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Evolution in the Arctic: a phylogeographic analysis of the circumarctic plant, *Saxifraga oppositifolia* (Purple saxifrage)

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Summary

- A survey of chloroplast DNA variation in the circumarctic-alpine plant, *Saxifraga oppositifolia*, has resolved two highly divergent cpDNA lineages with geographically widespread and mainly allopatric distributions that are largely concordant with those of two subspecies, that is, subspp. *oppositifolia* and *glandulisepala*. These subspecies differ for a single morphological trait and hence level of morphological divergence does not equate to molecular divergence within the species.
- The two cpDNA lineages were estimated to have diverged from their most recent common ancestor 5.37–3.76 Ma, that is, during the early to mid-Pliocene.
- A nested clade analysis was conducted in an attempt to determine how past episodes of range fragmentation, range expansion and long distance dispersal may have influenced the geographical distribution of cpDNA haplotypes.
- In Alaska – a known refugium for the species during the last ice-age – high levels of cpDNA diversity may be partly explained by divergence between populations that were isolated in different ice-free regions. It remains to be established whether the two subspecies of *S. oppositifolia* exhibit some form of reproductive isolation from each other under conditions of sympatry.

Key words: Arctic flora, plant evolution, phylogeography, molecular clock, nested clade analysis, glacial refugia, Quaternary, *Saxifraga oppositifolia* (Purple saxifrage).

Introduction

The highly dynamic nature of climate change in the Arctic during the late Tertiary (approximately 15–2 Ma) and throughout the Quaternary (2 Ma to the present) led to the origin of an entirely new flora in the region and created numerous scenarios for the potential origin of new plant species. The present-day arctic flora became established during the late Pliocene (*c.* 3 Ma) and is currently composed of approx. 1500 species (Murray, 1981; Murray *et al.*, 1995). Global temperatures decreased markedly from the mid-Miocene (15 Ma) until the beginning of the Pleistocene (approx. 2 Ma; Lear *et al.*, 2000; Zachos *et al.*, 2001), and by the late Pliocene a circumarctic belt of tundra was present (Matthews, 1979; Matthews & Ovenden, 1990; Murray, 1995). The arctic flora is derived partly from the pre-existing Arcto-Tertiary flora and also from ancestors that lived at high altitudes in mountain ranges to the south during the Tertiary (Hultén, 1937; Tolmachev, 1960; Weber, 1965, 2003; Hedberg, 1992; Murray, 1995). These mountain ranges extend north to the Arctic and are thought to have served as migration routes as global temperatures fell significantly from the mid-Miocene onwards. Hultén (1937) proposed that by the late Tertiary many arctic plants had achieved circumarctic distributions, which later were greatly affected by Pleistocene glaciations.

During Pleistocene ice-ages, species with previously wide and continuous distributions in the Arctic would have suffered marked reductions in range size, as a result of the massive extension of ice-sheets over large areas of north-west Europe and North America (Fig. 1). As species became restricted to refugia mainly at the periphery of ice-sheets, their distributions would have suffered fragmentation with populations

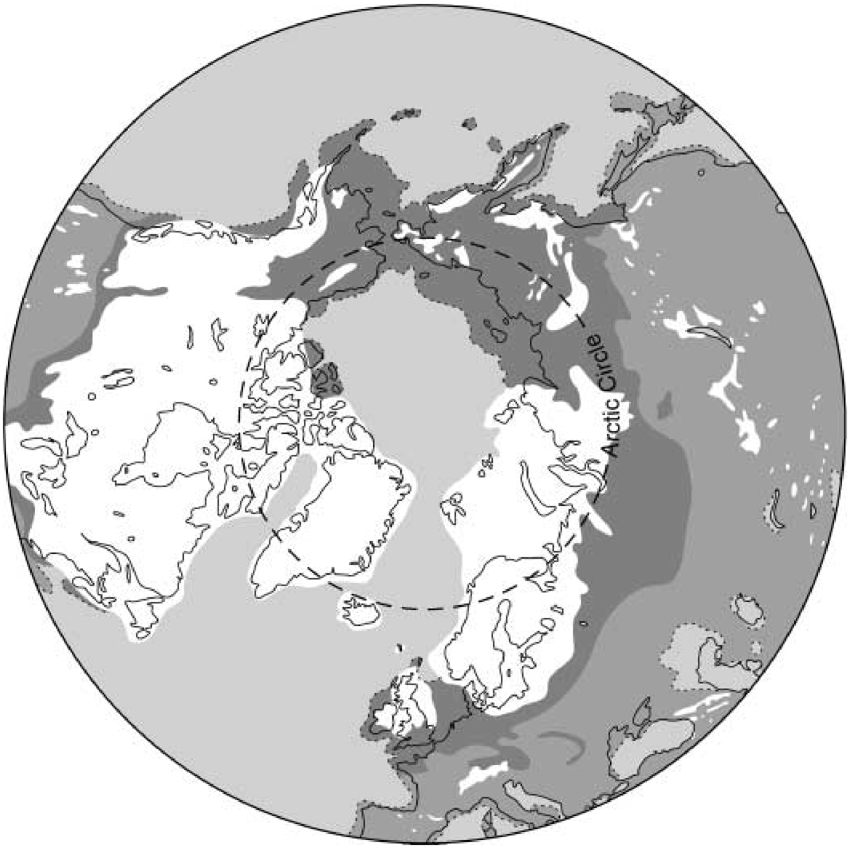

Fig. 1 Distribution of ice cover (white) and tundra (dark grey) in the Northern Hemisphere at the last glacial maximum (modified from Abbott & Brochmann, 2003).

becoming isolated from each other by long distances and large geographical barriers. Such populations would be expected to diverge over time because of the effects of chance and/or selection, causing molecular and possibly morphological differences to accumulate. Whether this process resulted in the origin of new species is debatable. In many instances different populations may have occupied a similar harsh environment, and might not have been subjected to strong divergent selection. However, changes in ploidy, the fixation of minor mutations and or chromosome repatterning could create strong postzygotic reproductive barriers between isolates, and thus lead to speciation (Abbott & Brochmann, 2003).

Following the retreat of ice sheets at the end of a glaciation, many previously isolated periglacial populations would have expanded their ranges into deglaciated areas, possibly reuniting with other conspecifics in 'secondary contact zones'. Such zones could be 'hotspots' for further evolution with several possible consequences. First, divergent material might interbreed without reduction of fertility in 'hybrid' offspring. Thus a genetically more varied population could form, and following recombination, differences between parent populations would be erased. Brochmann *et al.* (2003b) have argued that small populations of the most hardy arctic species which survived on ice-free mountain tops (nunataks) within glaciated regions, would have been genetically swamped by migrants from periglacial regions during the early postglacial period, thus erasing locally evolved differences in nunatak material.

A second possibility is that hybrid progeny in a contact zone might have reduced fertility and/or vigour allowing parent populations to remain distinct with reinforcement leading to the establishment of a prezygotic breeding barrier between them, and the formation of two new species. In the extreme, if hybrids were highly sterile, recombination might result in the origin of a new homoploid hybrid species (Rieseberg, 1997) or chromosome doubling could produce a new allopolyploid species (Stebbins, 1984; Brochmann *et al.*, 2003a). Many arctic plants reproduce asexually or are autogamous and Brochmann *et al.* (2003b) have documented that most North Atlantic arctic endemics are self-fertilizing or asexual polyploids, and some of these originated *in situ* during the Pleistocene interglacials following hybridization between non-endemic taxa. For example, there is strong molecular evidence that three new allopolyploid species of *Saxifraga* (*opdalensis*, *osloensis*, and *svalbardensis*) originated during the Holocene after the spread of their parent species into deglaciated areas of Scandinavia (Brochmann *et al.*, 1996, 1998; Steen *et al.*, 2000).

In summary, there are many potential scenarios in the arctic flora for speciation to be investigated that relate to Pleistocene cycles of glaciation and interglaciation. Indeed, there are already several well-documented examples of neospecies known to have originated during the Holocene (Abbott & Brochmann, 2003), and detailed investigation of these will clearly be informative about the speciation process.

In this paper, we focus on recent work that has investigated the phylogeography of the circumarctic-alpine plant, *Saxifraga oppositifolia* (Purple saxifrage). This has examined the geographical distribution of genealogical lineages within the species. Such an analysis can indicate how historical events, such as range fragmentation, range expansion and long-distance dispersal, as well as current levels of gene flow, have influenced the present-day distribution of lineages. It is therefore informative of a species' evolutionary history and provides a framework to an understanding of the speciation process if certain lineages evolve over time into new species (Avise, 2000). Here, we first briefly review previously published work on the phylogeography of *S. oppositifolia* based on surveys of cpDNA and ITS variation and attempt to date key processes of evolutionary divergence. We then examine more closely the possible causes of association between genealogical and geographic structure in the species using nested clade analysis of cpDNA variation (Templeton, 1998). Finally, we examine in some detail the pattern of cpDNA variation found in the species in Alaska. There is clear evidence from fossil (Goetcheus & Birks, 2001) and molecular studies (Abbott *et al.*, 2000), that Alaska was a refugium for *S. oppositifolia* during the last ice-age and it is possible that the species has occurred continuously in this region throughout the Pleistocene (Abbott & Brochmann, 2003). We examine therefore the possibility that the present-day geographic distribution of cpDNA variation in Alaskan material of *S. oppositifolia* may reflect, in part, population isolation during glacial periods of range fragmentation. Throughout this paper we attempt to examine events (historical and ongoing) that have led to cpDNA divergence within *S. oppositifolia*, and consider whether any of these events might be precursors of subspeciation or speciation.

Phylogeography of *Saxifraga oppositifolia*

Chloroplast DNA variation

Saxifraga oppositifolia L. (Saxifragaceae) is a long-lived, evergreen, runner-forming cushion plant (Webb & Gornall, 1989) that occurs throughout the Arctic and in many southern mountain ranges in Eurasia and North America. It occurs in both diploid ($2n = 26$) and tetraploid ($2n = 52$) forms and reproduces mainly by outcrossing (Stenström & Molau, 1992), although selfing is possible (Gugerli, 1997). Hultén (1973) described two widespread subspecies of *S. oppositifolia* – ssp. *oppositifolia* and ssp. *glandulisepala* – which are distinguished by the presence of glandular hairs on sepals of ssp. *glandulisepala*, and which have largely nonoverlapping geographical distributions (Fig. 2;

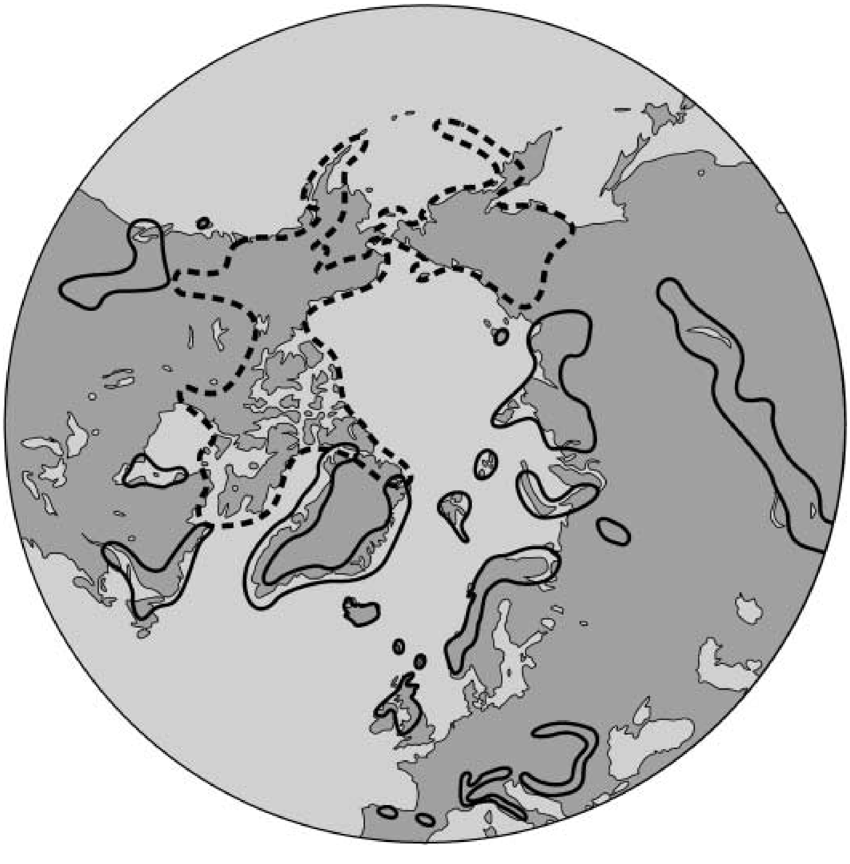

Fig. 2 Geographical distributions of two widespread subspecies of *Saxifraga oppositifolia* (ssp. *oppositifolia* (solid line), and ssp. *glandulisepala* (broken line), after Hultén & Fries, 1986).

Hultén & Fries, 1986). Subspecies *glandulisepala* is distributed from north Greenland, through arctic and subarctic North America into north-east Siberia, while ssp. *oppositifolia* ranges from central Siberia through northern Europe to Greenland and parts of Canada and the USA south of areas occupied by ssp. *glandulisepala.* Subspecies *oppositifolia* also occurs in the high mountains of central and southern parts of Eurasia. Several other subspecies are recognised with more restricted distributions (Webb & Gornall, 1989). Both diploid and tetraploid forms of *S. oppositifolia* have been recorded in Svalbard, Greenland, North America and East Siberia (Johnson & Packer, 1968; Webb & Gornall, 1989) and do not appear to be taxonomically correlated, although a detailed survey of ploidal variation in the species awaits to be conducted. Several population genetic and/or phylogeographic studies using cpDNA-RFLPs and RAPDs have been conducted on the species (Abbott *et al.*, 1995, 2000; Gabrielsen *et al.*, 1997; Gugerli *et al.*, 1999; Holderegger *et al.*, 2002; Abbott & Brochmann, 2003), and a phylogeographic analysis of cpDNA and ITS sequence variation has recently been completed (Holderegger & Abbott, 2003).

Abbott *et al.* (2000) surveyed cpDNA RFLP variation among 548 plants sampled from 90 different populations located throughout a large part of the geographical distribution of the species using *Saxifraga aizoides* as an outgroup. The analysis resolved 14 different cpDNA haplotypes in *S. oppositifolia* that were partitioned into two major clades using maximum parsimony. One clade had a mainly ‘Eurasian’ distribution, with transatlantic extensions into Greenland and eastern Canada, while the other showed an ‘American–Beringian’ distribution, and ranged from north Greenland, through North America to the Taymyr Peninsula in north-central Siberia. The Eurasian clade consisted of four haplotypes (*A-D*) and was separated from the American-Beringian clade (comprising 10 haplotypes, *E-N*) by a minimum of 13 site and length mutations. Seven of the 10 haplotypes within the American-Beringian clade occurred in Alaska and on this basis it was proposed that Alaska was a major refugium for the species during the last ice-age (Abbott *et al.*, 2000). Fossil evidence has subsequently confirmed that the species was present in Alaska at the time of the last glacial maximum (Goetcheus & Birks, 2001).

Estimates of divergence time from cpDNA data

We have attempted to estimate the divergence time between the two major lineages of *S. oppositifolia* from the cpDNA restriction site analysis of Abbott *et al.* (2000) based on 11 restriction endonucleases (six four-base and five six-base cutting enzymes). First, an assessment of rate-constancy of cpDNA evolution was evaluated by relative rate tests (Tajima, 1993) implemented in the program MEGA (vers. 2.1; Kumar *et al.*, 2001) using only those 12 cpDNA haplotypes of the species (i.e. *A*, *C-H*, *J-N*) that differed in restriction site mutations. For each test, the haplotype identified in the outgroup species, *S. aizoides*, was chosen as a reference. Genetic divergence between each haplotype pair was approximated as *p*-distance, that is the proportion (*p*) of restriction sites at which two haplotypes are different divided by the total number of sites surveyed (*c.* 1063 sites, representing sequence information for about 4382 nucleotides; R. J. Abbott & R. I. Milne, unpublished). Results of the relative rate tests (not shown) indicated that in only three of the 66 pairwise comparisons was a molecular clock (i.e. rate homogeneity) rejected at the 5% level. It should be noted that on average one out of 20 independent tests of the molecular clock will be rejected at the 5% level by chance (Sytsma & Schaal, 1985).

We next used the program RESTDATA (Ota, 1994) to calculate the number of nucleotide substitutions between pairs of haplotypes from the two major lineages following the maximum likelihood method of Nei & Tajima (1983) (Nei, 1987; pp. 96–110). Given the lack of any reliable extrinsic calibration point, we employed a slow (0.07%) and a fast (0.1%) average cpDNA divergence rate per million years following Wendel & Albert (1992) and Parks & Wendel (1990), respectively (Caujapé-Castells *et al.*, 2001). According to these rates, the average sequence divergence between the two lineages, 0.376%, would imply that they diverged from their common ancestor at about 5.37–3.76 Ma, that is, during the early to mid-Pliocene. Clearly, these estimates are dependent on a number of assumptions (e.g. there is no generally accepted divergence rate of the chloroplast genome) and it is difficult to make any definite conclusion at the present time. Nonetheless, the estimated sequence divergence between these two *S. oppositifolia* lineages from Eurasia and North America-Beringia (0.376%) falls well within the (positively skewed) distribution of values reported between conspecific populations in other plant species for which such estimates were derived from restriction site analysis of plastid DNA (0.028%–1.050%; median: 0.077%; Qiu *et al.*, 1995). These comparisons, however, also demonstrate that the two lineages have undergone a considerable amount of genetic differentiation since their divergence from a common ancestor.

Correlated molecular and taxonomic variation

Of particular interest was the finding that the different geographical distributions of the two major cpDNA lineages largely match those of ssp. *oppositifolia* and ssp. *glandulisepala*, respectively. The main disagreement concerns material from the Southern Rocky and the Cascade Mountains in north-west North America, which Hultén & Fries (1986) recognised as ssp. *oppositifolia* (Fig. 2), but which possesses a cpDNA that places it in the American-Beringian clade. This conflict between cytoplasmic and taxonomic status in material of ssp. *oppositifolia* from this part of north-west North America may result from unidirectional transfer (or capture) of cpDNA mediated by introgressive hybridzation, with ssp. *glandulisepala* acting as the maternal or 'donor' taxon. However, barring a selective sweep or a hitch-hiking effect, such range-wide and pervasive replacement of 'Eurasian' haplotypes by 'American-Beringian' ones appears unlikely. Rather, these north-west North American populations of ssp. *oppositifolia* may have lost their glandular sepal hairs (the only morphological character distinguishing them from ssp. *glandulisepala*) independently or may have retained this feature as an ancestral (plesiomorphic) character state. However, a well-resolved intra- and inter-specific phylogeny of *S. oppositifolia* and its close relatives is required before either of these latter two hypotheses can be rejected firmly.

Notwithstanding the above complications, it is evident that although considerable cpDNA divergence has occurred within the species, there is no equivalent morphological divergence. The two currently recognised subspecies are distinguished by a single character and therefore have apparently not been subjected to strong divergent selection for different morphologies in the two largely separate geographical regions they occupy. This is not surprising as a similar range of harsh environments is likely to be present in both of these regions. This is not to say that within each region ecotypic variation is absent: indeed, ecoclinal morphological variation has been documented in parts of the species distribution (Crawford *et al.*, 1995; Brysting *et al.*, 1996). However, no major morphological differences are diagnosable between the two geographically separated subspecies of *S. oppositifolia* recognised by Hultén (1973) other than presence/absence of sepal hairs.

A recent survey of sequence variation in the *psb*A-*trn*H intergenic spacer of chloroplast DNA (Holderegger & Abbott, 2003) revealed a similar geographical structure for the presence/absence of a 16-bp indel in *S. oppositifolia* with one notable exception. One sample from the North Cape of Norway (not examined previously) lacked the indel that was present in other Eurasian samples examined. This unexpected result may reflect a past episode of long distance dispersal from the geographical region occupied by the American-Beringian cpDNA lineage. Also of note was the finding that two other subspecies of *S. oppositifolia*, both of which have restricted geographical distributions, that is, ssp. *smalliana* in Alaska and ssp. *blepharophylla* in the Alps, plus a close relative, the Alpine *S. biflora*, shared the indel type of local material of *S. oppositifolia*. This could be due either to the recent origin of these taxa from *S. oppositifolia* in different parts of its range, ancient reticulation events, or incomplete lineage sorting. Holderegger & Abbott (2003) also surveyed sequence variation in the internal transcribed spacer region, ITS1-5.8S-ITS2, of nuclear ribosomal DNA among the same set of samples. Despite low levels of sequence variation and poor phylogenetic resolution, samples of *S. oppositifolia* with an American-Beringian cpDNA mainly grouped in an ITS clade that did not include samples with an Eurasian cpDNA, thus providing further support for the existence of major molecular lineages within *S. oppositifolia*.

cpDNA versus ITS sequence variation

The nuclear ITS sequences showed much weaker divergence and partly conflicting patterns of separation between the Eurasian and American-Beringian lineages of *S. oppositifolia* (Holderegger & Abbott, 2003), which were monophyletic with respect to each other on the basis of the cpDNA-RFLP data (Abbott *et al.*, 2000). This disagreement among nuclear vs cytoplasmic gene trees might be a consequence of the different transmission genetics of nuclear and organelle genes and their different rates of lineage sorting. Thus, under a neutral model of evolution, reciprocal monophyly of haplotypes (or alleles) is expected to evolve more quickly in cpDNA (as observed here) than in nuclear loci via random genetic drift and lineage sorting from a polymorphic ancestor (Avise, 2000; Hare, 2001; Palumbi *et al.*, 2001; but see Hudson & Turelli, 2003). This is because cytoplasmic DNAs tend to be effectively haploid and inherited uniparentally, and thus have a genetically effective population size that, in a hermaphrodite plant, is approximately two times smaller than that of most nuclear loci (Schaal *et al.*, 1998). As a consequence, cpDNA haplotype sorting is likely to be particularly pronounced in a species such as *S. oppositifolia* which may have suffered marked reductions in range size and/or bottlenecks in refugial populations during the glacial-interglacial cycles of the Pleistocene (see above). Under this scenario, time since origin of the two major lineages may have been insufficient for reciprocal monophyly to occur at nuclear ITS loci. In other words, these lineages are likely to occupy a 'mixed-monophyly' zone of divergence (*sensu* Hare, 2001) where cytoplasmic DNA will have a high probability of monophyly, but the average nuclear locus will not. It seems unlikely that the occurrence of (almost) identical ITS sequences in distant populations of *S. oppositifolia* from Eurasia/North America-Beringia (Holderegger & Abbott, 2003) and, hence, the weaker phylogeographic (subspecific) partition in the species inferred from nuclear markers results from differential patterns of contemporary gene flow, that is, a greater opportunity for the spread of nuclear ITS alleles via pollen than of maternally transmitted (Soltis *et al.*, 1990) cpDNA via seed. However, in regions where the distribution ranges of the two lineages meet, recent introgression of nuclear ITS genes cannot be rejected as an explanation for nuclear polyphyly. Thus, in both the Canadian high Arctic (Ellesmere Island) and north-east Siberia (Kotelny) accessions have been sampled that possess 'American-Beringian' cpDNA haplotypes but ITS sequences more similar to those characteristic of accessions from Eurasia (Abbott *et al.*, 2000; Holderegger & Abbott, 2003).

Nested clade analysis

In an attempt to obtain more detailed information on the causes of the present-day circumpolar distribution of chloroplast DNA variation within *S. oppositifolia*, and consequently the

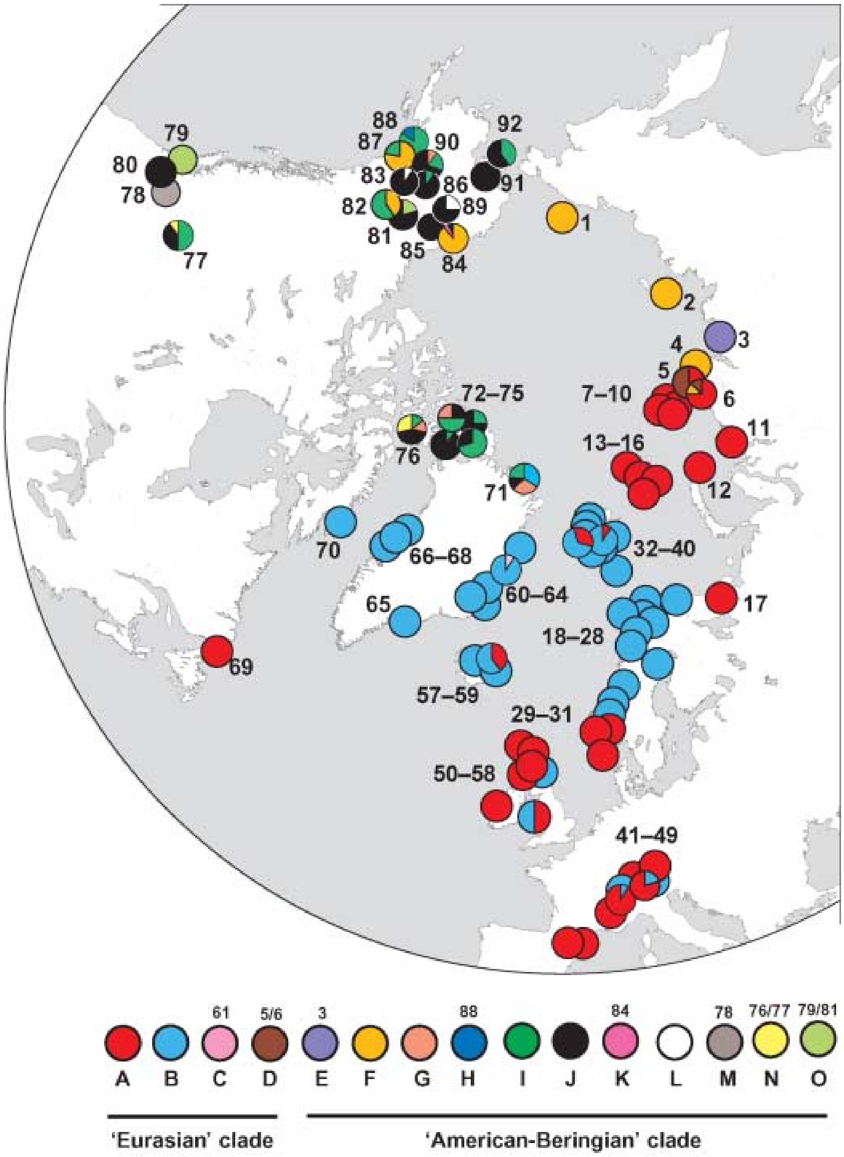

Fig. 3 Frequencies of cpDNA haplotypes (*A-O*) in each population of *S. oppositifolia* surveyed. Haplotypes are colour coded and their frequencies are expressed as sectors of pies. Numbers next to pies refer to population numbers. Sample sizes are given in Abbott *et al*. (2000).

evolutionary history of this species, we have conducted a nested clade analysis (NCA; Templeton, 1998) on cpDNA-RFLP haplotype variation within the species. ITS and cpDNA (*psb*A-*trn*H) sequence variation was not subjected to this form of analysis as only one individual per population from a subsample of populations examined for cpDNA-RFLP variation were surveyed for ITS and *psb*A-*trn*H variation. By integrating genealogical and geographical information, the NCA approach has the potential to differentiate between contemporary and historical processes, such as restricted gene flow, range expansion, or past fragmentation (Cruzan & Templeton, 2000; but see Knowles & Maddison, 2002 and further below). For the present analysis we used the data set of Abbott *et al.* (2000) plus information on one additional haplotype (*O*) recently discovered in the western USA and Alaska (R. J. Abbott, unpublished). Fig. 3 shows the relative frequency of each haplotype in each of the 92 populations that have now been surveyed. Relatedness among haplotypes was represented by an unrooted nested cladogram, generated using the program TCS (vers. 1.13; Clement *et al.*, 2000) and following nesting rules outlined in Templeton *et al.* (1987) and Templeton & Sing (1993).

The program GEODIS 2.0 (Posada *et al.*, 2000) was used to perform the following analyses. First, we conducted a permutational contingency analysis based on 10 000 resamples to test for the significant ($P < 0.05$) association of haplotypes or clades with locality. Together with information on the geographical distribution and frequency of haplotypes, two parameters were then estimated for each clade (and / or haplotype) showing significant geographic structure: the 'clade distance' (*Dc*), and the 'nested clade distance' (*Dn*). *Dc* is a measure of dispersion around the geographic center of a given clade, while *Dn* is a measure of displacement of the geographic center of a given clade from the geographic center of the next higher-level clade within which it is nested (Templeton, 1998). In addition, we calculated the average difference in *Dc* and *Dn* values between clades representing interior nodes in the nested cladogram and tip clades [abbreviated (I–T) *Dc* and (I–T) *Dn*, respectively,] to contrast the dispersion and displacement of recent (tip) clades relative to older (interior) ones. To determine whether any of these distance parameters were significantly small or large, haplotypes were permuted among localities (10 000 times) to generate a null distribution against which the observed value was tested. The inference key provided on the GEODIS web page, which is modified from Templeton (1998), was used to interpret significant ($P < 0.05$) parameters. We note that predictions made by the inference key need to be treated with caution as a result of its inability to distinguish statistically among alternative interpretations. Recently, this was highlighted by Knowles & Maddison (2002) who used computer-simulated populations to evaluate NCA's accuracy and validity. The authors concluded that the method did not adequately account for the stochasticity in the coalescent process to distinguish effectively between contemporary gene flow and historical processes, and in most cases, it did not accurately distinguish among the latter. As a consequence, it appears critical to examine the results of the inference key in the context of other evidence (e.g. biogeographical or paleoclimatic data), and to consider if alternative or additional hypotheses would be almost equally well supported by the data (Knowles & Maddison, 2002).

Results and interpretation of nested clade analysis

The nested cladogram (Fig. 4) connected the Eurasian and American-Beringian clades by 13 mutational steps, and contained no loops of ambiguity. Not unexpectedly, the two most common and widespread haplotypes of the American-Beringian clade, *J* and *I*, were placed close to the root of this

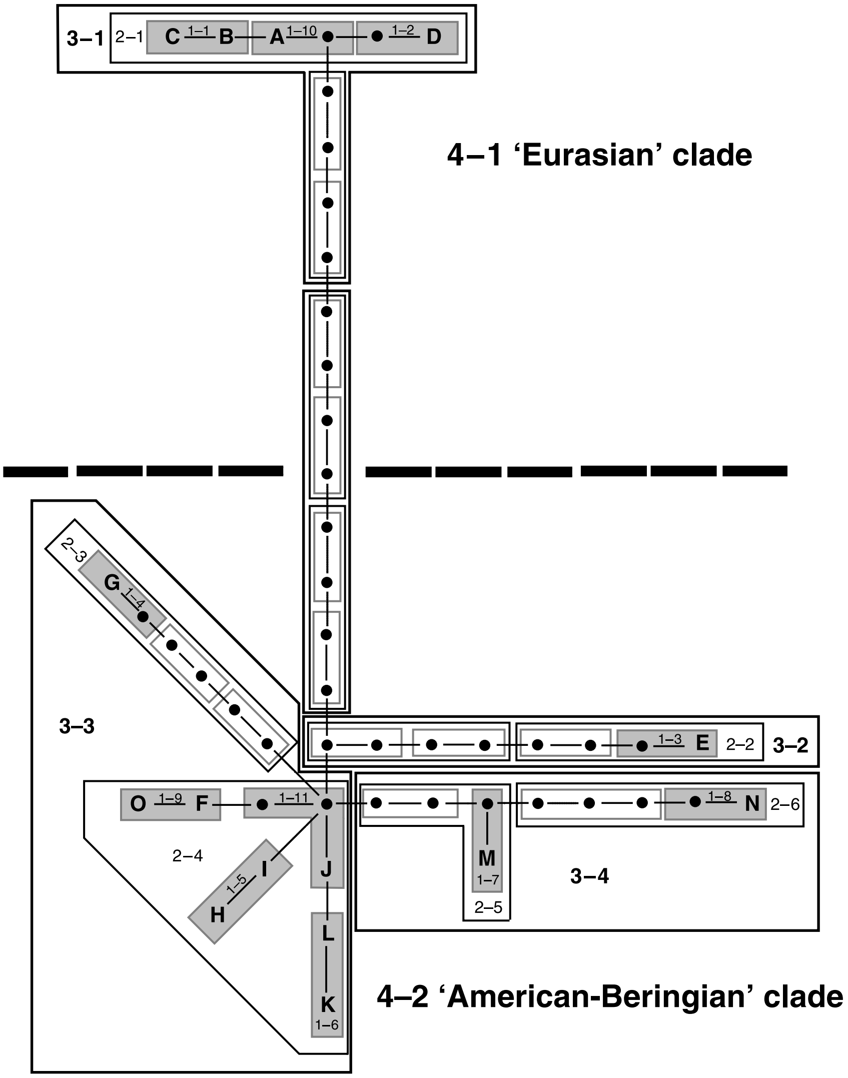

Fig. 4 The nested cladogram of cpDNA haplotypes (A-O). Sampled haplotypes are in capital letters. Each connection represents a mutational step with intermediate missing haplotypes represented by black dots. Nested haplotype groups (clades) with increasing number of steps are enclosed in rectangles or polygons and numbers for each nesting level and individual clade are indicated.

Table 1 Permutational chi-square probabilities for geographical structure of the clades identified in Fig. 4 from 10 000 resamples. Clades with a probability value less than 0.05 suggest a significant association of haplotypes or subclades with locality. *P* is the probability of a randomly generated chi-square statistic being greater than or equal to the observed chi-square. Note that clades with no genetic structure (e.g. 1–10; 2–3) were excluded

Clade (haplotypes or subclades involved)	Chi-square statistic	*P*
1–1 (C/B)	14.93	0.892
1–9 (O/F)	69.00	< 0.001
1–6 (K/L)	5.00	0.402
1–5 (H/I)	5.15	0.780
2–1 (C-B/A/D = Eurasia)	443.06	< 0.001
2–4 (O-F/K-L/J/H-I)	318.48	< 0.001
3–3 (O-F-K-L-J-H-I/G)	78.22	< 0.001
3–4 (N-M)	12.00	0.003
4–2 (O-F-K-L-J-H-I-G/N-M/E = North America-Beringia)	445.40	< 0.001
Total (C-B-A-D/O-F-K-L-J-H-I-G-N-M-E)	531.61	< 0.001

clade. Terminal haplotypes that differed by one mutation were grouped into one-step clades and numbered 1–1 through 1–9. Among all one-step clades, only group 1–9 (with haplotypes *O* and *F*) contained both genetic variation and significant geographical structure as inferred from the permutational contingency analysis (Table 1). The same applied to the higher-level nesting clades 2–1, 2–4, 3–3, 3–4, 4–2, and the total. Hence, for all these clades the null hypothesis of no clustering of genetic variation with geographic location was rejected. Therefore, they were subjected to the more complex statistical nested-clade tests of patterns of geographic/genetic association, incorporating estimation of geographic distance parameters (Fig. 5) and their interpretation (Table 2).

Within the Eurasian clade 2–1, containing haplotypes *A-D*, nested clade analysis inferred a scenario of past fragmentation. Significantly, one of the two tip subclades (1–1) within clade 2–1 contained haplotype *B*, which is widespread throughout Scandinavia, Svalbard, Iceland, Greenland, and east Baffin Island (Fig. 3). However, the statistical tests (Fig. 5) showed that this subclade, in fact, is more restricted in its distribution than the interior subclade, 1–10, which only contains haplotype *A* (fixed in material from north-west Russia and common in the Alps and the British Isles). Furthermore, these two subclades have mostly non-overlapping albeit parapatric ranges (Fig. 3), and are only connected by a single mutational step (Fig. 4). Overall, these results lend support to the previous notion (Abbott *et al.*, 2000) that haplotypes *A* and *B* were distributed in different (though still unknown) glacial refugia, and have come into 'secondary contact' only recently. Based on the present-day distribution of *S. oppositifolia* in Europe (Fig. 2) as well as relevant paleoclimatic data (Lang, 1994; Fig. 1) we may further assume that the species retreated postglacially into higher latitudes or high mountain regions, thus colonizing newly deglaciated areas, e.g. in the Alps or south of the retreating Eurasian ice-sheet. NCA, certainly, would have required higher levels of cpDNA diversity within subclades to register such regional processes. Altogether, there is much lower haplotypic diversity in the Eurasian clade compared to the American-Beringian clade (Figs 3 and 4). It is tempting to assume that this striking difference reflects a shortage of suitable long-term refugial sites

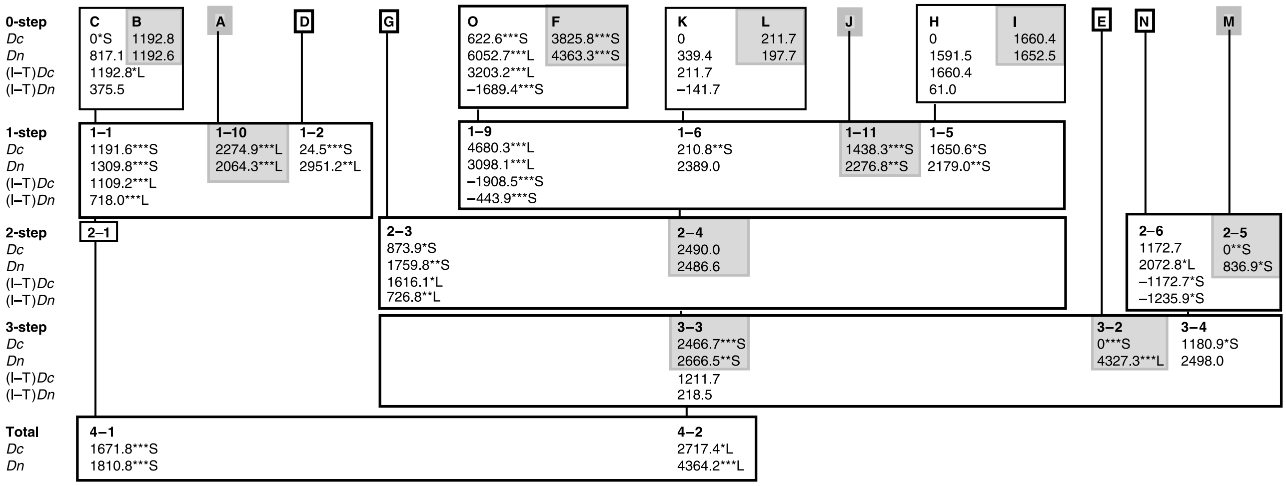

Fig. 5 Results of the nested clade analysis of the geographical distance for cpDNA haplotypes (A-O). Haplotypes (0-step) are given at the top and are boxed by the nested clade structure given in Fig. 4. Interior haplotypes/clades are shaded grey. Bold lines delimit clades showing significant geographical structure according to the permutational contingency analysis (Table 1). Proceeding down the diagram, higher level clade designations are given in bold directly below the nested clade. Significantly small or large values for *Dc*, *Dn*, (I–T) *Dc*, and (I–T) *Dn* are indicated by an 'S' or 'L', respectively, together with their level of significance: **P* < 0.05; ***P* < 0.01; *** *P* < 0.001, where *P* is the probability of a randomly generated value being equal to or smaller (larger) than the observed value.

Table 2 Chain of inference from the nested clade analysis (Fig. 5) following the inference key provided on the GEODIS (Posada *et al*., 2000) web page, which is modified from Templeton (1998). Note that only clades showing significant geographical structure according to the permutational contingency analysis (Table 1) were analysed

Clade	Inference chain	Inferred process
1–9	1–2^{c}–3–5–15–16–18–No	Inconclusive outcome†
2–1 (‘Eurasian’ clade)	1–2^{ac}–3–5–15–No	Past fragmentation
2–4	1–2–11^{abc}–12–No	Contiguous range expansion
3–3	1–2^{ac}–3–4–No	Restricted gene flow with isolation by distance
3–4	1–2–11^{bc}–12–No	Contiguous range expansion
4–2 (‘American-Beringian’ clade)	1–2–11^{b}–12–13–Yes	Long distance colonization
Entire cladogram	1–2	Inconclusive outcome††

†Geographical sampling scheme inadequate to discriminate between range fragmentation, expansion and isolation by distance. ††Tip/interior status cannot be determined.

for the species in Eurasia or, in general, more severe reductions in range size and bottlenecks brought about by the Pleistocene climatic changes.

Among all other, exclusively American-Beringian clades, mainly historical events were inferred from the analysis (Table 2). Most significant was evidence for contiguous range expansion within clade 2–4, which is mainly caused by a significantly large *Dc* value for the tip subclade 1–9 (Fig. 5). The latter contains haplotype *F*, which ranges from Alaska to the Taymyr Peninsula, and haplotype *O*, which is extremely rare in material from Alaska (Yukon) but fixed in a single population from the western United States (Olympic Peninsula). Since the interior subclade 1–11 (i.e. haplotype *J*) is present in Alaska, and apparently ancestral to subclade 1–9 (Fig. 4), these results suggest that *S. oppositifolia* expanded from Alaska in at least one direction, perhaps during the Pleistocene. The expansion of haplotype *F* westward to eastern Siberia and the Taymyr region could have occurred at any time during this period as these areas remained unglaciated throughout the Pleistocene (Hultén, 1937; Svendsen *et al.*, 1999; Abbott & Brochmann, 2003). More extensive sampling is required to exclude the possibility that haplotype *J* is absent from eastern Siberia and therefore rule out the possibility that haplotype *F* may have initially expanded from Siberia rather than Alaska. Given the present data, the apparently disjunct occurrence of tip haplotype *O* in Alaska and the western United States remains illusive. Because the mutation underlying this particular haplotype is a single length mutation (R. J. Abbott, unpublished), the possibility of parallelism cannot be ruled out with certainty. Furthermore, the species has a more or less continuous extant distribution from Alaska to the Western USA (Hultén & Fries, 1986). Because of inadequate geographical sampling along this range (Fig. 3), nested clade analysis was unable to discriminate between range fragmentation, expansion and isolation by distance within subclade 1–9 (Table 2).

It appears likely that *S. oppositifolia* also expanded from Alaska eastward to unglaciated parts of the Canadian high Arctic and to north Greenland (Abbott *et al.*, 2000). However, this is not readily apparent from the present analysis because the relevant tip subclade 1–5 (containing haplotypes *H* and *I*) had no significantly large value of *Dc* but was similarly restricted in range as the interior subclade 1–11 (Fig. 5).

Contiguous range expansion, however, was detected within clade 3–4 (Table 2). This contains interior subclade 2–5 (i.e. haplotype *M*) fixed in a single population from the western United States (Crescent Mine, Washington) as well as tip subclade 2–6 (i.e. haplotype *N*) from both western Canada (British Columbia) and the Canadian high Arctic (Devon Island). The latter two regions are separated by over 3400 kilometres. Since haplotype *M* is ancestral to haplotype *N* (Fig. 4), the analysis suggests that migration has also taken place from the Pacific north-west of North America northward into the Canadian high Arctic. The route taken may have utilised, in part, the high mountains connecting the Pacific north-west to Alaska. Alternatively, migration may have occurred along the western edge of the retreating Laurentide ice sheet, where north-flowing winds could have aided dispersal (Brown & Lomolino, 1998). Thus, in contrast to previous suggestions (Abbott *et al.*, 2000), the present analysis indicates that postglacial migration from further south has contributed, at least in part, to the high cpDNA diversity found in the Canadian high Arctic (Abbott *et al.*, 2000). However, because only a small number of populations and individuals have been examined, caution should be exercised in accepting this expansion scenario.

For the remaining American-Beringian clades, nested clade analysis provided less conclusive outcomes. Within clade 3–3, the analysis detected ‘restricted gene flow with isolation by distance’ (Table 2). Apparently, this was a result of the significantly restricted range of tip subclade 2–3 (i.e. haplotype *G*), mainly found in three fairly adjacent localities in the Canadian high Arctic (Ellesmere, Devon Island) and north Greenland (Pearyland; Fig. 3). In addition, however, haplotype *G* also occurred at very low frequency in a single population from central Alaska (Eielson, Denali National Park). Obviously, this sharing of haplotype *G* among these widely separated and disjunct populations cannot be easily ascribed to any current pattern of gene flow. Rather, this pattern may testify to an eastward spread of *S. oppositifolia* from Alaska to north Greenland (see above).

Finally, within clade 4–2, a long distance colonization event was inferred (Table 2). This clearly involved tip subclade 3–2 (i.e. haplotype *E*) that was fixed in a single population from the Taymyr Peninsula (Olenekshiy Bay) and significantly displaced from the geographical center of the American-Berigian clade 4–2 (Fig. 5). However, it is not possible to tell from the present data from where this haplotype (or its ancestor) was dispersed. The possibility that haplotype *E* is of central Asian (Sino-Himalayan) origin (Abbott *et al.*, 2000) cannot be excluded because samples of *S. oppositifolia* from this region are completely lacking.

Table 3 Populations surveyed in Alaska, number of individuals per cpDNA haplotype within each population and Nei's gene diversity (*H*)

	cpDNA haplotype								
Populations	F	G	H	I	J	K	L	O	*H*
Arctic coast:									
Prudhoe	25	–	–	–	2	1	–	–	0.204
Brooks Range									
Sagavanirktok River	–	–	–	–	9	–	–	–	0.000
Atigun	–	–	–	–	9	–	3	–	0.409
Central:									
Eagle	–	–	–	–	4	–	–	1	0.400
Tanana	2	–	–	3	–	–	–	–	0.600
Donnelly	–	–	–	–	9	–	1	–	0.200
Denali (Sugarloaf)	–	–	–	1	9	–	–	–	0.200
Denali (Eielson)	–	1	–	2	7	–	–	–	0.511
Kotzebue	–	–	–	–	4	–	–	–	0.000
Nome	–	–	–	2	3	–	–	–	0.600
Southern:									
Palmer	7	–	–	2	–	–	–	–	0.389
Anchorage	–	–	2	11	–	–	–	–	0.281

Geographical structure of cpDNA variation in Alaska

On the basis of fossil evidence and a high level of cpDNA diversity within Alaskan populations, it has been concluded that ice-free parts of this region served as a refugium for *S. oppositifolia* during the last ice-age and probably during earlier glacial periods throughout the Pleistocene (Abbott *et al.*, 2000; Abbott & Brochmann, 2003). Fig. 6 shows in more detail the distribution of glaciation in Alaska at the time of the last glacial maximum and the locations of populations surveyed for cpDNA variation. The most northerly population (Prudhoe – Pr) is located north of a glaciated area that covered much of the Brooks Range of mountains and adjacent areas from which two other populations (Sagavanirktok – Sag, and Atigun – At) were sampled. The majority of populations (i.e. from Eagle – Ea, Tanana – Ta, Donnelly Creek – Do, Denali Sugar Loaf Mountain – SL, Denali Eielson – Ei, Kotzebue – Ko, and Nome – N) were sampled from the central unglaciated region that stretches from the Yukon in the east to the Seward Peninsula in the west. Two further populations (Palmer – Pa, and Anchorage – An) were located in the glaciated area south of this region and the Alaska Mountain range. We wished to establish whether cpDNA variation found among these populations is randomly distributed or geographically structured.

All populations except Sag and Ko were polymorphic for cpDNA haplotype (Table 3). An analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) conducted using ARLEQUIN software (V2.0; Schneider *et al.*, 2000) showed that

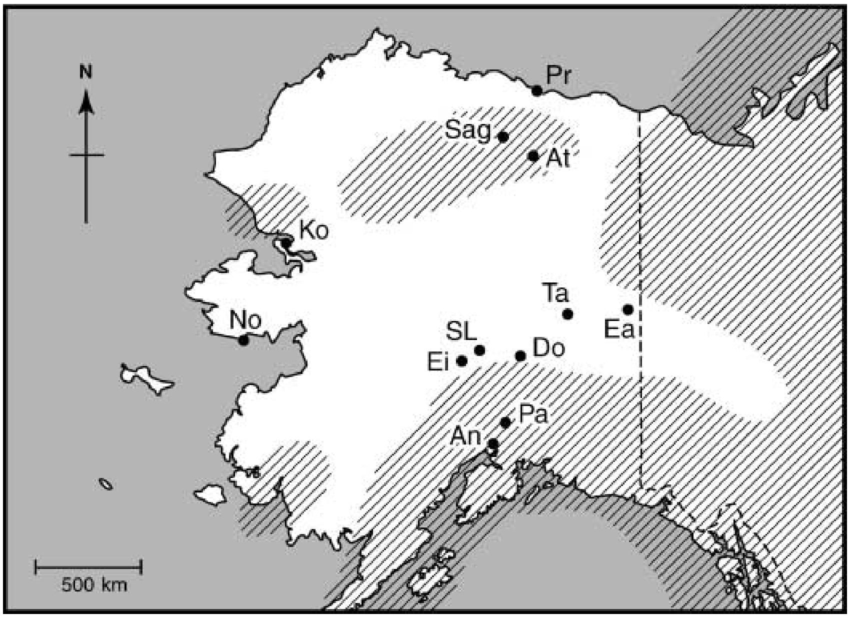

Fig. 6 Distribution of glaciated areas (hatched) in Alaska and Yukon at the time of the last glacial maximum. Also shown are the locations of populations from which samples of *S. oppositifolia* were surveyed for cpDNA variation: Pr (Prudhoe); Sag (Sagavanirktok); At (Atigun); Ea (Eagle); Ta (Tanana); Do (Donnelly Creek); SL (Denali Sugar Loaf Mountain); Ei (Denali Eielson); Ko (Kotzebue); N (Nome); Pa (Palmer); An (Anchorage).

Table 4 Analysis of molecular variance (AMOVA) for cpDNA haplotypes in *Saxifraga oppositifolia* sampled from Alaska. AMOVAS conducted among and within (i) all populations; (ii) three designated groups – Arctic vs Brooks Range plus Central vs Southern populations; (iii) three designated groups – Arctic plus Brooks Range vs Central vs Southern populations; and (iv) four designated groups – Arctic vs Brooks Range vs Central vs Southern populations

Source of variation	df	Sum of squares	Variance component	% Total variance	*P* (1023 permutations)
(i)					
Among populations	11	73.28	0.6518	64.01	< 0.001
Within populations	108	39.57	0.3664	35.99	
(ii)					
Among groups	2	53.31	0.6501	52.06	0.013
Among populations	9	19.97	0.2321	18.59	< 0.001
Within populations	108	39.57	0.3664	29.35	
(iii)					
Among groups	2	26.11	0.1452	13.70	0.280
Among populations	9	47.17	0.5480	51.72	< 0.001
Within populations	108	39.57	0.3664	34.58	
(iv)					
Among groups	3	54.96	0.4997	44.83	0.028
Among populations	8	18.32	0.2485	22.29	< 0.001
Within populations	108	39.57	0.3664	32.87	

Arctic group – Prudhoe; Brooks Range – Atigun, Sagavanirktok River; Central – Eagle, Tanana, Donelly, Denali (Sugarloaf), Denali (Eielson), Kotzebue, Nome; Southern – Palmer, Anchorage.

the difference between populations for cpDNA haplotypes was highly significant and explained 64% of the total variation (Table 4). However, genetic distances between populations, measured in terms of Phi_{st} were not correlated with geographical distance (Mantel's test with 10 000 permutations, $R^2 = 0.000335$, $P = 0.499$). AMOVAS which included an 'among group' source of variation showed that the greatest variance attributed to groups (52.1%) was produced when the groups compared comprised the coastal arctic population (Pr) vs Brooks Range and central Alaskan populations (Sag, At, Ea, Ta, Do, SL, Ei, Ko, No) vs southern Alaskan populations (Pa, An). Placing the two Brooks Range populations (Sag, At) into the coastal arctic group rather than combining them with central Alaskan populations reduced the total amount of variation explained by the among group term to 13.7% (Table 4). Also, treating the two Brooks Range populations as a separate group reduced the proportion to 44.8%.

From the above, it is concluded that cpDNA variation is geographically structured in Alaska and that *S. oppositifolia* was possibly fragmented into different refugia during past glacial periods. The arctic coastal population (Pr) contains the *F* haplotype at very high frequency (89%) among 28 plants surveyed (Table 3). The same haplotype was not present in the two Brooks Range populations and occurred only rarely in central Alaskan populations, but was present at high frequency (78%) in one of two southern populations (Pa). Interestingly, haplotype *F* was fixed in the only populations surveyed in north-east Siberia, that is, on Kotelny and Wrangel Islands (Abbott *et al.*, 2000; Fig. 3). It is feasible therefore that plants comprising the Prudhoe population are largely or entirely derived from ancestors that survived the last glaciation in a refugium north of the Brooks Range in Alaska. This refugium may have extended via the exposed ice-free coastal shelf westwards to north-east Siberia and beyond. By contrast, central Alaskan plants are possibly derived from ancestors that survived the last glaciation in ice-free central Alaska. These stocks may have dispersed both northwards and southwards at the end of the last ice age to found populations in the Brooks Range region and southern central Alaska, respectively. Founder effects could explain why the Brooks Range and southern Alaskan populations examined were composed of completely different haplotypes. These various scenarios are highly speculative, but would be worth investigating further if possible, for example should ancient DNA become available from plants that grew in Alaska at the time of the last glaciation.

A recent study of ITS and RAPD variation within and among *Oxytropis* (Fabaceae) taxa in Alaska (Jorgensen *et al.*, 2003) also recognised a geographical split dividing north-eastern arctic populations from most other populations. It was concluded that the split reflects the survival of populations in different ice-free glacial refugia north and south of the northern coastal ice shield of Alaska during glacial periods, which agrees with our conclusion based on the geographical structure of cpDNA variation in *S. oppositifolia*.

By contrast to what has been found in Alaska, a detailed study of cpDNA and RAPD variation in *S. oppositifolia* in the European Alps failed to detect significant geographical structure (Holderegger *et al.*, 2002). It was concluded from this study that if *S. oppositifolia* survived on ice-free nunataks in the Alps during the last glaciation, massive postglacial immigration from periglacial areas must have erased the genetic signature of such populations.

Conclusions

The survey of cpDNA variation within and among populations of *S. oppositifolia* throughout a large part of the species' geographical range has identified two major cpDNA lineages with discrete and largely non-overlapping distributions. A nested clade analysis of causes of geographical structure within these lineages highlighted past episodes of fragmentation, contiguous range expansion and occasional long distance dispersal as important historical processes. Caution must be exercised in accepting these conclusions as large parts of the species distribution have not been sampled sufficiently, e.g. eastern Siberia, central Asia, the Canadian high Arctic, and the Rocky Mountains in particular, and also because of the current uncertainty concerning the validity of Templeton's inference key used in nested clade analysis to determine alternative historical processes or contemporary gene flow as causes of genetic structure (Knowles & Maddison, 2002).

The two major cpDNA lineages are distinguished by a minimum of 13 mutations and are likely to have originated early in the evolution of the species, that is, during the early to mid-Pliocene, and remained geographically separated thereafter. The finding that their geographical distributions are largely concordant with those of two widespread subspecies recognised by Hultén (1973), that is, subspp. *oppositifolia* and *glandulisepala*, which are distinguished by a single morphological trait, suggests that morphological divergence does not reflect levels of molecular divergence. Crossing experiments are now required to determine levels of interfertility and to establish whether or not an apparent long period of geographical isolation might have resulted in the origin of postzygotic reproductive isolation between the two subspecies. It is possible that because both taxa occupy a similar range of harsh environments, selection has favoured the same morphology in each region. This situation resembles in some respects the morphological similarity that characterises closely related Tertiary relict taxa that are geographically separated in south-east Asia and south-east North America and which diverged from each other at least 5 Ma (Milne & Abbott, 2002). The morphological stasis exhibited by these taxa has been attributed to the similar environments they occupy and the action of strong stabilizing selection (Milne & Abbott, 2002).

It was previously speculated that *S. oppositifolia* may be derived from an ancestral stock native to the Sino-Himalayan region of central Asia where it currently co-occurs with several other closely related species possessing opposite leaves (Abbott *et al.*, 2000). It was also suggested previously that the species could have migrated northwards from this area to the Arctic along mountain ranges that connect the two areas as global temperatures fell during the late Tertiary. Given the estimated cpDNA-derived divergence times reported in the present paper, it seems possible that during or shortly after this stage the two major cpDNA lineages originated in geographical isolation. An important step in testing these hypotheses plus others related to the origin of all subspecies of *S. oppositifolia* and close relatives, would be to obtain a phylogeny that clearly shows which taxa are ancestral and which are derived. Whether there is sufficient molecular variation present in the complex for a clear phylogenetic resolution remains to be established. In this regard, it is of interest that Holderegger & Abbott (2003) found that two other subspecies of *S. oppositifolia*, both with restricted geographical distributions, that is, ssp. *smalliana* in Alaska, and ssp. *blepharophylla* in the Alps (Webb & Gornall, 1989), plus the closely related Alpine species, *S. biflora,* each possess cpDNA (*psb*A-*trn*H) and ITS sequences very similar to either *S. oppositifolia* ssp. *oppositifolia* or ssp. *glandulisepala.* As a result, it was not possible to obtain a clearly resolved phylogeny for this group based on variation of these sequences.

In summary, the phylogeographic analysis of cpDNA variation in *S. oppositifolia* has provided some relevant information to an understanding of the evolutionary history of this circumarctic-alpine plant and, in particular, to the origin of two of its geographically widespread and mainly allopatric subspecies. Moreover, it has become clear that a major northern refugium for the species in North America during Pleistocene glaciations was Alaska and the Yukon. This is in addition to refugia that occurred south and possibly also east and west of the main ice sheets that covered large parts of the continent during these periods (Abbott & Brochmann, 2003). In the Alaskan and Yukon area the species distribution was most probably fragmented into two or more large ice-free areas, allowing molecular divergence to proceed, thus promoting high cpDNA diversity within this region. It is not yet known whether any reproductive isolating barriers have evolved between populations that were isolated in these different regions or indeed between the two subspecies of *S. oppositifolia.* This needs to be examined to establish whether any of the divergent material is in the process of evolving into new species.

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