544	8	8	Karen Searcy
	Michigan	Orchis Fen	MIOF	45.400/84.860	8	5	Melanie Gunn
	Minnesota	Chippewa National Forest	MNCF	47.156/94.078	8	8	Ethan Perry
		Grand Portage	MNGP	48.036/90.037	8	7	Lynden Gerdes/Chel Anderson
	Montana	Glacier National Park	MTGA	48.485/113.357	8	7	Jennifer Hintz/Jennifer Asebrook
			MTGB	48.700/113.367	8	7	Jennifer Hintz/Jennifer Asebrook
			MTGC	48.974/114.325	8	6	Jennifer Hintz/Jennifer Asebrook
	Nevada	Mount Rose Ski Lodge	NYBG188324	39.315/119.899	4	2	New York Botanical Garden
		Schell Creek Range	NYBG188329	39.413/114.599	5	2	New York Botanical Garden
	New Hampshire	Waterville Valley	NHWV	43.950/71.500	8	8	Jean Howe
	Oregon	Hood River	ORHR	45.414/121.790	8	8	Diana Jolles
	Utah	Pine Valley Mountains	NYBG188334	37.540/113.602	6	6	New York Botanical Garden
		Eccles Canyon	NYBG188326	39.684/111.156	6	6	New York Botanical Garden
		Lily Lake	NYBG188303	40.695/109.962	3	3	New York Botanical Garden
	Vermont	Ewells Mills Swamp	VTEM	44.373/72.169	4	2	Brett Engstrom
		Greensboro	VTGR	44.576/72.296	4	4	Allison Stenger
	Washington	Okanogan National Forest	WAOF	47.379/121.123	8	7	Helen Lau
		Maverick	WAMA	47.243/120.695	8	8	Helen Lau
	Wisconsin	Chaffee Creek	WICC	43.950/89.362	8	8	Neil Harriman
Greenland	Qeqertarsuaq	Sletten	GRSL	69.254/53.525	8	8	Marianne Philipp
Siberia	Sakha	Anabas River	SIAR	72.400/113.500(E)	3	3	Olivier Gilg