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RAPID ADAPTIVE DIVERGENCE IN NEW WORLD *ACHILLEA*, AN AUTOPOLYPLOID COMPLEX OF ECOLOGICAL RACES

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Adaptive evolution is often associated with speciation. In plants, however, ecotypic differentiation is common within widespread species, suggesting that climatic and edaphic specialization can outpace cladogenesis and the evolution of postzygotic reproductive isolation. We used cpDNA sequence (5 noncoding regions, 3.5 kb) and amplified fragment length polymorphisms (AFLPs: 4 primer pairs, 1013 loci) to evaluate the history of ecological differentiation in the North American *Achillea millefolium*, an autopolyploid complex of "ecological races" exhibiting morphological, physiological, and life-history adaptations to diverse environments. Phylogenetic analyses reveal North American *A. millefolium* to be a monophyletic group distinct from its European and Asian relatives. Based on patterns of sequence divergence, as well as fossil and paleoecological data, colonization of North America appears to have occurred via the Bering Land Bridge during the Pleistocene (1.8 MYA to 11,500 years ago). Population genetic analyses indicate negligible structure within North American *A. millefolium* associated with varietal identity, geographic distribution, or ploidy level. North American populations, moreover, exhibit the signature of demographic expansion. These results affirm the "ecotype" concept of the North American *Achillea* advocated by classical research and demonstrate the rapid rate of ecological differentiation that sometimes occurs in plants.

KEY WORDS: *Achillea*, adaptation, ecotype, niche, phylogeography, polyploidy, speciation.

Botanists have historically emphasized intraspecific ecological races as primary examples of adaptive evolution (Turesson 1925; Clausen et al. 1939, 1940, 1948; Stebbins 1950; Clausen 1951; Kruckeberg 1951). Classical transplant studies by Göte Turesson and Jens Clausen demonstrated dramatic examples of adaptation—including fundamental changes in anatomical, life-history and physiological characteristics—occurring within widespread plant species. Such ecotypic differences exist at small spatial scales and involve populations isolated by few intrinsic reproductive barriers. As a consequence, botanists often regard ecological differentiation as a rapid process that can be decoupled from speciation (Antonovics et al. 1971; Schemske 1984; Linhart and Grant 1996). In contrast, discussions of ecological differentiation in animals have tended to emphasize niche conservatism, developmental constraints, and the coincidence of adaptation and speciation (Futuyma 1987; Gould and Eldridge 1993; Schluter 2000; Wiens 2004).

Surprisingly, few molecular studies have tested the phylogenetic hypotheses of early plant evolutionists, in particular, that so-called ecotypes do not in fact represent well-differentiated cryptic species with distinct evolutionary histories. Here we evaluate the phylogenetic and demographic history of the North American *Achillea millefolium*, a complex of "ecological races" that is a textbook example of plant ecotypic differentiation. In their studies, Clausen and his colleagues at the Carnegie Institute of Washington (Division of Plant Biology, Stanford University) investigated phenotypic characteristics of *Achillea* populations

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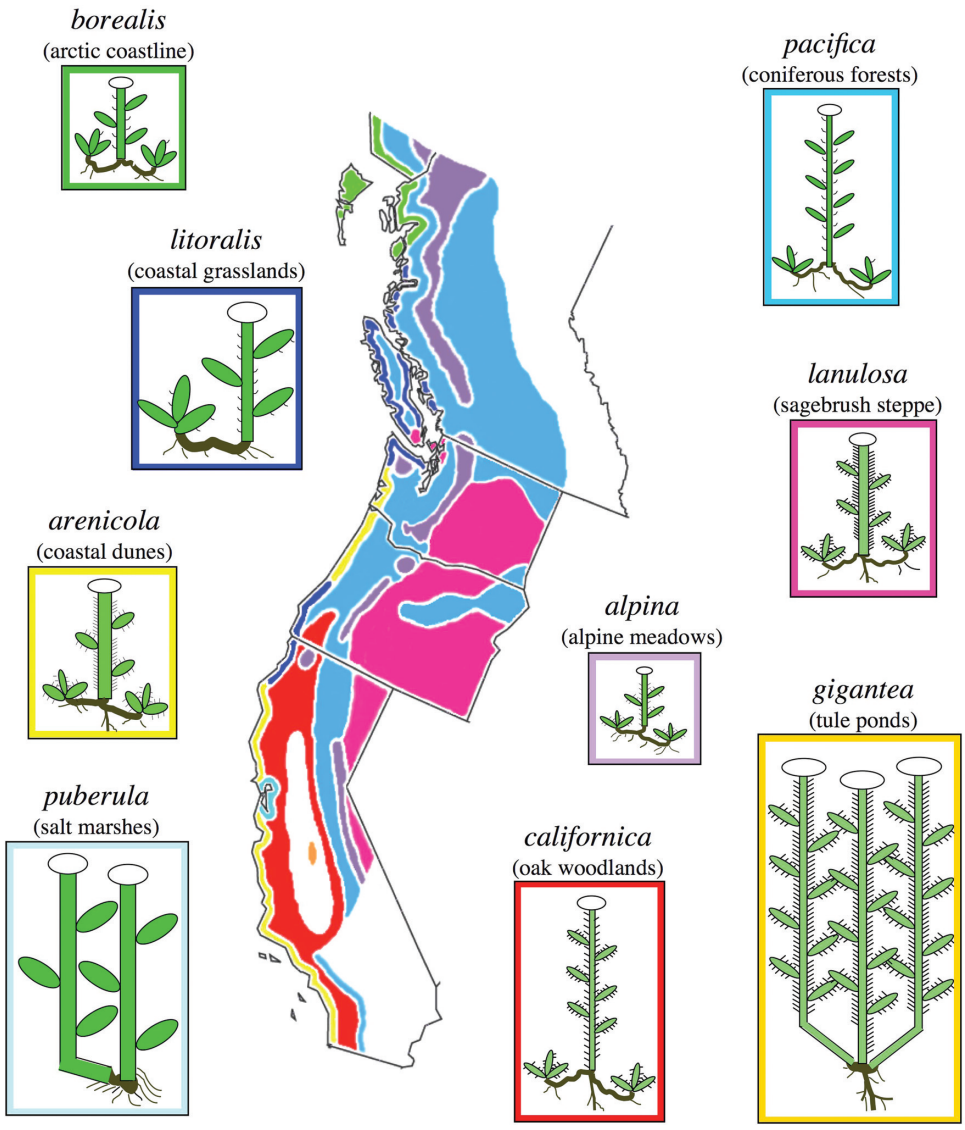

Figure 1. **Geographic distribution of the ecological races of North American *A. millefolium*. Pictograms show characteristics of the sampled varieties, including stem size, root structure, pubescence, and vegetative growth (see Table 1).**

along an altitudinal transect in California that ran from sea level to alpine regions (Clausen et al. 1940, 1948). Using experimental transplant gardens, the Carnegie group documented local adaptation of *A. millefolium* populations to their native environments. In total, nine ecological races were described from western North America, including forms adapted to coastal habitats, salt marshes, oak woodlands, coniferous forests, sagebrush steppe, and alpine meadows (Nobs 1960). The varieties are distinguished primarily on the basis of stem features (height and diameter), leaf characteristics (pubescence, color, fleshiness), phenology (dormancy, flowering times), and habitat associations (Fig. 1; Table 1).

The North American *A. millefolium* is an autopolyploid complex of tetraploid ($2n = 4x = 36$) and hexaploid ($2n = 6x = 54$) populations that exhibit multisomic inheritance (random association of homologous chromosomes during meiosis) and recurrent neopolyploid formation via unreduced gametes (Clausen et al. 1940, 1948; Lawrence 1947; Kruckeberg 1951; Hiesey and Nobs 1970; Ehrendorfer 1973; Tyrl 1975; Purdy and Bayer 1996; Ramsey 2007). The North American *Achillea* belongs to the circumpolar "*A. millefolium* aggregate," and ecological races are generally recognized as varieties of *A. millefolium* (Nobs 1960; Hiesey and Nobs 1970; Hickman 1993; Table 1). Eurasian members of the *A. millefolium* aggregate are geographically widespread but phenotypically less diverse than North American populations and are largely restricted to grasslands and forest margins (Tutin et al. 1976; Stace 1997; Ehrendorfer and Guo 2006; Table 1). Here we use phylogenetic and population genetic analyses of chloroplast sequence and amplified fragment length polymorphisms (AFLPs) to evaluate historical relationships among the North American *A. millefolium* and related Eurasian taxa. We

Table 1. Distribution, ecology, and phenotypic characteristics of North American *A. millefolium* and related Eurasian taxa.

Taxon	Ploidy level	Geographic distribution	Habitat	Stems	Foliage	Roots	Vegetative reproduction	Flowering phenology
A. millefolium var. *alpicola*	4*x*	w. North America (mountains)	alpine meadows	10–30 cm; slender	gray, villous	fibrous, along rhizomes	extensive	July–Sept.
A. millefolium var. *arenicola*	6*x*	w. North America (coasts of CA, OR, WA)	sand dunes, grasslands	20–80 cm; stout	gray, densely villous, fleshy	fibrous plus central taproot	extensive	April–June
A. millefolium var. *borealis*	6*x*	w. North America (coasts of AK, BC)	sand dunes, forest margins	20–40 cm	dark green, lightly villous	fibrous, along rhizomes	extensive	June–Sept.
A. millefolium var. *californica*	6*x*	w. North America (interior CA, OR)	oak woodlands	30–120 cm	gray green, villous	fibrous plus central taproot	extensive	March–June
A. millefolium var. *gigantea*	6*x*	w. North America (Central Valley, CA)	freshwater "tule" ponds	100–220 cm; robust	gray, wooly	deep taproot	absent (all stems reproductive)	May–July
A. millefolium var. *lanulosa*	4*x*	w. North America (Great Basin, Palouse)	sagebrush steppe, dry grasslands	30–70 cm; stout	gray, wooly	fibrous plus central taproot	extensive	April–June
A. millefolium var. *litoralis*	4*x*	w. North America (coasts of CA, OR, WA, BC)	mesic grasslands, forest margins	30–90 cm; robust	dark green, sparsely villous, fleshy	fibrous plus central taproot	extensive	June–Sept.
A. millefolium var. *pacifica*	4*x*	w. North America (Pacific lowlands)	open coniferous forest	30–90 cm; slender	green, sparsely villous	fibrous, along rhizomes	extensive	June-Sept.
A. millefolium var. *puberula*	4*x*	w. North America (coast of central CA)	salt marshes	80–180 cm; robust	dark green, ±hairless, very fleshy	fibrous, at stem base	absent (all stems reproductive)	July–Sept.
A. millefolium var. *mandshuricum*	2*x*, 4*x*	China, Russia	grasslands, riparian areas	20–60 cm;	green, sparsely villous	fibrous, along rhizomes	extensive	June-Sept.
A. millefolium var. *ceretanica*	2*x*	Spain, France (Pyrenees Mountains)	open coniferous forest	20–40 cm; slender	green, sparsely villous	fibrous, along rhizomes	extensive	July–Sept.
A. millefolium var. *millefolium*	6*x*	Europe	grasslands, riparian areas	20–60 cm	green, sparsely villous	fibrous, along rhizomes	extensive	June–Sept.

seek to identify the timeframe of adaptation and ask whether the North American plants represent (1) a monophyletic grouping of recently diverged but ecologically distinct forms; (2) a monophyletic grouping of highly divergent evolutionary lineages; or (3) a polyphyletic assemblage of historically Eurasian lineages that now co-occur in the western United States and Canada.

Materials and Methods

SAMPLING

We sampled the nine varieties of western North American *A. millefolium* described by the Carnegie group (Clausen et al. 1940, 1948; Nobs 1960) (Fig. 1; Tables 1 and 2). Most plants were collected by the authors as seed from Alaska, British Columbia, Washington, Oregon, and California during September 2003. A total of 43 North American populations were sampled; in some cases, we were able to include sites described in the Clausen et al. monographs (Table 2). Populations were identified to the varietal level based on morphological features, habitat associations and geographic distribution. Ploidy level of sampled individuals was confirmed using flow cytometry, as described by Ramsey (2007). We also included related Eurasian members of the *A. millefolium* aggregate based on findings by Guo et al. (2004, 2005) (Tables 1 and 2). F. Ehrendorfer and Y. Guo provided genomic DNA of *A. millefolium* var. *ceretanica* from France and *A. millefolium* var. *mandshuricum* from central Russia and China. Live plants of *A. millefolium* var. *millefolium* from Sweden were provided by D. Schemske and J. Agren.

DNA EXTRACTION, AMPLIFICATION, AND SEQUENCING

We used the DNeasy 96 plant kit (Qiagen, Valencia, CA) to isolate genomic DNA from two greenhouse-grown individuals from different maternal parents in each sampled population. Approximately 10 mg of fresh tissue was used from newly expanded leaves. We used universal primers to amplify five noncoding chloroplast regions: the *trnL-trnF* intergenic spacer (Taberlet et al. 1991), *atpB-rbcL* intergenic spacer (Golenberg et al. 1993), *rpS16* intron, *trnH-psbA* intergenic spacer, and *trnC-ycf6* intergenic spacer (Shaw et al. 2005). We performed 25 μl polymerase chain reactions with 1 μl genomic DNA, 0.5μl of each primer (10 mM stock), 0.5 μl $MgCl_2$ (25 mM stock), 0.5 μl dNTPs (10 mM stock), 0.05 μl taq polymerase (5000 U/mL), and 2.5 μl PCR buffer. Thermocycling was performed as follows: (1) denaturation at 95˚C for 3 min; (2) "touchdown" program for 10 cycles that decreased the annealing temperature by 1˚C per cycle, with initial values of 95˚C for 45 sec, 55˚C for 45 sec, and 72˚C for 45 sec; and (3) 20 cycles of 95˚C for 45 sec, 50˚C for 45 sec, and 72˚C for 45 sec. A random sample of PCR products was visualized on agarose gels to confirm amplification. Products were purified with Montáge™ plates (Millipore, Billerica, MA).

Cycle-sequencing reactions were performed in a 12.5-μl volume that included 1 μl cleaned PCR product, 1 μl of each primer (10 mM stock), 1μl Big Dye (ver. 3.1; Applied Biosystems, Foster City, CA) and 1 μl Big Dye buffer (5× Terminator; Applied Biosystems). Samples were sequenced in forward and reverse directions. Sequence products were cleaned using sephadex columns in combination with HV Plates (Millipore) and sequenced on an ABI 3730 capillary sequencer. Sequences were manually aligned using Sequencher™ (ver. 4.1; Gene Codes, Ann Arbor, MI). We retained indels for analysis when inserted/deleted regions did not contain microsatellites, here defined as tandems of simple sequence (1–4 base pairs) repeated 10 or more times (Queller et al. 1993). All DNA sequences used in this study can be found in GenBank accessions EU128982–EU129456.

AMPLIFIED FRAGMENT LENGTH POLYMORPHISMS

Because of potential effects of DNA quality on fragment generation, we only evaluated AFLPs for live plants growing in the greenhouse (North American *Achillea* and *A. millefolium* var. *millefolium* from Sweden). We included two to three individuals from a subset of populations used for cpDNA sequencing, with representative inclusion across varieties (80 individuals from 30 populations). AFLP protocol followed the digestion–ligation–amplification scheme outlined by Vos et al. (1995). Restriction products were visualized on agarose gels to confirm full digestion. For selective amplification, we used "+3" primers with randomly selected base pair composition. *Eco*RI primers were fluorescently labeled with "vic" (green, for -ACA); "pet" (red, for -ACT); "fam" (blue, for -ACG); or "ned" (yellow, for -AAG). *Mse* primers were unlabelled and all -CAC. Products of selective amplification were pooled such that each cell of a 96-well plate combined four different primer pair combinations, each with a different fluorescent color. Fragments were visualized on the ABI 3730 capillary sequencer using standard fragment protocols. The GeneMapper™ software application (ver. 3.7; Applied Biosystems) was used to score alleles. We used an automated allele binning protocol (bin width of 0.9; minimum relative fluorescence units of 200). Manual review of allele bins revealed few apparent errors. Repeated preselective and selective amplifications of eight randomly selected individuals indicated an average genotype similarity of 92.8% (range: 90.8–94.8) whereas mean pairwise similarity across individuals was 67.7% (range: 62.2–73.3).

PHYLOGENETIC ANALYSES

Chloroplast sequence data were evaluated using maximum parsimony and distance methods with PAUP* (ver. 4.0b10; Swofford 2003). Partition homogeneity tests did not indicate rate heterogeneity ($P > 0.50$) so all phylogenetic analyses were based on

Table 2. Location and cytotype information for *Achillea* included in phylogenetic and population genetic analyses.

Taxon	Location	Population	Code	Ploidy	El. (m)
A. millefolium var. *alpicola*	U.S.A. (Washington)	Hurricane	1	4*x*	1620
—	U.S.A. (California)	Lassen	2	4*x*	2040
—	U.S.A. (Oregon)	Mt. Hood	3	4*x*	1800
—	U.S.A. (Washington)	Rainier	4	4*x*	2020
—	U.S.A. (Arizona)	Rustler Peak	5	4*x*	2500
—	U.S.A. (California)	Tuolumne	6	4*x*	2620
A. millefolium var. *arenicola*	U.S.A. (California)	Asilomar	7	6*x*	10
—	U.S.A. (California)	Bodega	8	6*x*	30
—	U.S.A. (Washington)	Damon Point	9	6*x*	10
—	U.S.A. (Oregon)	Ecola	10	6*x*	10
—	U.S.A. (Washington)	Nahcotta Tidelands	11	6*x*	10
—	U.S.A. (Oregon)	Oregon Dunes	12	6*x*	20
—	U.S.A. (California)	Stone Lagoon	13	6*x*	10
A. millefolium var. *borealis*	U.S.A. (Alaska)	Cohoe Dunes	14	6*x*	10
—	U.S.A. (Alaska)	Tonsina Point	15	6*x*	20
—	U.S.A. (Alaska)	Turnagain Dunes	16	6*x*	10
A. millefolium var. *californica*	U.S.A. (California)	Bald Hills	17	6*x*	800
—	U.S.A. (California)	Wantrup	18	6*x*	230
A. millefolium var. *gigantea*	U.S.A. (California)	Selma	19	6*x*	80
A. millefolium var. *lanulosa*	U.S.A. (Oregon)	Crooked River	20	4*x*	1360
—	U.S.A. (Washington)	Deception Pass	21	4*x*	40
—	U.S.A. (Washington)	Horsethief	22	4*x*	60
—	U.S.A. (Washington)	Leavenworth	23	4*x*	1200
—	U.S.A. (California)	Lee Vining	24	4*x*	2100
—	Canada (British Columbia)	Mill Hill	25	4*x*	180
—	U.S.A. (Washington)	Mima Mounds	26	4*x*	80
A. millefolium var. *litoralis*	U.S.A. (Washington)	Birch Bay	27	4*x*	10
—	U.S.A. (Oregon)	Cape Arago	28	4*x*	20
—	U.S.A. (Oregon)	Cape Blanco	29	4*x*	30
—	U.S.A. (California)	Damnation Creek	30	4*x*	20
—	U.S.A. (California)	Patrick's Point	31	4*x*	30
—	Canada (British Columbia)	Rathtrevor Beach	32	4*x*	10
—	U.S.A. (Washington)	Ruby Beach	33	4*x*	10
—	Canada (British Columbia)	San Josef Bay	34	4*x*	10
—	U.S.A. (Washington)	Semiahmoo	35	4*x*	10
—	U.S.A. (Washington)	Wolfe Beach	36	4*x*	10
A. millefolium var. *pacifica*	U.S.A. (Washington)	Box Canyon	37	4*x*	950
—	U.S.A. (California)	Castle Creek	38	4*x*	640
—	U.S.A. (Alaska)	Denali	39	4*x*	530
—	U.S.A. (California)	Groveland	40	4*x*	1000
—	U.S.A. (Oregon)	Ochoco Divide	41	4*x*	1380
—	U.S.A. (Oregon)	Wahkeena Falls	42	4*x*	180
A. millefolium var. *puberula*	U.S.A. (California)	Napa Marsh	43	4*x*	0
A. millefolium var. *mandshuricum*	China (Inner Mongolia)	Huiliang Valley	44	2x	1600
—	Russia	Altai	45	2x	1100
—	Russia	Altai	46	4*x*	220
A. millefolium var. *ceretanica*	France	Pyrenees	47	2x	1700
A. millefolium var. *millefolium*	Sweden	Rödåsen	48	6*x*	300

the combined dataset. We treated gaps (nonmicrosatellite indels) as a fifth character state and weighted all nucleotide substitutions as equal and unordered. Indels were coded as a single character irrespective of actual length. For maximum parsimony analysis, heuristic searches were conducted using TBR branch-swapping with 1000 random additions, and we estimated bootstrap support for nodes on the trees using 1000 replicates. For distance analysis, we used the neighbor-joining algorithm in PAUP*. Distances were based on total character differences (including transitions, transversions, and indels) and bootstrap support for nodes was estimated with 1000 replicates. We used "two-cluster" tests (Takezaki et al. 1995) to determine consistency of cpDNA data with a clock-like model of evolution.

For analysis of AFLP data, we used a neighbor-joining algorithm, following Albertson et al. (1999). Distances were based on total character differences, as calculated by PAUP*. We assessed bootstrap support for nodes using 1000 replicates.

POPULATION GENETIC ANALYSES

Arlequin (ver. 2.0; Schneider et al. 2000) was used for population genetic analyses of cpDNA haplotypes and AFLPs among the North American *Achillea*. We performed analysis of molecular variance (AMOVA; Excoffier et al. 1992) with three grouping schemes: geographic (populations grouped by state or province; Arizona was excluded because only a single location was sampled), taxonomic (populations grouped by variety; *gigantea* and *puberula* were excluded because only single populations were sampled), and chromosomal (populations grouped by ploidy level). In each analysis, significance of variance components among groups (ϕ_{CT}), among populations within groups (ϕ_{SC}), and within populations (ϕ_{ST}) was evaluated using 1000 permutations. Historical demography of North American *Achillea* was assessed using mismatch distributions, which are unimodal in lineages experiencing demographic expansion (Slatkin and Hudson 1991; Rogers and Harpending 1992). For cpDNA sequences, parametric bootstraps were used to compare the actual frequency distribution of pairwise differences versus an expected distribution generated by a stepwise population expansion model for a haploid locus (Schneider and Excoffier 1999). We also estimated demographic expansion parameters τ (modal value of mismatch distribution), θ_0 ($2Nu$ before population expansion), and θ_1 ($2Nu$ after population expansion) as defined for a haploid, nonrecombining locus. Because explicit models of population expansion are not parameterized for multisomic polyploids and comparisons across ploidy levels, for AFLPs we simply computed the mean, variance, and modality of mismatches between fragment profiles of sampled individuals.

For cpDNA data, we also used DnaSP (ver. 4.0; Rozas et al. 2003) to estimate Tajima's *D*, a statistical descriptor of the frequency spectrum of alleles at a locus (Tajima 1989). For statistical analysis, we performed coalescent simulations assuming a neutral model with constant population size and no recombination. We defined 95% confidence intervals for the coalescent simulations based on 1000 replicates.

Results

The combined cpDNA sequence dataset contained 3500 nucleotide base pairs, including 36 variable and 28 parsimony-informative sites. Indels were nonoverlapping and did not obscure base pair differences among sampled haplotypes (Tables 3 and 4). The five noncoding regions contributed similar numbers of informative sites (variable sites, including indels: *trnL-trnF*, 6; *atpB-rbcL*, 3; *rpS16*, 6; *trnH-psbA*, 9; *trnC-ycf6*, 12). In the combined dataset, the North American *A. millefolium* possessed a single major haplotype (66% of samples) and 16 minor haplotypes that were one or two mutation steps from the major haplotype (via an indel or base pair difference) (Table 4). Eurasian taxa contributed five additional haplotypes that were distinguished from the North American plants by 7–12 base pair differences and indels. Heuristic search of the cpDNA sequence dataset generated three most parsimonious trees that differed in placement of several North American populations (haplotypes *m* and *o*; Table 4) but were otherwise identical (length = 38 steps; CI = 0.947; RI = 0.978). The consensus phylogeny exhibited strong bootstrap support (Fig. 2A). Neighbor-joining generated a similar tree topology.

Phylogenetic analyses of cpDNA provided strong support for a monophyletic clade of North American *A. millefolium* that was distinct from Eurasian taxa (Fig. 2A). Structure within North America was generally limited to grouping of individuals within populations, because most of the minority haplotypes were either unique to the dataset (and hence autopomorphic) or specific to a single population (Table 4). The genealogy of North American haplotypes was distinctly star-shaped (Fig. 2A). There was no phylogenetic structure associated with geographic distribution, variety, or ploidy level. Eurasian taxa were distinguished from each other in the chloroplast phylogeny (Fig. 2A). In contrast to the lack of phylogenetic structure within the North American *A. millefolium*, Chinese and Russian populations of *A. millefolium* var. *mandshuricum* were strongly differentiated. Chinese *A. millefolium* var. *mandshuricum* had a close relationship with the North American *Achillea* (Fig. 2A) with 0.20% uncorrected sequence divergence (seven sites in 3.5 kb). Two-cluster tests failed to reject the null hypothesis of a "clock-like" accumulation of cpDNA differences, suggesting branch lengths could be interpreted as relative time since divergence.

The AFLP dataset consisted of 1013 loci, including 838 informative fragments. Phylogenetic analysis revealed limited structure among the North American samples of *Achillea* (Fig. 2B).

Table 3. Indels inferred from alignment of five noncoding cpDNA regions. Status of indels as insertions (+) or deletions (−) made by assignment of *A. millefolium* var. *millefolium* as an outgroup (Guo et al. 2004, 2005).

Indel	Region	Position (bp)	Size	Taxon (population code)
I	*trnL-trnF*	66	−1	North American *A. millefolium* (1–43)
II	–	88	+21	*A. millefolium* var. *alpicola* (6)
III	–	702	+1	*A. millefolium* var. *gigantea* (19)
IV	*atpB-rbcL*	657	−1	*A. millefolium* var. *mandshuricum* (44); North American *A. millefolium* (1–43)
V	*rpS16*	45	−6	*A. millefolium* var. *mandshuricum* (46);
VI	–	154	−7	*A. millefolium* var. *mandshuricum* (44–46); North American *A. millefolium* (1–43)
VII	–	235	−3	*A. millefolium* var. *borealis* (16)
VIII	–	744	+1	*A. millefolium* var. *mandshuricum* (44)
IX	*trnH-psbA*	30	+1	*A. millefolium* var. *alpicola* (4)
X	–	82	+11	*A. millefolium* var. *californica* (18)
XI	–	186	+5	*A. millefolium* var. *mandshuricum* (45–46)
XII	–	380	+15	*A. millefolium* var. *alpicola* (4)
XIII	*trnC-ycf6*	336	+1	North American *A. millefolium* (1–43)
XIV	–	427	+6	*A. millefolium* var. *alpicola* (3)
XV	–	479	+21	*A. millefolium* var. *litoralis* (36)

Internal branches on the neighbor-joining phylogeny were very short, generating a star-shaped topology (Fig. 2B). Bootstrap support was restricted to nodes grouping individuals from single populations. North American plants were clearly distinguished from Swedish *A. millefolium* var. *millefolium*.

For both cpDNA and AFLPs, analysis of molecular variance among North American *A. millefolium* indicated negligible genetic structure associated with geography, variety, and ploidy level (Tables 4 and 5). In contrast, variance was strongly associated with differences among populations and differences among individuals within populations. Analysis of cpDNA haplotypes indicated an equal split between among- and within-population differences whereas variance in AFLPs was primarily tied to within-population differences (Table 5). Mismatch distributions for cpDNA haplotypes and AFLPs exhibited the characteristic unimodal pattern of lineages experiencing demographic expansion (Fig. 3) and did not differ significantly from that expected by a stepwise population expansion model for a haploid locus ($P = 0.9$). Estimates of demographic parameters and confidence intervals ($\alpha = 0.05$) for cpDNA haplotypes were as follows: modal value, τ, 0.967 [0–2.451]; $2Nu$ before expansion, θ_0, 0 [0–0.713]; $2Nu$ after population expansion, θ_1, 4.182 [0.156–4754.182]. Mismatch distributions for AFLPs exhibited a mean and variance of 206.130 and 1048.897, respectively.

The observed value of Tajima's *D* for the North American *A. millefolium* was −1.929. Coalescent simulations based on a neutral model with constant population size and no recombination had a 95% confidence interval of [−1.398, 2.179], indicating that the excess occurrence of rare cpDNA sequences in the North American *A. millefolium* was unlikely to have been observed by chance ($P < 0.0001$).

Discussion

ORIGIN OF NORTH AMERICAN *A. MILLEFOLIUM*

The close phylogenetic relationship between North American and Asian *Achillea* suggests a recent origin of North American plants (Guo et al. 2004, 2005; Fig. 2). Identifying a specific timeframe for the colonization of North America, however, is difficult. The Bering Land Bridge, the principle conduit for species migration between North America and Eurasia, has existed for most of the past 70 million years (Hopkins 1967a). Marine fossils reveal a major break ~5.5 million years ago (MYA), an event that generated the Bering Strait (Gladenkov et al. 2002). However, water depth reaches only 50 m below current sea level along the eastern Siberian and western Alaskan continental margins (Creager and McManus 1967). Fluctuations in sea level associated with glacial cycles during the Pliocene and Pleistocene led to repeated emergence and submergence of Beringia over the past five million years (Hopkins 1967a, b; Elias 1996). Because of the close association that Beringia has maintained between Asia and North America, it is unrealistic to define a single window of time when colonization of North America could have occurred—we must rely on other information.

During the Miocene (23.8–5.3 MYA) and Pliocene (5.3–1.8 MYA), mesic forests dominated the northern hemisphere (Tiffney 1985). Global cooling and the submergence of Beringia fragmented continuous forest habitat, remnants of which are now

Table 4. Major and minor cpDNA haplotypes observed in North American *A. millefolium* (86 individuals from 43 populations).

		cpDNA sequence																							
		trnL-trnF		*atpB-rbcL*	*rpS16*		*trnH-psbA*				*trnC-ycf6*														
Haplotype	Mutation steps[1]	0 8 8	7 0 2	5 1 6	2 3 5	5 3 3	0 3 0	0 8 2	1 7 9	3 8 0	0 3 7	3 2 7	3 5 0	3 5 2	4 2 7	4 4 9	4 7 9	5 8 4	Variety (population)	No. individuals (percentage)
a	–	–	–	G	G	A	–	–	C	–	T	G	T	A	–	T	–	G	*alpicola* (1, 2, 4, 5); *arenicola* (7, 8, 9, 11, 12, 13); *borealis* (14, 15); *californica* (17); *lanulosa* (20, 22, 23, 24, 25, 26); *litoralis* (27, 28, 29, 30, 31, 32, 33, 34, 35); *pacifica* (37, 38, 41, 42)	57 (66.3%)
b	1	–	–	G	G	A	–	–	C	–	T	G	T	A	**T**	T	–	G	*alpicola* (3)	2 (2.3%)
c	2	–	–	G	G	A	**A**	–	C	**A**	T	G	T	A	–	T	–	G	*alpicola* (4)	1 (1.2%)
d	1	**T**	–	G	G	A	–	–	C	–	T	G	T	A	–	T	–	G	*alpicola* (6)	2 (2.3%)
e	1	–	–	G	G	**C**	–	–	C	–	T	G	T	A	–	T	–	G	*arenicola* (7)	1 (1.2%)
f	1	–	–	G	G	A	–	–	C	–	T	G	**G**	A	–	T	–	G	*arenicola* (10)	2 (2.3%)
g	1	–	–	G	G	A	–	–	C	–	T	G	T	**C**	–	T	–	G	*arenicola* (9, 11); *lanulosa* (26)	3 (3.5%)
h	1	–	–	G	**–**	A	–	–	C	–	T	G	T	A	–	T	–	G	*borealis* (16)	1 (1.2%)
i	1	–	–	G	G	A	–	–	C	–	**G**	G	T	A	–	T	–	G	*borealis* (16); *litoralis* (35)	2 (2.3%)
j	1	–	–	G	G	A	–	**A**	C	–	T	G	T	A	–	T	–	G	*californica* (18)	1 (1.2%)
k	1	–	–	G	G	A	–	–	C	–	T	**T**	T	A	–	T	–	G	*californica* (18)	1 (1.2%)
l	1	–	**T**	G	G	A	–	–	C	–	T	G	T	A	–	T	–	G	*gigantea* (19)	2 (2.3%)
m	2	–	–	G	G	A	–	–	**T**	–	T	G	T	A	–	**A**	–	G	*lanulosa* (21, 22); *litoralis* (32)	4 (4.7%)
n	1	–	–	G	G	A	–	–	C	–	T	G	T	A	–	T	**T**	G	*litoralis* (36)	1 (1.2%)
o	1	–	–	G	G	A	–	–	C	–	T	G	T	A	–	**G**	–	G	*pacifica* (39)	2 (2.3%)
p	1	–	–	G	G	A	–	–	C	–	T	G	T	A	–	T	–	**T**	*pacifica* (40)	2 (2.3%)
q	1	–	–	**T**	G	A	–	–	C	–	T	G	T	A	–	T	–	G	*puberula* (43)	2 (2.3%)

[1] Mutational steps separating minor haplotypes (*b*-*q*) from the major haplotype (a).

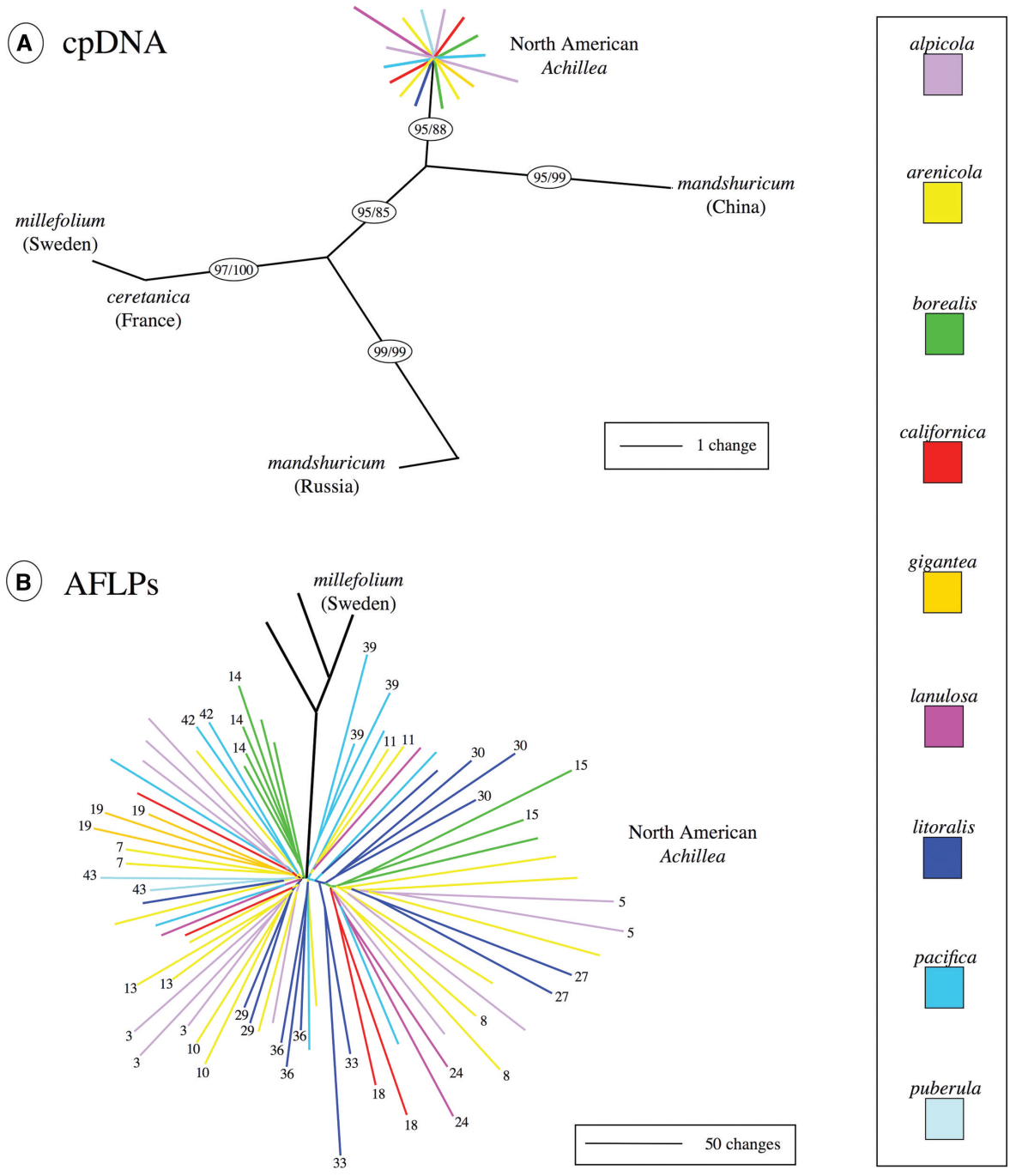

Figure 2. Unrooted phylograms depicting relationships among North American *A. millefolium* and related Eurasian taxa. Branches are color-coded to identify varieties. (A) Strict consensus of three most-parsimonious trees generated from cpDNA sequence (5 noncoding regions totaling 3.5 kb; 36 variable characters observed from 95 individuals in 48 populations). Circled numbers on branches indicate bootstrap support based on parsimony and distance-based analyses, respectively. (B) Neighbor-joining analysis of amplified fragment length polymorphisms (4 primer pairs; 838 informative alleles observed from 80 individuals in 30 populations). Numbers identify populations for individuals that cluster to form monophyletic groupings (see Table 2). Bootstrap support was found for nodes uniting individuals in populations 5 (74%), 14 (51%), 15 (68%), 18 (59%), 27 (72%), 36 (57%), 39 (77%), and 48 (98%).

restricted to North America, eastern Asia, and central Eurasia (Milne and Abbott 2002). Divergence of Asian–North American taxa dating from the Miocene and Pliocene generally involves plants of moist woodlands (Wen 1999; Xiang et al. 2000). In contrast, *Achillea* species occur in open, relatively xeric environments (Clausen et al. 1940, 1948; Guo et al. 2004, 2005) such as those that dominated Beringia during the Pleistocene (1.8 MYA to 11,500 years ago; Colinvaux 1996; Elias 1996). Hence, from a

Table 5. Analysis of molecular variance for North American *A. millefolium* with groupings defined by geography, variety or ploidy level.

Data	Analysis	Percentage of variation	Fixation index	Statistical significance
cpDNA	Groups defined by geography			
	Among groups	0	Φ_{CT}=0	P=0.57, df=4
	Among populations within groups	49.7	ϕ_{SC}=0.50	P<0.001, df=37
	Within populations	50.7	ϕ_{ST}=0.49	P<0.001, df=42
	Groups defined by variety			
	Among groups	2.4	ϕ_{CT}=0.02	P=0.16, df=6
	Among populations within groups	40.7	ϕ_{SC}=0.42	P<0.001, df=34
	Within populations	56.9	ϕ_{ST}=0.43	P<0.001, df=41
	Groups defined by ploidy level			
	Among groups	1.4	ϕ_{CT}=0.01	P=0.109, df=1
	Among populations within groups	48.4	ϕ_{SC}=0.49	P<0.001, df=41
	Within populations	50.2	ϕ_{ST}=0.50	P<0.001, df=43
AFLPs	Groups defined by geography			
	Among groups	1.5	ϕ_{CT}=0.02	P=0.005, df=3
	Among populations within groups	10.7	ϕ_{SC}=0.11	P<0.001, df=24
	Within populations	87.8	ϕ_{ST}=0.12	P<0.001, df=47
	Groups defined by variety			
	Among groups	0.8	ϕ_{CT}=0.01	P=0.16, df=6
	Among populations within groups	11.2	ϕ_{SC}=0.11	P<0.001, df=20
	Within populations	88.0	ϕ_{ST}=0.12	P<0.001, df=45
	Groups defined by ploidy level			
	Among groups	0.1	ϕ_{CT}=0	P=0.393, df=1
	Among populations within groups	11.9	ϕ_{SC}=0.12	P<0.001, df=27
	Within populations	88.0	ϕ_{ST}=0.12	P<0.001, df=48

paleoecological perspective, a recent colonization of North America seems likely.

Fossil data also suggest a Pleistocene origin of North American *Achillea*. Fossilized *Achillea* are known from Beringia at 35,000 years ago (seeds in rodent middens; Zuzula et al. 2005) and 11,000 years ago (pollen in marine sediments; Colinvaux 1964) but not from samples dated to the Pliocene or early Pleistocene (Muhs et al. 2001; Matheus et al. 2003; Kienast et al. 2005). In the continental United States and southern Canada, there are no *Achillea* fossils known from shell middens, rodent middens, or pollen sediments (Sawbridge and Bell 1972; Cole 1983; Jennings and Elliot-Fisk 1993; Lyford et al. 2002; Sharp 2002) despite its current abundance in these regions and the propensity of middens to accurately catalog surrounding vegetation (Nowak et al. 2000). The fossil record of North American *Achillea* is thus recent, geographically limited, and consistent with rapid expansion across the continent.

Low sequence divergence between Chinese *A. millefolium* var. *mandshuricum* and North American *Achillea* also indicates a recent colonization event. Although there is no universal chloroplast molecular clock, comprehensive studies of cpDNA differences between plants with well-defined divergence times suggest that, at neutral sites, chloroplast divergence rates are on the order of 0.1–0.3% per million years (Wolfe et al. 1987; Muse 2000). Application of these rates to *Achillea* suggests that divergence of *A. millefolium* var. *mandshuricum* and North American *A. millefolium* from their most recent common ancestor occurred in the range of 1.0–0.3 MYA. We emphasize that divergence estimates outlined here are based on indirect evidence and should not be considered conclusive. Nonetheless, paleoecological data, the fossil record, and cpDNA divergence support the inference of a Middle or Late Pleistocene origin of North American *Achillea*.

RAPID ECOLOGICAL DIFFERENTIATION

Despite the morphological and ecological diversity of North American *A. millefolium*, the complex exhibits a striking uniformity of cpDNA haplotypes. We observed the major cpDNA haplotype from central Alaska to southeast Arizona, in fleshy, almost hairless plants on moist seashores, wooly tap-rooted plants in sagebrush deserts, and diminutive plants on alpine meadows (Figs. 1, 2; Tables 1 and 4). Transplant studies indicate that phenotypic differences between the ecological races of *A. millefolium* have a strong genetic component, and that ecotypes are highly adapted to their native environs (Clausen et al. 1940, 1948; Ramsey 2003). Eurasian *A. millefolium* present a contrasting pattern in which phenotypically similar taxa, occurring in grassland and riparian habitats, are strongly distinguished phylogenetically. This juxtaposition indicates that the North American plants have undergone

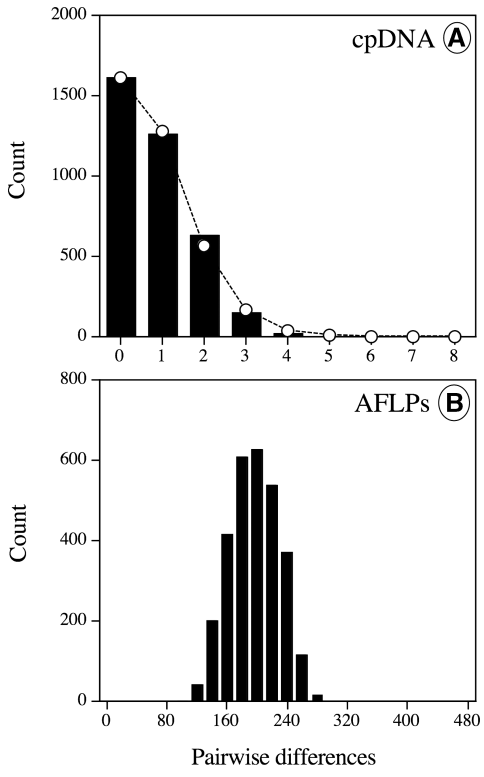

Figure 3. Mismatch distributions for (A) cpDNA haplotypes and (B) AFLP profiles of the North American *A. millefolium*. The line shows values for cpDNA haplotypes expected by a model of population expansion for a haploid locus.

rapid ecological differentiation—the proliferation of ecological races in the New World has outpaced the timeframe of cladogenesis exhibited by Old World *Achillea*.

The pattern of cpDNA variation among North American *A. millefolium* suggests that rapid ecological differentiation was accompanied by demographic expansion. For a given neutral allele, the timeframe for coalescence is proportional to population size. The probability of coalescence in a lineage thus decreases precipitously following demographic expansion; looking backwards in time, coalescence events will cluster immediately prior to expansion (Wakeley 2003; Hein et al. 2005). Haplotype frequencies among extant populations will consequently be unequal, composed of a dominant major haplotype (fixed prior to population growth) and rare, independently derived minor haplotypes (generated following demographic expansion). Internal branches on resulting genealogies will be short, generating a star-shaped topology (Slatkin and Hudson 1991; Rogers and Harpending 1992). Another signature of demographic expansion is a unimodal frequency distribution of haplotype differences (Slatkin and Hudson 1991). In contrast, lineages in demographic equilibrium produce a multi-modal mismatch distribution due to underlying phylogenetic structure and staggered coalescence events (Slatkin and Hudson 1991; Schneider and Excoffier 1999; Hein et al. 2005). Haplotype variation among the North American *A. millefolium* proves a good fit to the population genetic outcomes of demographic expansion (Figs. 2, 3; Tables 4 and 5).

AFLPs corroborate cpDNA data in implying rapid ecological divergence and demographic expansion. Despite the large number of polymorphic fragments identified, there are few fixed differences and negligible phylogenetic structure delineating the nine North American varieties of *A. millefolium* (Fig. 2B; Tables 4 and 5). Phylograms generated by AFLP data have short internal branches and star-shaped topology (Fig. 2B). The frequency distribution of pairwise fragment differences is also unimodal (Fig. 3B). Despite these similarities, important differences do exist between the cpDNA and AFLP datasets. For example, variability of cpDNA sequence is less than that of AFLP fragment profiles, and branch lengths are shorter in phylograms generated by cpDNA than AFLPs (Figs. 2, 3). Although cpDNA and AFLPs both indicate negligible genetic structure associated with geography and ploidy level, there is less between-population differentiation for AFLPs than cpDNA (Table 5).

As revealed in comparisons of human mtDNA versus nuclear markers (Hey 1997; Hey and Harris 1999), differences between cpDNA and AFLPs observed in this study probably reflect intrinsic features of organelle versus nuclear genomes. First, the expected time (in generations) for coalescence of chloroplast haplotypes is equal to the effective population size, N_e, whereas for nuclear genes in an autotetraploid or autohexaploid the expected time is $4N_e$ or $6N$, respectively (Hein et al. 2005). Second, mutation rates for nuclear genes are on the order of 5–10 times greater than mutations rates for cpDNA (Wolfe et al. 1987; Muse 2000). Third, nuclear regions experience frequent recombination whereas cpDNA does not. For these reasons, we anticipate cpDNA to exhibit relatively rapid fixation in local populations with limited overall variation whereas AFLPs will exhibit fewer fixed differences with more overall variability (Figs. 2, 3; Table 5).

Selective sweeps, population bottlenecks, and demographic expansions are alternate interpretations for the results of this study (Wakeley 2003; Hein et al. 2005). However, several lines of evidence support the hypothesis of demographic expansion. First, it is unlikely that selective sweeps would generate a unimodal mismatch distribution and star-shaped genealogies for AFLPs, which reflect many unlinked regions of the nuclear genome (Vos et al. 1995). Second, an expected outcome of population decline is large-scale variability in coalescence times across loci, but we observe many similarities between cpDNA and AFLPs. Assuming that the genealogy of cpDNA reflects a history of population growth, we can use expansion parameters to estimate expansion time (Schneider and Excoffier 1999). For the chloroplast, the modal value of the mismatch distribution (τ) is equal to $2\mu kt$, where μ is the per site mutation rate and k is the total number of base pairs studied. Based on direct study (GuhaMajumbdar and

Sears 2005) and inference from sequence divergence over time (Muse 2000), mutation rates in cpDNA are probably on the order of 1.0×10^{-8} to 1.0×10^{-9} per site per generation. Application of these parameters to the North American *A. millefolium* suggests a timeframe in the range of 15,000–150,000 generations.

Why was population growth associated with rapid adaptive divergence in historic populations of North American *A. millefolium*? Invasion of a new continent presented colonists with unique ecological opportunities that may have fueled both demographic explosion and ecotypic differentiation. For example, North American populations escaped competition with >130 congeneric species that occur across Europe and Asia. Additionally, the topographically and edaphically varied environments of western North America present numerous avenues for ecological specialization in plants (Hickman 1993; Ornduff et al. 2003). Studies of adaptive radiations suggest that island habitats frequently drive ecological divergence because of competitive release and the availability of novel habitats (Givnish and Sytsma 1997; Schluter 2000). Colonization of North America by *A. millefolium* can perhaps be understood in the same context on a shorter time scale.

INCIPIENT SPECIATION

Polyploidy is commonly regarded as a mechanism of instantaneous and complete reproductive isolation (Ramsey and Schemske 1998, 2002). On this basis, North American *A. millefolium* was originally described as two species, the tetraploid *A. lanulosa* and hexaploid *A. borealis* (Clausen et al. 1940, 1948). Later work cast doubt on this scheme (Nobs 1960; Hiesey and Nobs 1970; Ehrendorfer 1973; Tyrl 1975; Purdy and Bayer 1996; Ramsey 2007). First, parallel patterns of phenotypic differentiation were observed between tetraploids and hexaploids, indicating that field identification of "*A. lanulosa*" and "*A. borealis*" is not straightforward. Second, cytogenetic study revealed the autopolyploid nature of the complex—including multisomic inheritance and recurrent hexaploid formation in tetraploid populations—suggesting that "*A. borealis*" could be a polyphyletic assemblage of independently derived autohexaploid cytotypes. Third, pentaploid F_1 hybrids between *A. lanulosa* and *A. borealis* are semifertile, producing fertile euploid progeny in backcrosses to tetraploids and hexaploids. For these reasons, North American plants were lumped taxonomically into a broadly defined and circumpolar taxonomic species (*A. millefolium*).

Phylogenetic analyses confirm the close evolutionary relationship between North American cytotypes. There are no consistent cpDNA sequence differences between tetraploids and hexaploids (Table 4). Two of the three minor cpDNA haplotypes occurring in multiple locations were found in tetraploid and hexaploid individuals. For both cpDNA and AFLPs, analysis of molecular variance indicates that there is negligible genetic structure associated with ploidy level (Table 5). If tetraploid and hexaploid cytotypes represent monophyletic lineages isolated by strong reproductive barriers, there has been too little time for this to be reflected by coalescence of the cpDNA regions and AFLPs studied here. Given the available data, polyploidy is probably best viewed as a mechanism of partial reproductive isolation that may contribute to incipient speciation within the North American *Achillea*.

Aside from polyploidy, there is little postzygotic reproductive isolation within the North American *A. millefolium*. Ecological races are fully intercrossable, and hybrids exhibit few meiotic aberrations and high fertility (Clausen et al. 1940, 1948, 1955; Ehrendorfer 1952; Hiesey and Nobs 1970; J. Ramsey, unpubl. data). Climate and edaphic specialization thus appears to be evolving more rapidly than intrinsic reproductive barriers and may be driving ecological speciation, as has been postulated in some other plant groups (see Abbott and Comes 2007). For example, habitat differences probably provide strong reproductive isolation to ecological specialists like varieties *gigantea* (endemic to sandy bottomlands of California's Central Valley) and *puberula* (endemic to salt marshes in the San Francisco Bay) (Nobs 1960; Table 1). Similarly, phenological isolation is probably well developed between ecological races that are spring flowering (e.g., varieties *arenicola* and *lanulosa*) versus summer flowering (e.g., varieties *litoralis* and *alpicola*). On the other hand, clinal variation and intergradation between widespread races like *californica* and *pacifica* suggest that reproductive isolation may be poorly developed between some varieties.

TAXONOMIC IMPLICATIONS

Based on studies of the California flora, Jens Clausen described three concepts of species, roughly corresponding to stages of evolutionary divergence: ecotypes (ecologically differentiated populations that lack intrinsic genetic barriers and typically intergrade in sympatry); ecospecies (ecologically differentiated populations, generally isolated by partial postzygotic reproductive barriers, that maintain their integrity in sympatry); and cenospecies (complexes of ecospecies isolated by absolute genetic incompatibility) (Clausen et al. 1939, 1940; Clausen 1951). The New World *A. millefolium* is perhaps best understood as a recent arrival to North America that has diverged ecologically (i.e., varieties represent ecotypes) and may be in the early stages of speciation (e.g., incipient speciation via polyploidy may be creating ecospecies). Recognition of a single polymorphic North American species appears the most justified approach on the basis of the biological, phylogenetic, and taxonomic species concepts.

What species epithet should identify the North American *Achillea*? In recent floristic treatments North American plants are generally described simply as "*A. millefolium*." This is unsatisfactory given (1) genetic incompatibility between the Eurasian *A. millefolium* sensu strictu (*A. millefolium* var. *millefolium*) and

North American *A. millefolium* (Clausen et al. 1940, 1948, 1955); (2) substantial DNA sequence divergence between *A. millefolium* var. *millefolium* and the North American *A. millefolium* (Guo et al. 2004, 2005; Fig. 2); and (3) recent recognition of "*A. asiatica*" (*A. millefolium* var. *mandshuricum*) and "*A. ceretanica*" (*A. millefolium* var. *ceretanica*) by European botanists as species separate from *A. millefolium* (Ehrendorfer and Guo 2006). The North American *A. millefolium* clearly deserve a name that distinguishes them from Eurasian relatives. Of the competing species epithets, precedence is for "*borealis*," a name ascribed in 1832 by Bongard to plants collected near Sitka, Alaska.

Although not widely appreciated by North American botanists, another *Achillea* species occurs naturally in far northern regions of the continent. *Achillea alpina* Linnaeus (= *A. sibirica* Ledebour) is a genomic allotetraploid derived in Eurasia from hybridization between a diploid member of the *A. millefolium* aggregate and the distantly related *A. acuminata* (sect. *Ptarmica*) (Guo et al. 2004, 2005). *Achillea alpina* is widespread in Asia (Ohwi 1965; Schischkin and Bobrov 2000) but also occurs in parts of Alaska and Canada, where it tends to be much less common than the North American *A. millefolium* (Anderson 1959; J. Ramsey, pers. obs.). We therefore recommend recognition of two *Achillea* species in North America: *A. borealis* (referencing autopolyploid members of the *A. millefolium* aggregate, which are common throughout Canada, the United States and highland areas of Mexico); and *A. alpina* (referencing the allotetraploid species, which is most abundant in eastern Asia but occurs sporadically throughout Canada and Alaska).

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