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ORIGIN OF THE FLAX CULTIVAR 'APPAR' AND ITS POSITION WITHIN THE *LINUM PERENNE* COMPLEX

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The 'Appar' flax germplasm was originally released by the USDA Soil Conservation Service as a cultivar of *Linum lewisii*. The observation that 'Appar' is heterostylic, a key taxonomic character distinguishing Lewis flax from other members of the *Linum perenne* complex, created a need for further study in order to resolve the taxonomic position of both Lewis flax and the 'Appar' cultivar. We grew 10 plants each of nine European, nine North American, and three putative 'Appar' seed sources, measured 18 morphologic variables on each plant, and examined genetic variation using randomly amplified polymorphic DNA (RAPD). We also performed self- and cross-pollinations within and among source groups. North American collections differed from European collections for 12 of the 18 morphologic variables. 'Appar' collections were morphologically similar to European collections and differed from North American collections for 13 of the 18 variables. RAPD data also showed that 'Appar' sources clustered with European, and both were distinct from North American sources. European and 'Appar' collections were self-sterile and interfertile, whereas North American collections were self-fertile but reproductively isolated from European and 'Appar' collections. We conclude that *L. lewisii* is a distinct species within the *L. perenne* complex and that 'Appar' should be classified as *L. perenne*. This study provides an example of how questions regarding origin and/or taxonomy of plant materials developed for restoration can be resolved.

Keywords: heterostyly, *Linum lewisii*, native plants, revegetation, morphometrics, RAPDs.

Introduction

North American Lewis flax (*Linum lewisii* Pursh) is a short-lived, nonclonal native perennial that has wide distribution over the western half of the United States, Canada, and northern Mexico (Mosquin 1971). The broad adaptability of this species, along with the showiness of its blue flowers, recommends it as a suitable candidate for use in restoration plantings and xeriscaped gardens (McArthur 1988; Kitchen 1995). In 1980, the USDA Soil Conservation Service released the cultivar 'Appar' for commercial production of seed, believing it to be a selection of Lewis flax (Howard and Jorgensen 1980). The original seed collection for 'Appar' was made in South Dakota by Perry Plummer, then project leader at the Great Basin Research Center of the U.S. Forest Service, Intermountain Forest and Range Experiment Station. Development and release of the cultivar followed common-garden evaluations of multiple Lewis flax seed sources. The 'Appar' release has good seedling vigor, excellent seed production, a deep blue petal color, and has been used in a number of revegetation and restoration plantings. It is also available commercially under the name of perennial blue flax.

In 1989, we observed that 'Appar' plants were heterostylous in nature. Heterostyly is a reproductive system thought to promote outcrossing in which populations have two (distyly) or three (tristyly) plant morphotypes that differ in the heights of their stigmas and anthers (Barrett 1992). 'Appar' is distylous; that is, approximately half of the plants have flowers with short stamens and long styles, while the other half have flowers with short styles and long stamens (fig. 1). Subsequent reference to taxonomic keys revealed that North American Lewis flax is reported to be homostylous, having only long-styled plants. We wondered whether there could be populations of Lewis flax that were heterostylous or whether the 'Appar' cultivar belonged to a species other than *L. lewisii*. Determining the origin of 'Appar' (native or not) would affect the type of plantings for which the cultivar could be used, with some situations calling for the exclusive use of native species.

A further complication comes from the disagreement among taxonomists regarding the position of Lewis flax within the *Linum perenne* L. complex. The *L. perenne* complex consists of over 20 recognized taxa that comprise a morphologically and genetically distinct group of species and subspecies widely distributed throughout Europe and Asia (Ockendon 1968; Mosquin 1971; Heitz 1973; Coates and Cullis 1987). Most members of the group are heterostylous and occur in central Europe. Homostylous species are found toward the margins of the distribution, i.e., France, central Asia, western Siberia, and North America (Ockendon 1968). Lewis flax is the sole perennial member of this group endemic to North America. A closely related annual species, *Linum pratense*, also occurs in the southwestern United States and, presumably, adjacent Mexico. Many taxonomists (e.g., Munz 1968; McGregor et al. 1986;

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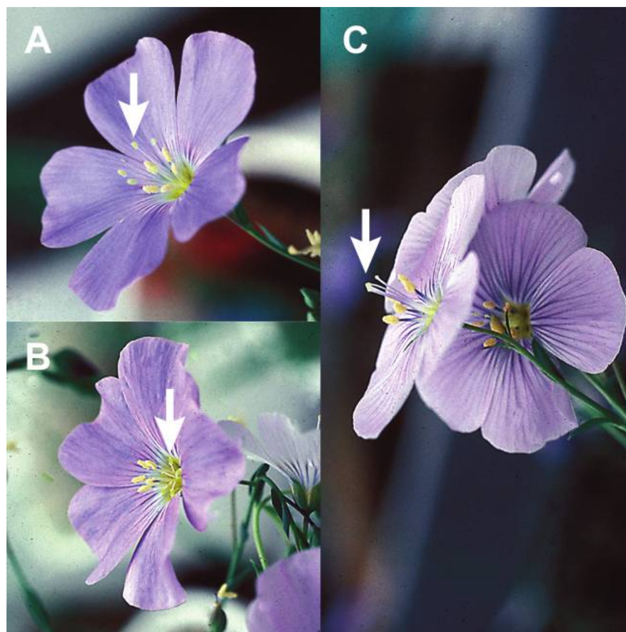

Fig. 1 Flowers of native North American Lewis flax (*Linum lewisii*) and the 'Appar' blue flax release showing relative positions of anthers and stigmas. *A*, Long-styled 'Appar' plant. *B*, Short-styled 'Appar' plant. *C*, Long-styled North American plant. Note also differences in the shapes of the stigmatic surface (arrows).

Welsh et al. 1993) consider Lewis flax to be a subspecies of *L. perenne*, while others (e.g., Kearney and Peebles 1969; Martin and Hutchins 1980; Cronquist et al. 1997) recognize it as a separate species (i.e., *L. lewisii*). Most currently, Kartesz and Meacham (1999) and the USDA PLANTS database (USDA NRCS 2006) list Lewis flax as a separate species.

Before beginning this study, we made reconnaissance trips to the putative original collection sites in Montana and South Dakota looking for heterostylous populations of *L. lewisii*. We also examined all pressed specimens in the herbarium at Brigham Young University (BRY). No native heterostylous plants were found. Subsequently, we initiated the following study with the goal of determining the most probable origin of the 'Appar' cultivar. In this study, we compared 'Appar' to European collections of *L. perenne* and North American collections of *L. lewisii*, using a number of morphological measurements, self- and cross-fertilization experiments, and genetic analyses using randomly amplified polymorphic DNA (RAPD) markers. Genetic analyses were used to supplement more traditional methods because a combined approach has proved to be a powerful tool in low-level taxonomy (Hansen et al. 2000; Casiva et al. 2002). We address the following questions: (1) are *L. lewisii* and *L. perenne* separate species, and (2) how should the 'Appar' cultivar be classified taxonomically?

Material and Methods

We obtained seed of eight accessions of *Linum perenne* and one accession of *Linum austriacum* (also within the *L. perenne* complex) from botanical gardens in Europe (table 1). We hand collected seed of *Linum lewisii* from nine natural populations of diverse geography and elevation. Three purported 'Appar' collections were also used in the study. One was certified 'Appar' seed. The other two were old seed collections used in the initial common-garden evaluation of multiple collections of Lewis flax that resulted in the subsequent development and release of the 'Appar' cultivar. The collection from South Dakota is thought to be the original source for Appar; however, the collection from Montana is virtually identical, causing some confusion as to which collection was truly the original source.

Seeds were moist-stratified at 4°C for 2 wk before planting. Stratified seeds were germinated and grown for 12 wk in a potting soil mixture and then transplanted to 25-cm-diameter pots containing locally obtained sandy loam topsoil. We grew 10 plants per accession for a total of 210 plants. Plants were grown outside under shade cloth for the first summer and overwintered in a sawdust pit. Plants were then transferred to a greenhouse for the second growing season. Pots were randomized on the greenhouse benches to minimize bench effects. Artificial crosses and data collection for morphological analysis occurred during the second growing season. Some mortality occurred during the course of the experiment. No morphological data were collected from three European and eight North American plants that died, and limited data were collected from another six North American plants.

Morphological Data

The following variables were measured or scored for each plant after flowering had commenced during the second growing season. Plant height was measured to the nearest centimeter. Flower diameter and length of the longest internode at midstem were measured to the nearest millimeter. Stem diameter, leaf length, leaf width, stamen length, and pistil length were measured to the nearest 0.1 mm under a dissecting scope at ×20 magnification, using an ocular micrometer. Scored or counted variables were (1) flower type (long styled or short styled), (2) angle of the leaf to stem (scored 1–5, with 1 being appressed and 5 reflexed), (3) number of days from transfer to the greenhouse to first flowering, (4) flower color (1 = blue, 2 = lavender, 3 = white), (5) basal stem color (1 = red, 2 = green, 3 = yellow), (6) number of flowering stems, and (7) number of nodes per 3 cm of stem length. In addition, we measured length and width to the nearest 0.05 mm of 10 seeds per accession, using a micrometer at ×20 magnification. We also measured the weight of two to four replicate samples of 25 seeds from each accession.

Morphological data were analyzed using the GLM, MIXED, and FREQ procedures of SAS, version 9.1 (SAS Institute 2004). Continuous data were transformed using power and log functions to achieve normality of residuals as determined by an iterative Box-Cox procedure. Univariate comparisons among the three geographic source groups were made using a likelihood-based estimation approach to account for existing between-group heterogeneity in variances. We used the MIXED procedure of SAS to compare pairwise differences in least squares source-group means, using the Tukey-Kramer adjustment. Residuals were examined for normality. Transformed data were converted back for reporting. Categorical (flower color, stem color) and ordinal (leaf angle) data were analyzed using χ^2 contingency tables

Table 1

Source Information for Flax Seed Collections Used in the Study

Collection number	Seed source	Collection type
European collections:		
E1 *Linum austriacum*	Eisenstadt, Burgenland, Austria	Natural population
E2 *Linum perenne*	Vienna, Vienna, Austria	Botanical garden
E3 *L. perenne*	Meise, Flemish Brabant, Belgium	Cultivated plants
E4 *L. perenne*	Turku, Western Finland, Finland	Botanical garden (cultivated)
E5 *L. perenne*	Freiburg, Saxony, Germany	Unknown
E6 *L. perenne*	Warsaw, Mazowieckie, Poland	Botanical garden (cultivated)
E7 *L. perenne*	Berlin, Berlin, Germany	Botanical garden (known wild origin)
E8 *L. perenne*	Vácrátót, Pest, Hungary	Cultivated plants
E9 *L. perenne*	Brno, South Moravia, Czech Republic	Cultivated plants
North American collections:		
U1 *Linum lewisii*	Black Hills, Pennington Co., South Dakota	Natural population
U2 *L. lewisii*	Fort Collins, Larimer Co., Colorado	Natural population
U3 *L. lewisii*	Asotin, Asotin Co., Washington	Natural population
U4 *L. lewisii*	Provo, Utah Co., Utah	Natural population
U5 *L. lewisii*	Confusion Range, Millard Co., Utah	Natural population
U6 *L. lewisii*[a]	Elk Knoll, Sanpete Co., Utah	Natural population
U7 *L. lewisii*	Cache Valley, Cache Co., Utah	Natural population
U8 *L. lewisii*	Potosi Pass, Clark Co., Nevada	Natural population
U9 *L. lewisii*	Little Antelope Summit, White Pine Co., Nevada	Natural population
Appar collections:		
A1	Aberdeen Plant Materials Center, Bingham Co., Idaho	Certified 'Appar' seed
A2	Helena, Lewis and Clark Co., Montana	Original collection (maintained seed line)
A3	Black Hills, Pennington Co., South Dakota	Original collection (maintained seed line)

[a] Many plants of this accession appeared to be in poor health.

(PROC FREQ). Differences among the three source groups were determined by subdividing the tables.

Additionally, we constructed a classification tree using plant morphological variables of European and North American origin (Breiman et al. 1984; Therneau and Atkinson 1997). Classification trees are similar to discriminant analysis or logistic regression in that a model is constructed that attempts to predict a categorical response variable (such as European or North American origin) from one or more predictor variables. The advantage of a classification tree is that it does not have to conform to a given probability distribution, predictor variables can be a mixture of continuous and categorical, predictor variables may be associated in a nonlinear fashion, and predictor variables may appear in more than one place in the tree. The resulting classification model was then applied to the 'Appar' data.

Seed data were analyzed using a GLM procedure on ranked data (equivalent to a Kruskal-Wallis test) because of the presence of outliers and because standard data transformations failed to improve normality. Differences among source groups were determined using accession means. Group mean separations were accomplished using a Tukey-type nonparametric analog (Hollander and Wolfe 1999). As a check, we also compared European and North American accessions in a separate test because balanced designs are less susceptible to Type I error.

Artificial Crosses

Artificial crosses were made among accessions of each source group, as well as between groups, using a variety of pollen donors. Flax flowers typically last 1 d, opening in early morning and closing in early to midafternoon, depending on temperature (Addicott 1977). Flowers to be pollinated were emasculated in the evening or very early morning before opening. Pollination was accomplished by taking dehiscing anthers from fully opened flowers and gently rubbing them across the stigmatic surface. Color-coded threads tied around each pedicle were used to designate pollen donor. Each of the three source groups was used as both an ovule and a pollen donor, for a total of nine cross-pollination types. We used from seven to 11 plants for each cross type and an average of five flowers per plant. We also carried out geitonogamous self-pollinations among flowers of the same plant, using an average of three recipient flowers per plant and a total of 44 plants. Fruit development was monitored several times weekly, and the dry fruits were collected before shattering. For each cross, we determined the number of pollinated flowers that set fruit and the number of viable seeds per fruit. As previously noted by Ockendon (1968), seeds were of three types: black and hard (obviously good), pale brown and papery (aborted), and brown and moderately hard. Viability of the latter group was determined using a standard tetrazolium assay (Grabe 1970).

RAPD Analysis

RAPD was chosen as the means for determining genetic relatedness among collection sources because it provides fast, interpretable, and inexpensive DNA-based markers (Andersen and Fairbanks 1990; Williams et al. 1993). Banding patterns can be compared among samples to determine genetic similarity.

Young leaves collected from approximately eight greenhouse-grown plants of each of the North American and European populations were bulked to give eight European and eight North American samples. Populations U2 and E1 were not used in the analysis because of insufficient plant material. Leaves were also collected from a total of 14 individual plants of putative 'Appar' sources; two from the greenhouse-grown collections of A1, three each of A2 and A3, two from another planting of the Montana source (M), and four from another planting of the Black Hills source (B). The latter two (M, B) sources represent plants of known seed origin growing outside of the Shrub Sciences Laboratory building in Provo, Utah. Fresh tissue was immediately frozen in liquid nitrogen and stored at −80°C until DNA was extracted.

The extraction method used was adapted from Delaporta et al. (1983). Tissue was first ground to a powder in liquid nitrogen using a mortar and pestle. About 1–1.5 mL extraction buffer (10 mM Tris, pH 8.0; 50 mM EDTA, pH 8.0; 50mM NaCl), 100 μL 20% SDS, and 5 μL 2-β-mercaptoethanol per gram of plant tissue were added, and the mixture was ground further. One-milliliter portions of the homogenate were transferred to 1.5-mL centrifuge tubes and incubated for 20 min at 68°C in a water bath. Following incubation, 500 μL of 5 M potassium acetate was added to each tube, and the tubes were mixed thoroughly. The samples were then incubated at 4°C for 20 min and centrifuged at 14,000 rpm for 5 min. The supernatant was transferred through Miracloth to a clean microcentrifuge tube containing 500 μL of isopropanol. The tubes were mixed by gently inverting, and the samples were left overnight at 4°C to help precipitate the DNA (Fairbanks et al. 1993).

Because of the high amount of carbohydrate obtained after the above extraction, we performed a carbohydrate wash. Tubes containing DNA from a particular plant or bulked sample were combined to form single pellets. Each pellet was resuspended in 700 μL of 1 M NaCl and vortexed gently (DNA dissolves in the salt, but carbohydrates do not). The samples were incubated at 4°C for 20 min and then centrifuged at 14,000 rpm for 5 min. The resulting supernatant was added 1 : 1 to isopropanol and incubated overnight at 4°C to aid DNA precipitation. The DNA was pelleted and washed with 70% ethanol, dissolved in TE (10 mM Tris, pH 8.00; 1 mM EDTA, pH 8.0), and stored at −20°C until use.

DNA concentration was estimated using a Hoeffer TKO 100 DNA fluorometer (Hoeffer Scientific Instruments, San Francisco, CA). The extracted DNA was then diluted with TE buffer to achieve a final concentration of approximately 5 ng/μL. Samples were amplified using random 10-mer primers following procedures from Williams et al. (1990), as modified by Mudge et al. (1996). Fifteen-microliter reactions were prepared containing the following: 5–10 ng DNA, 1.5 μL 10X Stoffel buffer (100 mM KCl; 100 mM Tris HCl, pH 8.3), 0.1 mM each of four deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP) obtained from either Perkin Elmer–Cetus (Norwalk, CT) or Promega (Madison, WI), 3.5 mM $MgCl_2$, 0.4 μM primer (Operon Technologies, Alameda, CA), and 1.2 U AmpliTaq DNA Polymerase Stoffel Fragment (Perkin Elmer–Cetus). Amplification was carried out using a Perkin Elmer–Cetus DNA thermal cycler, with the following cycling regime: (1) 3-min initiation step at 94°C; (2) 40 cycles: 94°C for 1 min, 35°C for 45 s, and 72°C for 1 min 45 s; and (3) 7 min at 72°C. Samples were stored at 4°C until undergoing electrophoresis (Fairbanks et al. 1993).

Amplified products were separated by electrophoresis using 1.4% agarose gels in TAE buffer (40 mM Tris-acetate, pH 7.5–7.8; 1 mM EDTA). Bands were stained with ethidium bromide and visualized on a transilluminator with 302-nm UV light. Photographs were taken through a red filter, using Polaroid 107C film (Fairbanks et al. 1993). Each sample was scored for presence or absence of bands of the same molecular weight. Ambiguous bands were scored as missing data. Bands common to all samples were not scored; therefore, genetic distance between samples is relative. RAPDs were first tested on bulks and then on individual samples, thereby ensuring repeatability.

We used the NTSYS-pc (ver. 1.80) statistical software package to analyze amplified DNA products (Rohlf 1993). The E6 bulk sample consistently did not amplify well enough to score and was omitted from statistical analysis. Presence or absence of specific bands was analyzed for percent similarity using Jaccard's coefficient of similarity (Jaccard 1912; Sneath 1957). UPGMA clustering analysis was performed with the SAHN matrix subroutine. A phenetic tree with midpoint rooting (TREE, NTSYSpc) was generated to graphically show similarity among samples. A cophenetic value matrix obtained from similarity values in the tree cluster was compared with the original Jaccard's matrix (MXCOMP, NTSYS-pc) in order to see how well the tree cluster fit the data.

Results

Morphology

Morphological variables of pistil length, stamen length, and shape of the stigmatic surface were closely associated with reproductive system, whether heterostylous or homostylous. Pistil and stamen lengths of short-styled European plants did not differ from those of 'Appar' plants (table 2). Neither did pistil and stamen lengths of long-styled plants. Long-styled North American plants did differ significantly from long-styled European and 'Appar' plants, averaging approximately 2.5 mm more in stamen length and 2.6–3.4 mm in pistil length. Also, stigmas of North American plants were all more linear in shape as opposed to the capitate shape of European and 'Appar' plants (fig. 1).

North American plants were morphologically distinct in other characters as well (table 2). Overall, North American sources had longer internodes, somewhat reflexed leaves, and fewer, thicker flowering stems that were green in color, as opposed to European and 'Appar' plants, which were suffused with red near the base. Petal color of 'Appar' sources was consistently a deep blue (mean score 1.0), whereas European plants were either blue or lavender (mean score 1.4). North American plants were mostly lavender (score 2.0) but ranged from blue (U2 and U6) to almost white (U8). 'Appar' plants were among the smallest in height, leaf length, stem diameter, and flower diameter.

Morphological data for 87 European plants and 76 North American plants were used in the construction of a classification tree (fig. 2). Classification criteria were then applied to 'Appar' plant data to see how they would be classified by the model. The output of the classification routine is a decision tree. Each node in the tree is a decision point that partitions the data. The tree is read much like a dichotomous key. Cross-validation (reapplication

Table 2

Means and Attained Significance Values for 16 Morphological Characters from 21 Accessions of *Linum*

Character	North American accessions		European accessions		'Appar' accessions		Attained significance
	n	Mean	*n*	Mean	*n*	Mean	
Plant height (cm)	81	47.7^{A}	87	43.5^{A}	30	39.2^{B}	**<.0001**
Leaf angle (rank 1–5)	83	3.6^{A}	87	2.0^{B}	30	1.6^{B}	**<.0001**
Leaf length (mm)	81	16.2^{A}	87	14.4^{B}	30	12.3^{C}	**<.0001**
Leaf width (mm)	81	1.6^{A}	87	1.6^{A}	30	1.3^{B}	**.0002**
Longest internode length (mm)	81	6.7^{A}	87	4.7^{B}	30	5.2^{B}	**<.0001**
No. nodes per 3 cm	79	8.6^{A}	87	10.8^{B}	30	10.2^{AB}	**.0005**
Basal stem color (score 1–3)	82	2.2^{A}	87	1.4^{B}	30	1.1^{C}	**.0052**
Stem diameter (mm)	79	1.5^{A}	87	1.3^{B}	30	1.1^{C}	**<.0001**
No. flowering stems	79	11.2^{A}	87	17.6^{B}	30	16.7^{B}	**<.0001**
No. days to first flowering	77	23.6^{A}	87	24.1^{A}	30	23.6^{A}	.6041
Flower diameter (mm)	77	29.6^{AB}	87	31.1^{A}	30	29.4^{B}	**.0007**
Petal color (score 1–3)	76	2.0^{A}	87	1.4^{B}	30	1.0^{C}	**.0001**
Pistil length of long-styled plants (mm)	76	10.9^{A}	40	8.3^{B}	16	7.5^{B}	**.0029**
Stamen length of long-styled plants (mm)	76	7.1^{A}	40	4.7^{B}	16	4.5^{B}	**.0001**
Pistil length of short-styled plants (mm)	...	...	47	4.9^{A}	14	4.8^{A}	.2531
Stamen length of short-styled plants (mm)	...	...	47	7.4^{A}	14	7.1^{A}	.5054

Note. Significant model effects are in bold. Letters following means denote significant differences among source groups at $P \leq 0.05$. The number of plants measured for each source group is shown. Note that no data were collected from three European and eight North American plants that died. Limited data were collected from an additional six North American plants that appeared unhealthy due, in part, to a severe aphid infestation. Four of the six were from the Elk Knoll accession (U6).

of the model to the original data) resulted in correct classification of 76 of the 87 European plants and 60 of the 76 North American plants. Five of the North American plants that were incorrectly classified belonged to the sickly U6 collection. If all U6 plants are omitted from cross-validation, the number of correctly classified plants is 75 of 87 European and 60 of 71 North American, or 86% and 85%, respectively. Applying this tree model to the 'Appar' data resulted in 28 plants classified as European and two plants classified as North American. Many 'Appar' plants were classified down the left side of the tree and had leaf angles of 1 or 2 and stem color code 1 (red).

Seeds from the three sources differed in size, shape, and weight (table 3). All four seed variables varied greatly among accessions ($P < 0.0001$). When seed variables were analyzed in a completely nested design, the accession term accounted for 43% of the variance in seed length, 60% of the variance in seed width, 39% of the variance in width/length ratio, and 62% of the variance in seed weight. Overall, however, seeds from North American accessions were significantly greater in length and smaller in width/length ratio than were European collections. 'Appar' seeds were significantly shorter in length and lighter in weight than North American accessions. Seed weights for 'Appar' accessions ranged from 29 to 36 mg for 25 seeds, whereas North American accessions ranged from 39 to 67 mg for 25 seeds. European collections had the greatest variation in weight, ranging from 23 to 65 mg per 25 seeds. 'Appar' accessions were not significantly different from European accessions for any of the four seed variables.

Artificial Crosses

Results from cross- and self-pollination experiments demonstrated that North American plants were self-fertile, averaging 7.9 seeds per fruit (10 possible) with 100% fruit set (table 4). The 'Appar' sources were completely self-incompatible, producing no fruit and no viable seed. European sources were largely self-incompatible. One plant of accession E3 produced two fruit and a total of four seeds from the two flowers that were self-pollinated. This may indicate limited self-compatibility in some European collections or experimental error on our part.

Plants of European and 'Appar' sources appear to be fully cross-fertile. Fruit set and seed numbers for European × 'Appar' crosses were similar to those obtained from European × European crosses, averaging 92% when 'Appar' was used as the pollen receptor and 100% when 'Appar' was used as the pollen donor. The mean numbers of seeds per fruit from these crosses were 7.4 and 6.5, respectively. In contrast, few fruits and no viable seeds were produced when North American plants were used as pollen donors for either European or 'Appar' pistils. Fruit set was greater (near 40%) when North American accessions were used as the maternal parent with pollen from either European or 'Appar' accessions. However, only a small number of potentially viable seeds were produced (nine and two seeds from 22 and 19 fruits, respectively).

RAPD Analysis

Nineteen primers polymorphic in the bulks produced 51 scorable markers that were used to determine similarity among population and individual plant samples. Due to a distortion in the gel, one of these primers, OP-U-06, produced no scorable bands. The other 18 primers (OP-AA-06, OP-AA-09, OP-AA-11, OP-AA-16, OP-AA-18, OP-AA-19, OP-AI-04, OP-F-09, OP-U-01, OP-U-03, OP-U-08, OP-V-01, OP-V-07, OP-V-08, OP-W-01, OP-W-07, OP-W-08, OP-W-09) produced between one and five scorable bands. In addition, the following primers were tested on the bulks: OP-A-09, OP-AA-01, OP-AA-04, OP-AA-05, OP-AA-07, OP-AA-08, OP-AA-10, OP-AA-13, OP-AA-15, OP-AA-20, OP-AB-05, OP-AB-07, OP-U-02, OP-U-04, OP-U-05, OP-U-

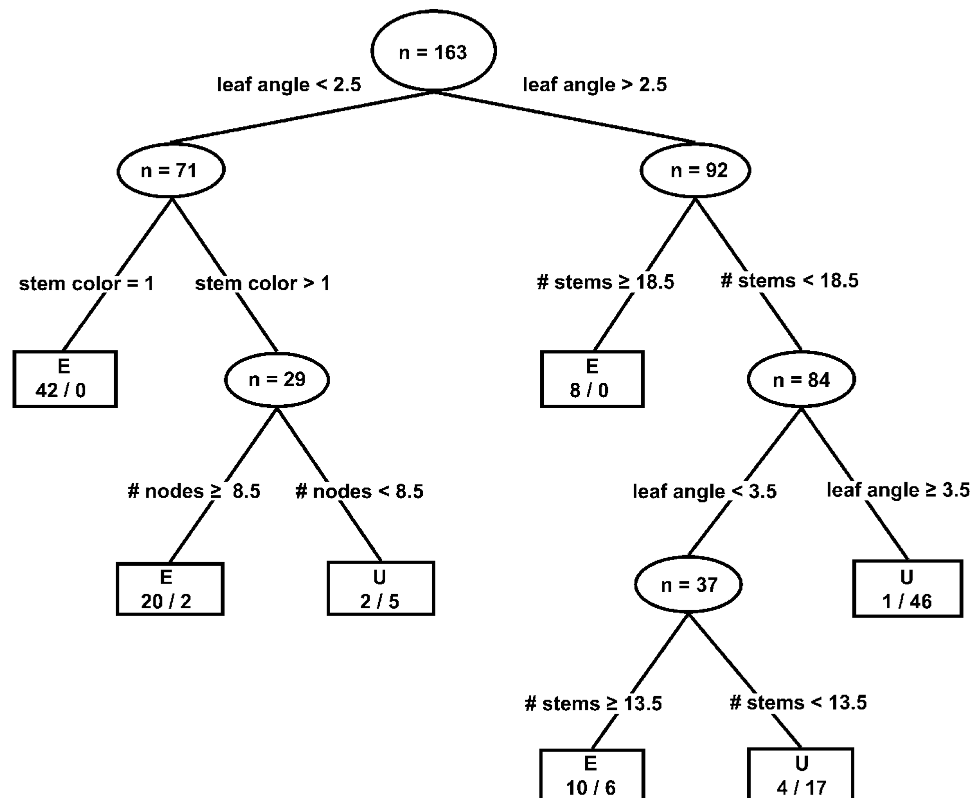

Fig. 2 Classification tree for E and U sources based on morphological data of 87 European and 76 North American plants. Each node is a decision point, partitioning the plants into one of two groups, and is read much like a dichotomous key. The number of plants to be partitioned is given at the top of each node. The numbers at the bottom of each branch are the numbers of European (*E*) and North American (*U*) plants classified as either *E* or *U* by the tree (no. E/no. *U*).

09, OP-U-12, OP-U-13, OP-V-02, OP-V-03, OP-V-04, OP-V-05, OP-V-06, OP-V-09, OP-V-10, OP-V-11, OP-V-12, OP-V-13, OP-V-14, OP-V-15, OP-V-16, OP-V-17, OP-V-18, OP-V-19, OP-W-02, OP-W-03, OP-W-06, OP-W-10, OP-W-11, OP-W-12, OP-W-13, OP-W-14. The resulting phenogram clearly distinguishes between North American and European populations (fig. 3). All plants of putative 'Appar' origin (identified in fig. 3 as *A*, *M*, or *B*) clustered together and were most closely aligned with European accessions E3, E9, E5, E8, and E7. Collections E2 and E4 clustered somewhat independently from other European populations but were still more similar to the European-'Appar' group than to North American sources.

The comparison between the tree cluster values and the original Jaccard's matrix yielded a product-moment correction (cophenetic correlation) of $r = 0.950$, indicating an excellent fit of the original similarity matrix and the similarity data obtained from the tree cluster. The tree was rooted with midpoint rooting, which makes the assumption of equal mutation rates along each branch. Violation of this assumption may affect the topology of the tree. Nevertheless, the tree corresponds well to the similarity matrix, indicating that the 'Appar' plants show more similarity to European populations than to North American ones. Indeed, both the morphological and molecular data suggest that European and North American populations are each more similar

Table 3

Means and Attained Significance Values for Four Seed Characters from 21 Accessions of *Linum*

	North American accessions		European accessions		'Appar' accessions		
Character	*n*	Mean	*n*	Mean	*n*	Mean	Attained significance
Seed length (mm)	90	4.0^{A}	90	3.5^{B}	30	3.3^{B}	**.010**
Seed width (mm)	90	2.1^{A}	90	2.0^{A}	30	1.9^{A}	.128
Seed width/length ratio	90	.52^{A}	90	.58^{B}	30	.57^{AB}	**.011**
Weight of 25 seeds (mg)	36	49^{A}	31	42^{AB}	12	34^{B}	**.047**

Note. Significant model effects are in bold. Letters following means within a row denote significant differences among source groups at $P \leq 0.05$.

Table 4

Percentage Seed Set and Number of Viable Seeds Per Fruit from Self- and Cross-Pollination Experiments Carried Out Within and Among *Linum* Accessions

Source (no. accessions)	No. plants	No. fruits/no. flowers	% fruit set	Seeds per fruit
Self-pollinations:				
North American (6)	19	56/56	100	7.9
European (5)	16	2/87	2	2.0
'Appar' (3)	9	0/31	0	. . .
Cross-pollinations:				
North American (5) × North American (6)	8	36/41	88	6.8
North American (6) × European (4)	9	22/50	44	.4
North American (6) × 'Appar' (2)	11	19/49	39	.1
European (6) × North American (5)	9	3/50	6	0
European (6) × European (4)	9	48/50	96	5.4
European (6) × 'Appar' (2)	8	50/50	100	6.5
'Appar' (3) × North American (5)	7	1/43	2	0
'Appar' (3) × European (5)	7	35/38	92	7.4
'Appar' (3) × 'Appar' (3)	8	34/37	92	7.2

Note. For cross-pollinations, maternal plant source is listed first. Numbers in parentheses indicate the numbers of accessions of each source that were used as maternal or paternal parent.

within than between populations and that 'Appar' plants show more similarity to European than North American accessions.

Discussion

The *Linum perenne* complex is a group of more than 20 closely related species, subspecies, or varieties and many geographical races, representing considerable morphological variation (Ockendon 1968; Mosquin 1971). All European members of the complex are heterostylous, except for *Linum leonii*. Heterostylous species of the complex are completely self-incompatible but have retained some degree of interfertility among taxa (Ockendon 1968). Morphologic variation within the group is often almost continuous, leading to difficulties in classification (Ockendon 1968, 1971). In addition to geographic variation, evolutionary trends within the *L. perenne* group appear to be the development

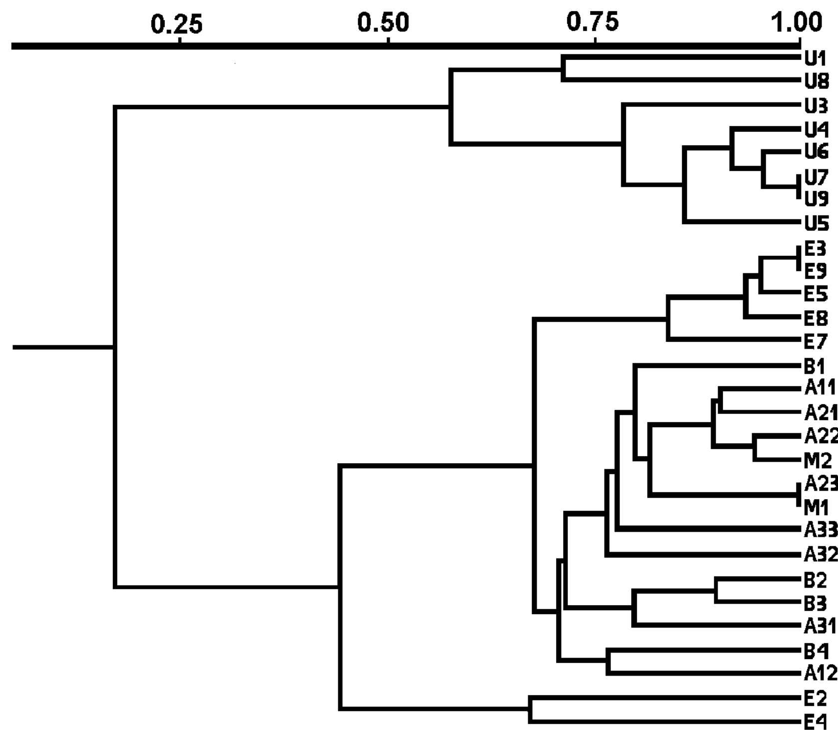

Fig. 3 Phenogram produced using UPGMA clustering analysis (NTSYS-pc; Rohlf 1993) for 29 individual or bulked tissue samples from the *Linum perenne* complex. *U* = North American bulked population samples; *E* = European bulked population samples; *A*, *M*, and *B* = individual plants from putative 'Appar' sources.

of homostyly from heterostyly and the production of polyploids (Ockendon 1968). *Linum leonii* is homostylous and self-fertile, as are other homostylous non-European members of the group (e.g., *Linum mesostylum* and *Linum pallescens*). Homostylous species tend to occur on the periphery of the group's distribution, where the ability to self-fertilize assists in range expansion and colonization of new sites (Allard 1965; Ockendon 1968). Heterostyly is controlled by a supergene composed of genes coding for anther and stigma heights, as well as a sporophytic self-incompatilibity system. Homostyly can arise through a mutation or crossover in the supergene (Barrett 1992) and may have occurred several times in the *L. perenne* group (Ockendon 1968). North American Lewis flax most likely originated from heterostyled *L. perenne* through a crossover in the supergene (Baker 1961, 1975; Ockendon 1968).

The question arises, then, of how to define separate species within the complex and just what should constitute a species. Multiple concepts for defining species have been used, and the issue is a source of much debate among biologists (George and Mayden 2005). Among higher organisms, the biological species concept (Mayr 1992) is one of several well-recognized concepts, although most typically applied to animals rather than plants. This concept defines a species as a reproductive unit that can successfully interbreed only within itself. Our data show that North American Lewis flax and European perennial blue flax are reproductively isolated and therefore, by this definition, would be classified as separate species. The phylogenetic species concept, another commonly invoked concept, relies on a "unique combination of character states" (Nixon and Wheeler 1990, p. 218). These can be morphologic, genetic, reproductive, or some combination of these. We have demonstrated that *L. perenne* and *Linum lewisii* are distinct both morphologically and genetically. In our opinion, the best theoretical framework for defining species is the evolutionary species concept, defined by Wiley and Mayden (see p. 392 in George and Mayden 2005) as "an entity composed of organisms that maintains its identity from other such entities through time and over space and that has its own independent evolutionary fate and historical tendencies." Lewis flax is geographically isolated from the European complex and appears to have arisen as a result of a genetic mutation that set it on a separate evolutionary pathway. As noted by Baker (1961, p. 881), "speciation often follows relatively quickly after a change has been made in the breeding system." He also stated that inbreeding forms often show geographical separation from the outcrossing parental form, thus allowing adaptation or drift to produce the morphological changes we associate with speciation. Morphological speciation then follows. It would appear, therefore, that Lewis flax fulfills most definitions of a species and should, without question, be considered distinct from other members of the *L. perenne* complex.

Although originally released as a cultivar of Lewis flax, 'Appar' is apparently of central European origin and likely escaped from garden cultivation somewhere in the northern plain states near South Dakota. Both morphologically and genetically, 'Appar' plants clustered with European collections of *L. perenne*. Crosses between 'Appar' and all European collections were fully fertile and produced viable seed. Therefore, we advise seed producers to designate all seed from the 'Appar' cultivar as perennial blue flax (*L. perenne* Pursh). Furthermore, care should be taken regarding the true source of seed currently labeled as Lewis flax (*L. lewisii* or *L. perenne* ssp. *lewisii*). Seed of 'Appar' origin has been marketed and used in research in the belief that it was native Lewis flax.

Appar seed has also been used in many soil stabilization projects. As information regarding the source of the 'Appar' cultivar began to circulate, the question of gene flow between 'Appar' and Lewis flax became an issue. Concerns over possible introgression of 'Appar' genes into native flax populations prompted members of the Colorado Weed Management Association to consider listing the 'Appar' germplasm as a noxious weed (Kitchen 2002). The question of introgression is important and needs to be addressed. Our data from cross-pollination studies indicate that little or no gene flow between 'Appar' plantings and native populations of Lewis flax could occur. However, cross-pollination studies between other homostylic and heterostylic *Linum* species have found that incompatibility between the two groups is not complete when the homostylic species is used as the mother plant (Ockendon 1968; Ghosh and Shivanna 1984). In our experiments, what little seed was produced by North American × 'Appar' crosses (two seeds from a possible 190) did come from crosses where the maternal plant was of North American origin. Baker (1961) also reported limited seed production when flowers of Lewis flax were pollinated with pollen of the short-styled morph of *L. perenne*. In most crosses of this type, however, embryos failed to develop (Baker 1975). Some additional new research results (Johnson et al. 2006) provide further evidence that 'Appar' (*L. perenne*) and *L. lewisii* do not hybridize. In this work, analysis of intersimple sequence repeat DNA markers and of morphological characteristics of sympatric indigenous *L. lewisii* and seeded 'Appar' (*L. perenne*) showed no evidence of hybridization.

At times, during the development of plant materials for restoration of degraded habitat, questions arise regarding the origin of specific native germplasms. Originally, the 'Appar' release was assumed to be that of our native Lewis flax. Careful examination followed by systematic study revealed otherwise, as described above. This study thus provides an example of how such questions regarding origin or taxonomy of plant materials developed for restoration might be resolved.

In summary, the 'Appar' flax cultivar should be classified as *Linum perenne*, a species of European origin that is separate from our native Lewis flax (*Linum lewisii*). Although not native to North America, 'Appar' may continue to have a place in revegetation of areas, such as xeriscaped gardens and highway rights-of-way, where color, diversity, and ecosystem stabilization are primary considerations and the exclusive use of native seed is not mandated. It is not likely to pose a threat to the genetic integrity of our native flaxes. A new Lewis flax cultivar, 'Maple Grove,' is now available for native restoration projects (Kitchen 2006).

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