Systematics of *Alnus maritima* (Seaside Alder) Resolved by ISSR Polymorphisms and Morphological Characters

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ABSTRACT. *Alnus maritima* (Marsh.) Muhl. ex Nutt. is a rare woody plant species that exists as three subspecies found in widely disjunct locations in the United States. Although there is a growing interest in the phylogeography, ecology, conservation, and landscape potential of this species, the phylogeny of *A. maritima* has not yet been resolved by using molecular methods. We have combined a relatively new method of genome fingerprinting, ISSR-PCR, and the automated imaging capabilities of GeneScan technology to investigate the molecular systematics of *A. maritima*. Based on the molecular evidence from 108 ISSR loci, we confirm that the three disjunct populations of *A. maritima* have diverged sufficiently to be classified as subspecies. Our molecular phylogeny of the three subspecies of *A. maritima* agreed in topology with a phylogeny produced from morphological data and showed that subsp. *oklahomensis* is the most distinct of the three subspecies and was the first to diverge. The simultaneous analysis of molecular and morphological data provides a detailed and balanced phylogeny reconstruction for the three subspecies. Our results support the theory that *A. maritima* originated in Asia, migrated into North America across the Bering land bridge, and was established over a large range in the New World before being forced into its present meager disjunct distribution.

Alnus maritima is a rare species of *Alnus* P. Mill from the subgenus *Clethropsis* (Spach) Regel. While the other members of subg. *Clethropsis*, *Alnus nepalensis* D. Don and *Alnus nitida* (Spach) Endl., are sympatric and occur in southern Asia, *A. maritima* is found only in the United States and has a peculiar distribution consisting of three disjunct subspecies separated by ≥ 960 km. *Alnus maritima* subsp. *oklahomensis* Schrader & Graves occurs naturally in only two counties in south-central Oklahoma, *A. maritima* subsp. *georgiensis* Schrader & Graves is isolated to one county in northwestern Georgia, and *A. maritima* subsp. *maritima* is found only on the Delmarva Peninsula in four counties in Maryland and two counties in Delaware (Schrader and Graves, 2002).

There are two popular models explaining the phylogeny and phylogeography of *A. maritima*. Some believe that *A. maritima* evolved on the Delmarva Peninsula, and that the subspecies in Georgia and Oklahoma were established from Delmarva germplasm by Native Americans or by some natural form of long-distance dispersal (Schrader and Graves, 2002; Stibolt, 1981). Others believe the theory proposed by Furlow (1979), that subg. *Clethropsis* evolved in southern Asia, that *A. maritima* diverged from other members of subg. *Clethropsis* in Asia, and that ancestors of *A. maritima* migrated into North America via the Bering land bridge and spread across North America before being forced into the present, disjunct range (Furlow, 1979; Schrader and Graves, 2000a, 2002). Some circumstantial evidence supports the possibility of long-distance dispersal of *A. maritima* by Native Americans (Stibolt, 1981), but most of the evidence, including that from paleobotany, ecology, phylogenetic analyses of morphological characters, and the comparative stress physiology of *A. maritima* and *Clethropsis*, supports the second model

(Furlow, 1979; Schrader and Graves, 2000a, 2000b, 2002, 2003; Stibolt, 1981). Molecular evidence is needed to complete the systematics of *A. maritima*, but no studies have been published that utilize molecular approaches to resolve the systematics of this species.

The goals for this study were to determine the molecular phylogenetic relationships among the three subspecies of *A. maritima*, to measure the divergence and diversity among the three subspecies, and to investigate the origin of the remarkable distribution of *A. maritima* by using the techniques of molecular systematics. To accomplish these goals we have used the genome fingerprinting methods of inter-simple sequence repeats–polymerase chain reaction (ISSR–PCR) (Zietkiewicz et al., 1994), the automated imaging capabilities of GeneScan technology, and the phylogeny reconstruction tools of the PHYLIP software package (Felsenstein, 1995). We also examined the morphometric phylogeny, assessed cross-matrix disparity (Bateman, 1999), and performed a simultaneous analysis (Nixon and Carpenter, 1996) of morphological and molecular data, producing an infraspecific phylogeny for *A. maritima* that is derived from all the available evidence.

Along with resolving molecular differences at and below the infraspecific level, ISSRs sample a large portion of the genome and therefore avoid the bias accompanying phylogenies based on the sequence of only one or a few genes. Although some researchers object to the use of ISSRs and other PCR-based techniques for phylogenetic analyses because bands of the same size from different genotypes may not be homologous (Harris, 1999), alternative DNA-centered techniques capable of consistently resolving infraspecific variation have not been developed sufficiently (Fang et al., 1997; Wolfe et al., 1998; Wolff and Morgan-Richards, 1999). With randomly amplified polymorphic DNA (RAPD) markers, a technique similar to ISSR–PCR, potential for the occurrence of nonhomologous PCR fragments of the same size increases with genetic distance (Harris, 1999). This problem is considered minimal at the infraspecific level (Wolff and Morgan-Richards, 1999), and it has been demonstrated that 91% of comigrating products

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Table 1. Vouchers for the 20 individuals sampled for ISSR-PCR analysis. Source abbreviations: (NCRPIS) = North Central Regional Plant Introduction Station, Ames, Iowa; (S&G) = Schrader and Graves *Alnus* collection at Iowa State University. Latitude and longitude are according to Global Positioning System (GPS) and are included when known.

Species or subspecies	Accession	Source	Origin	Latitude	Longitude
<i>A. maritima</i>					
subsp. <i>oklahomensis</i>	OK 4	S&G	Connerville, Okla.	34° 20' 11.33387' N	96° 35' 39.47116' W
	OK 7	S&G	Connerville, Okla.	34° 20' 11.33387' N	96° 35' 39.47116' W
	OK 22	S&G	Tishomingo, Okla.	34° 15' 38.06' N	96° 41' 0.67' W
subsp. <i>georgiensis</i>	GA 103	S&G	Euharlee, Ga.	34° 07' 45.09' N	84° 56' 50.877' W
	GA 104	S&G	Euharlee, Ga.	34° 07' 45.09' N	84° 56' 50.877' W
	GA 107	S&G	Euharlee, Ga.	34° 07' 44.48' N	84° 56' 53.814' W
	GA 109	S&G	Euharlee, Ga.	34° 07' 44.48' N	84° 56' 53.814' W
	GA 111	S&G	Euharlee, Ga.	34° 07' 44.48' N	84° 56' 53.814' W
	GA 116	S&G	Euharlee, Ga.	34° 07' 59.12' N	84° 57' 34.65' W
	GA 117	S&G	Euharlee, Ga.	34° 08' 09.99' N	84° 57' 29.16' W
	GA 121	S&G	Euharlee, Ga.	34° 08' 09.99' N	84° 57' 29.16' W
	GA 123	S&G	Euharlee, Ga.	34° 08' 05.69' N	84° 57' 30.11' W
	GA 125	S&G	Euharlee, Ga.	34° 08' 05.69' N	84° 57' 30.11' W
subsp. <i>maritima</i>	MA 205	S&G	Snow Hill, Md.	38° 10' 06.78774' N	75° 26' 00.40549' W
	MA 209	S&G	Sharptown, Md.	38° 32' 53.358' N	75° 43' 22.718' W
	MA 216	S&G	Bethel, Del.	38° 36' 1.98329' N	75° 38' 44.40750' W
	MA 219	S&G	Ellendale, Del.	38° 50' 13.767' N	75° 26' 19.466' W
	MA 221	S&G	Ellendale, Del.	38° 50' 13.767' N	75° 26' 19.466' W
	MA 226	S&G	Millsboro, Del.	38° 35' 36.96466' N	75° 17' 27.82579' W
<i>A. japonica</i>	PI 479297	NCRPIS	Kushiro-shi, Japan		

are homologous at the species level in the genus *Helianthus* L. (Rieseberg, 1996). In studies comparing the two techniques, ISSRs were shown to be more reliable and more effective than RAPDs (Moreno et al., 1998; Wolfe et al., 1998), and ISSRs have been used to determine the genetic relationships among species, subspecies, populations, and cultivars of numerous plant taxa (Fang et al., 1998; Huang and Sun, 2000; Iruela et al., 2002; Joshi et al., 2000; Maunder et al., 1999; Potter et al., 2002; Raina, 2001; Wolfe and Randle, 2001). When coupled with other systematic data, such as the morphometric characters utilized in our study, ISSRs can be used to produce strong, consistent phylogenies for plant taxa at or below the interspecific level.

Materials and Methods

ISSR-PCR techniques (Zietkiewicz et al., 1994) and ABI PRISM GeneScan (Perkin Elmer: Applied Biosystems, Foster City, Calif.) technology were used to fingerprint and compare the genomes of the three subspecies of *A. maritima*. DNA samples were extracted with CTAB (Doyle and Doyle, 1987) from the roots of greenhouse-grown seedlings (*A. maritima*) or rooted stem cuttings [*Alnus japonica* (Thunb.) Steud.]. A total of 20 DNA samples were analyzed, one for *A. japonica* and 19 for *A. maritima*, which included three for subsp. *oklahomensis*, 10 for subsp. *georgiensis*, and six for subsp. *maritima* (Table 1). After completion of the basic CTAB extraction, all DNA samples were purified further with GENECLAN (Qbiogene, Inc., Carlsbad, Calif.).

DNA samples were amplified for three replications with each of three fluorescent 3'-anchored ISSR primers [(CA)₆RG, (AC)₈G, and (AG)₈YT], which were synthesized at the DNA Sequencing and Synthesis Facility at Iowa State University. These primer sequences were chosen based on their successful use with other woody plant species (Fang et al., 1997; Fang et al., 1998; Moreno et al., 1998; Tsumara et al., 1996). Optimization reactions under the range of conditions employed by recent authors were run to

determine proper reaction conditions and reagent concentrations for consistent PCR amplification. Thermocycler conditions for ISSR-PCR were 94 °C for 5 min (initial denaturing), 94 °C for 30 s (denaturing), primer-specific temperatures (see below) for 45 s (annealing), and 72 °C for 2 min (extension), for 30 cycles with the final extension at 72 °C for 5 min. Annealing temperatures for the three primers were 47 °C for (CA)₆RG, 50 °C for (AC)₈G, and 54 °C for (AG)₈YT. In our 25-μL reaction mixes, we used 30 ng of template DNA, 1.2 μM of primer, 300 μM dNTP mix (SIGMA, St. Louis, Mo.), 1× reaction buffer containing Mg(OAc)₂, and 1.5 units of KlenTaq LA DNA polymerase (SIGMA).

Amplification products were processed at the DNA Sequencing and Synthesis Facility at Iowa State University. Applied Biosystems (ABI) 377 automated DNA sequencing systems separated the DNA by electrophoresis and collected the gel image (Fig. 1). Image data were analyzed by using the facility's ABI PRISM GeneScan software that accurately resolves DNA fragment length differences as small as one base pair. ISSR bands (loci) were scored as 1 for band presence and 0 for band absence. Only bands that appeared in at least two of the three replications were considered present. A locus was any fragment length that was present in at least one sample for a given taxonomic level. The resulting two-state (1 • 0) data matrices for the three primers were combined to form a cumulative data set for assessing molecular relationships among and within the three subspecies of *A. maritima*.

The UPGMA analysis of all individuals was performed by using JMP software (SAS Institute Inc., Cary, N.C.). Cladistic and phenetic analyses were performed by using PHYLIP (Phylogeny Inference Package; Felsenstein, 1995). The Seqboot program was used for bootstrap (Felsenstein, 1985) and jackknife (Farris et al., 1996) analyses, the Mix program for Wagner parsimony analysis, the Consense program for consensus trees, and the Neighbor program for neighbor-joining dendrograms (Felsenstein, 1995). Genetic distances for neighbor-joining analyses were Euclidean distances (Sneath and Sokal, 1973). We compared and contrasted our morphometric and molecular phylogenies, a procedure termed

Fig. 1. Gel image produced by GeneScan and showing banding patterns from ISSR-PCR (inter-simple sequence repeats-polymerase chain reaction) of total DNA from four *Alnus* taxa. Lanes are shown divided into groups reading from left to right: *A. maritima* subsp. *oklahomensis* (OK), subsp. *georgiensis* (GA), subsp. *maritima* (MA), and *A. japonica* (A. jap). Some taxon-specific bands and bands shared by only two of the subspecies have been highlighted with boxes. Primer for this run was a fluorescent-labeled, 3'-anchored simple sequence repeat [(CA)₆RG]. This figure may be viewed in color at <http://www.hort.iastate.edu/graves/>.

cross-matrix disparity by Bateman (1999), then merged the two data sets for a simultaneous analysis (Nixon and Carpenter, 1996). Morphological data were standardized, unweighted, and derived from the measurements of Schrader and Graves (2002). *Alnus japonica* was chosen as the outgroup for cladistic analyses because it is considered to be monophyletic with *A. maritima* and is a member of the most primitive section of *Alnus* (Furlow, 1979).

Results

ISSR-PCR produced ample molecular markers for assessing the variability and phylogenetic relationships among the subspecies of *A. maritima*. ISSR banding patterns were clearly resolved with the ABI 377 automated sequencing system, and allele calling was easily accomplished with GeneScan software

(Fig. 1). Using three primers, our amplifications produced 108 bands (loci) total. Abundant polymorphisms and taxon-specific markers (Table 2) illustrated the resolving power of ISSR-PCR and the three chosen primers. UPGMA analysis of banding patterns from all samples placed all individuals within the cluster of their subspecies (Fig. 2).

An exhaustive search produced a single most parsimonious tree of 89 steps for the molecular relationship among the three subspecies of *A. maritima*. Bootstrap and jackknife frequencies for the molecular phylogeny (Fig. 3I) were 92% and 90%, respectively, for the clade of subspp. *georgiensis* and *maritima*, values that strongly support this topology. Molecular and morphometric analyses produced trees with the same topology (Fig. 3I and II). Both phylogenies show subspp. *oklahomensis* diverging first, followed by the divergence of the two subspecies from the eastern United States, subspp. *georgiensis* and *maritima*. Although the branch lengths for the molecular and morphometric phylogenies were not identical, comparison of the two trees show them to be similar and proportional (Figs. 3I and II). Simultaneous analysis of the combined molecular and morphological data (Fig. 4) again gave the same topology and provided reasonable adjustments in branch lengths, balancing the phenotypic and genotypic information concerning the subspecies.

Euclidean distances between subspecies given by molecular data were 15 for *oklahomensis* to *georgiensis*, 19 for *oklahomensis* to *maritima*, and 12 for *georgiensis* to *maritima*. Euclidean distances between subspecies given by unweighted morphological data were 15.2 for *oklahomensis* to *georgiensis*, 18.1 for *oklahomensis* to *maritima*, and 10.3 for *georgiensis* to *maritima*. The total Euclidean distances between subspecies given by simultaneous analysis were 30.2 for *oklahomensis* to *georgiensis*, 37.1 for *oklahomensis* to *maritima*, and 22.3 for *georgiensis* to *maritima*. These pairwise comparisons indicate that, even though subspp. *oklahomensis* is not the most derived, it is the most unique of the three subspecies at both the molecular and morphological levels.

Discussion

The parallel topology of the morphometric and molecular phylogenies of the three subspecies of *A. maritima* (Fig. 3I and 3II) provides strong evidence supporting the classification and systematics of these taxa described by Schrader and Graves (2002). The striking similarity between the trees in both topology and branch length illustrates the effectiveness of the morphometric and ISSR characters for estimating the phylogenetic history of these subspecies. In addition to these parallel phylogenies, the UPGMA clustering of all individuals within their subspecies (Fig. 2) and the discovery of taxon-specific markers at the subspecies level (Table 2, Fig. 1) provide strong molecular evidence that the three disjunct populations of *A. maritima* have differentiated sufficiently to be considered separate subspecies.

Along with demonstrating that subspp. *oklahomensis* was the first of the subspecies to diverge, all three dendrograms also show a relatively short branch for the line leading to subspp. *georgiensis*, indicating a lesser degree of differentiation for subspp. *georgiensis* after divergence than the differentiation for the other two subspecies. Another indication that subspp. *georgiensis* has undergone less differentiation than the other two subspecies is its lower number of subspecies-specific markers (three compared to four and six for subspp. *oklahomensis* and *maritima*, respectively) shown in the ISSR analysis (Table 2).

Table 2. Percentage of polymorphic loci among and within the three subspecies of *A. maritima* and taxon-specific bands resolved by using three, 3'-anchored ISSR primers.

	Primer			
	(CA) ₆ RG	(AG) ₈ YT	(AC) ₈ G	All three primers
% of polymorphic loci				
Total ^z	81	75	86	80
Among subspecies	41	55	46	48
within <i>oklahomensis</i>	16	25	17	20
within <i>georgiensis</i> ^y	17	9	39	20
within <i>maritima</i>	30	22	23	25
Taxon-specific loci				
subsp. <i>oklahomensis</i>	1	3	0	4
subsp. <i>georgiensis</i>	0	1	2	3
subsp. <i>maritima</i>	2	4	0	6
<i>A. japonica</i>	9	16	12	37

^zValues include loci for *A. japonica*.

^yTo achieve a consistent comparison among subspecies, polymorphic loci for subsp. *georgiensis* and *maritima* were determined for three randomly chosen samples each.

The conclusion that subsp. *oklahomensis* is the most unique of the subspecies is reasonable because it was the first of the subspecies to diverge and has therefore been isolated from subsp. *georgiensis* and *maritima* longer than these two subspecies have been isolated from each other. Another factor that may have contributed to the unique character of subsp. *oklahomensis* could be the uniqueness of its environmental niche. Plants of subsp. *oklahomensis* grow in and along swiftly flowing rivers and streams, while plants of the other two subspecies inhabit ponds or slow-flowing waterways. Soil potassium, calcium, and

magnesium concentrations of the Oklahoma provenance are over twice those of the other two provenances, and soil pH in the Oklahoma habitat is alkaline, while the pH of the other two habitats is acid (Schrader and Graves, 2002). The Oklahoma provenance also has the warmest winter and hottest summer temperatures of the three locations. Its early isolation and adaptation to the most unique niche of the three subspecies probably have contributed to the unique character of subsp. *oklahomensis*.

While ISSR fingerprinting showed clear differentiation between the three subspecies, the methods were also sensitive enough to detect diversity within each subspecies. Of the loci detected for each subspecies individually, 20%, 20%, and 25% were polymorphic for subsp. *oklahomensis*, *georgiensis*, and *maritima*, respectively, across all three primers (Table 2). This substantial degree of ISSR polymorphism reinforces the morphological findings of Schrader and Graves (2002) that sufficient genetic diversity exists within each subspecies and that the degree of genetic diversity should not be a hindrance to the long-term survival of the three subspecies.

Our molecular phylogeny of *A. maritima* provides further support for Furlow's model of divergence and migration, while almost directly opposing the other model, that *A. maritima* originated on the Delmarva Peninsula and underwent long-distance dispersal to the other two locations. This new evidence, appearing in its most complete form in the simultaneous analysis (Fig. 4), confirms that subsp. *oklahomensis* was the first of the three subspecies to diverge and that subsp. *maritima* is the most derived of the subspecies, rather than the most primitive.

There is a growing interest in the use of *A. maritima* as an ornamental for managed landscapes (Schrader and Graves, 2000a), and the first cultivar was recently selected (Graves and Schrader, 2004). Because of the disjunct distribution of *A. maritima*, the subspecies have already differentiated sufficiently to provide genotypic resources on which to begin plant-breeding efforts. Our discovery of subspecies-specific ISSR markers and detection of polymorphisms within each subspecies indicate that ISSRs could be a valuable tool for breeding of this species. If desired characteristics could be linked to specific ISSR markers, plants would not have to reach full maturity, potentially several years, to screen for successful crosses. *Alnus maritima* can flower within two to three years, but requires about 10 years to attain full size. ISSR markers could facilitate the sequential crossing of plants in a fraction of the time that would otherwise be required.

Fig. 2. UPGMA dendrogram characterizing the molecular relationship among all 20 individuals sampled and illustrating the resolving power of the three inter-simple sequence repeats (ISSR) primers. This figure may be viewed in color at <http://www.hort.iastate.edu/graves/>.

Fig. 4. Inferred phylogenetic relationship among the three subspecies of *Alnus maritima* based on the simultaneous analysis (Nixon and Carpenter, 1996) of inter-simple sequence repeats (ISSR) polymorphisms together with 23 characters of morphology derived from the results of Schrader and Graves (2002). Molecular and morphological data were unweighted and analyzed by using Euclidean distances and the neighbor-joining method. *Alnus japonica* was the designated outgroup. Numbers indicate the branch lengths from a point of divergence to the position of an extant taxon or the distance between successive points of divergence (A to B). The total Euclidean distances between subspecies were 30.2 for *oklahomensis* to *georgiensis*, 37.1 for *oklahomensis* to *maritima*, and 22.3 for *georgiensis* to *maritima*.

Fig. 3. (I) The single most parsimonious tree of 89 steps characterizing the inferred phylogenetic relationship among the three subspecies of *Alnus maritima* based on molecular data (polymorphisms in 108 ISSR [inter-simple sequence repeats] loci total). Bootstrap and jackknife percentages for clade B were 92 and 90, respectively, based on 1000 resamplings each. (II) Inferred phylogeny for the three subspecies of *Alnus maritima* based on unweighted morphological data (23 characters), derived from the measurements of Schrader and Graves (2002). *Alnus japonica*, an Asian species from a primitive section of subg. *Alnus* (Furrow, 1979), was designated as the outgroup for both cladograms. Numbers indicate the branch lengths from a point of divergence to the position of an extant taxon or the distance between successive points of divergence (A to B). Euclidean distances between subspecies given by molecular data were 15 for *oklahomensis* to *georgiensis*, 19 for *oklahomensis* to *maritima*, and 12 for *georgiensis* to *maritima*. Euclidean distances between subspecies given by unweighted morphological data were 15.2 for *oklahomensis* to *georgiensis*, 18.1 for *oklahomensis* to *maritima*, and 10.3 for *georgiensis* to *maritima*. Both phylogenies were produced using the neighbor-joining algorithm of PHYLIP (Phylogeny Inference Package; Felsenstein, 1995).

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