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Scott Nissen

University of Nebraska-Lincoln

Robert A. Masters

University of Nebraska-Lincoln, rmasters1@unl.edu

Donald Lee

University of Nebraska-Lincoln, dlee1@unl.edu

Martha Rowe

University of Nebraska-Lincoln

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Comparison of Restriction Fragment Length Polymorphisms in Chloroplast DNA of Five Leafy Spurge (*Euphorbia* spp.) Accessions¹

SCOTT J. NISSEN, ROBERT A. MASTERS, DONALD J. LEE, and MARTHA L. ROWE²

Abstract. Chloroplast DNA (cpDNA) restriction fragment length polymorphisms (RFLPs) were analyzed to assess genetic variation and relatedness among selections of North American and Eurasian leafy spurge. Leafy spurge accessions from Nebraska, Montana, Russia, Italy, and Austria were evaluated. Total DNA was extracted from young leaves and digested with the restriction endonuclease, *EcoRI*. CpDNA fragment patterns were determined by Southern blot analysis using mung bean cpDNA probes. Colinearity between the mung bean and leafy spurge chloroplast genomes was indicated by the observation that common fragments were hybridized by adjacent probes. Minimum estimates of chloroplast genome size for the five leafy spurge accessions, which ranged in kilobase size from 130 to 132, were within the size range of most terrestrial plants. Structural collinearity and reasonable estimates of chloroplast genome size provided evidence that the mung bean cpDNA library was suitable for characterizing leafy spurge cpDNA. Seven of the 13 mung bean probes hybridized to polymorphic leafy spurge cpDNA fragments. Based on number of polymorphisms unique to each Eurasian accession, the Austrian accession appeared to be most divergent followed by the Italian and Russian. The North American accessions seem to be most closely related to each other and to the Russian leafy spurge accession. Nomenclature: Leafy spurge, *Euphorbia esula* (L.) #³ EPHES; mung bean, *Vigna radiata* L. **Additional index words:** Chloroplast restriction fragment length polymorphism, genetic diversity, genetic relatedness, EPHES.

INTRODUCTION

Leafy spurge has been recognized as the most serious threat to the productivity and quality of rangeland in the northern Great Plains of the United States and Prairie Provinces of Canada (39). This nonendemic species has continued to spread during the past 40 yr despite intensive research and management efforts. Lack of economical chemical control strategies and absence of natural enemies

have made leafy spurge in North America a candidate for biological control.

Biological control of leafy spurge has had limited success because of an incomplete understanding of the Eurasian origins of leafy spurge found in North America (7, 11, 28). Researchers became aware of the importance of establishing this relationship when it was discovered that insects collected in Austria and Switzerland rejected North American leafy spurge as a host (11). This observation suggested that candidate biological control organisms should be collected from areas of Eurasia with the same genotype(s) of leafy spurge found in North America.

The genetic relationship between leafy spurge in North America and Eurasia has not been established. There has been little success using morphological traits (7), cytogenetic measurements (33), and analysis of secondary chemical compounds (12, 14, 16, 37) as criteria to determine this relationship.

Attempts to classify leafy spurge using morphological traits have confused, rather than clarified, the relatedness of North American and Eurasian leafy spurge. The same leafy spurge specimen was identified as *E. virgata* Wald. & Kit. (17), as *E. esula* L. (40), and *E. waldsteinii* (Sojak) (27). It was suggested that the characteristics used to differentiate between *E. esula* and *E. virgata* merged such that the species were indistinguishable (1). Most of the 19 morphological traits used to distinguish between 39 accessions of leafy spurge from the United States, Canada, and Austria were inadequate as criteria to establish relatedness (7).

Cytogenetic studies conducted to determine the relationships within *Euphorbia* spp. in North America and Eurasia also were found to be unsatisfactory (33). Classification of the polyploids present within the North American complex of leafy spurge was weakened because the progenitors were unknown. Progenitors must be known to establish chromosome associations before cytogenetic classification can be utilized (10).

Micromolecular chemosystematic criteria used to differentiate among members of the leafy spurge complex include variation in epicuticular waxes (16) and latex chemical composition (12, 14, 37). Five leafy spurge biotypes had similar hydrocarbon composition and differed mainly in triterpenoid content (16). Latex samples analyzed by Curie-point pyrolysis-gas chromatography coupled with multivariate analysis techniques have been used in an attempt to characterize accessions of leafy spurge (37). This method was able to separate *E. esula* from *E. cyparissias* but did not clearly differentiate North American biotypes of leafy spurge. Latex triterpenoid composition was highly variable and could not be used to discriminate between 27 Montana and 15 European leafy spurge accessions (12). The basis of triterpenoid inheritance and influence of environment on

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²Authors are Asst. Prof., Dep. Agron., Univ. Nebraska; Range Sci., U.S. Dep. Agric., Agric. Res. Serv.; Asst. Prof., and Res. Technol., Dep. Agron., Univ. Nebraska, Lincoln, NE 68583.

³Letters following this symbol are a WSSA-Approved computer code from Composite List of Weeds, Revised 1989. Available from WSSA, 309 West Clark Street, Champaign, IL 61820.

Table 1. Sources of leafy spurge accessions evaluated in this study.

USDA-ARS accession number ^a	State or country	Town	Contributor	Location
FD-6	Nebraska	Lincoln	R. Masters	Agronomy Department, University of Nebraska, Lincoln, NE
FD-7	Montana	Bozeman	P. Fay	Plant & Soil Science, Montana State University, Bozeman, MT
FD-38	Russia	Stavropol	R. Bennet	USDA-ARS Foreign Disease, Weed Science Research Unit, Fort Detrick, MD
FD-39	Italy	Pisa	R. Bennet	USDA-ARS Foreign Disease, Weed Science Research Unit, Fort Detrick, MD
FD-13	Austria	Krems	R. Lym	Agronomy Department, North Dakota State Univ., Fargo, ND

^aAccessions maintained in a nursery by the USDA-ARS, Foreign Disease, Weed Science Laboratory, Ft. Detrick, Frederick, MD.

triterpenoids must be understood before latex triterpenoid composition could be reliably used as criteria to distinguish among leafy spurge biotypes (14).

A macromolecular chemosystematic technique, restriction fragment length polymorphism (RFLP)⁴ analysis of chloroplast DNA (cpDNA)⁴, can be used to assess intra- and interspecific genetic variability and has been particularly useful where multiple hybridizations have occurred (19). The utility of cpDNA polymorphism analysis for purposes of classification has been demonstrated with *Pisum* (20), *Lycopersicon* (25), *Nicotiana* (13, 31), *Triticum-Aegilops* (3, 36, 38), *Brassica-Raphanus* (8, 22), *Cucumis* (26), *Linum* (4), and *Clarkia* and *Heterogaura* (35).

Several factors make cpDNA simpler to study than nuclear DNA (nDNA)⁴ and mitochondrial DNA (mtDNA)⁴. The chloroplast genome, in contrast to nuclear and mitochondrial DNA (19), is smaller, has a highly consistent gene order (18, 41), contains a relatively small amount of repetitive DNA, and is highly conserved. Nucleotide substitutions occur at a relatively slow rate in cpDNA compared to nDNA (5, 18, 24, 34).

Analysis of cpDNA polymorphisms is an effective tool for classifying plant species where introgression has occurred (19). Size and stability of the chloroplast genome of land plants, and its intermediate rate of nucleotide substitution, make it suitable for investigations mainly at and above the species level. Therefore, the objectives of this study were to establish the existence of cpDNA polymorphisms in Eurasian and North American leafy spurge and to determine if cpDNA polymorphisms could be used in establishing the Eurasian origin of North American leafy spurge.

MATERIALS AND METHODS

Plant material. Leafy spurge accessions from Nebraska,

Montana, Russia, Italy, and Austria were obtained for evaluation in this study (Table 1). The nonendemic North American and endemic Eurasian accessions were single clones representing geographically separate populations of leafy spurge. Plants were propagated from adventitious crown buds planted in a 1:1 mixture by vol of Sharpsburg silty clay loam and sand soil contained in 12-L plastic pots. Plants were placed in a greenhouse, surface watered as needed, and provided with 500 μ E m⁻² s⁻¹ supplemental lighting from mercury vapor lamps to supply 12 h of light. Every 14 d 200 mg of a complete fertilizer⁵ was applied to each plant in 250 ml of water. Plant topgrowth was removed at 45-d intervals to stimulate growth of the young tissue needed for analysis. DNA extraction, restriction endonuclease digestion, and gel electrophoresis. Young leaves were removed from plants and lyophilized, and total DNA was extracted (30). Total DNA samples were quantified in a fluorometer⁶ with DNA specific dye Hoechst 33258⁷. DNA samples were digested to completion with *EcoRI*⁸ following supplier's instructions. Approximately 10 μ g of digested total DNA was loaded into each lane of a 25-cm gel containing 8 g L⁻¹ agarose. Electrophoresis was conducted on a horizontal apparatus⁹ at 1 V cm⁻¹ for 23 h in TBE gel buffer [(0.089 M Tris, 0.089 M boric acid, and 0.002 M ethylenediaminetetracetic acid (EDTA)] (15). Gels were stained with ethidium bromide and photographed under ultraviolet light. Mobilities of *HindIII*-digested Lambda DNA markers were measured from the photographs.

Southern blot transfers. Transfer of restriction fragments from agarose gels to nylon membranes was performed according to Reed and Mann (29). After transfer, membranes were baked at 80 C for 2 h.

Hybridization probes. A library of mung bean cpDNA *PstI* and *SalI* restriction fragments cloned in the plasmid vector pBR322 served as hybridization probes to the filter-bound leafy spurge DNA fragments. This library, which covers almost the entire mung bean chloroplast genome (23) (Figure 1), has been thoroughly characterized (21) and used to study cpDNA of other species (6). Plasmids containing cpDNA inserts were isolated from *E. coli* hosts using a miniprep procedure (2). CpDNA inserts were removed from their plasmid vectors by restriction endonuclease digestion and were isolated on low melting point agarose gels. Probe bands were cut from the gels and incubated with 10 mM Tris, 1 mM EDTA buffer (pH 8) at 68 C for 10 min. The cpDNA was

⁴Abbreviations: RFLP, restriction fragment length polymorphism; cpDNA, chloroplast deoxyribonucleic acid; nDNA, nuclear deoxyribonucleic acid; mtDNA, mitochondrial deoxyribonucleic acid.

⁵RA-PID-GRO. Chevron Chem. Co., San Ramon, CA.

⁶TKO-100. Hoefer Scientific Instruments, San Francisco, CA.

⁷American Hoechst Corp., Somerville, NJ.

⁸Promega Biotech, Madison, WI.

⁹Model SGE-014. C.B.S. Scientific, Delmar, CA.

* Probe not available for this segment of mung bean chloroplast genome.

Figure 1. Arrangement of cpDNA probes along the mung bean chloroplast genome [shown in linear fashion for clarity of illustration; normally a circular molecule (21)]. Striped blocks show regions containing the inverted repeat. Regions of the leafy spurge cpDNA that are collinear with the mung bean chloroplast genome are shown.

labeled with digoxigenin-11-2'-deoxyuridine-5'-triphosphate (dUTP) using a random priming method (9). A commercially available nonradioactive labeling and detection kit¹⁰ was used for analysis of Southern Blots. Final membrane washes were conducted at 68 C with 2 times SSC buffer (35.0 gm NaCl and 17.6 gm sodium citrate L⁻¹). Sizes of labeled filter-bound probes were determined by regression analysis using *HindIII*-digested Lambda virus DNA as molecular weight markers (32).

RESULTS AND DISCUSSION

Total leafy spurge DNA, digested with *EcoRI*, yielded cpDNA fragments that strongly hybridized to each of the mung bean cpDNA probes. A total of 60 cpDNA restriction fragments from each accession were visualized using the thirteen labeled mung bean cpDNA probes. *EcoRI* fragments that share regions of homology to adjacent mung bean probes were visualized twice in this analysis. Eight fragments hybridized by adjacent probes were judged to be overlapping fragments. Therefore, we estimate that each accession has 52 unique *EcoRI* cpDNA fragments.

Analysis of cpDNA restriction fragment patterns provided evidence for colinearity between the mung bean and leafy spurge chloroplast genomes. The arrangement of probes in the mung bean chloroplast genome and regions of colinearity between mung bean and leafy spurge cpDNA are shown in Figure 1. Colinearity was indicated by the observation that common fragments were hybridized by each pair of adjacent probes: 4a and 4b, 7 and 8, 8 and 9, 11 and 12.

Leafy spurge cpDNA size was estimated by summing the sizes of all nonoverlapping fragments (fragments that were not hybridized by two adjacent mung bean probes). Minimum

estimates of chloroplast genome size for the five leafy spurge accessions examined ranged from 130 to 132 kb. This is within the size range of 120 to 160 kb that has been established for most land plants (18). Structural colinearity and reasonable estimates of chloroplast genome size provided evidence that the mung bean cpDNA library was suitable for characterizing the leafy spurge cpDNA.

Polymorphisms among leafy spurge accessions. Examples of monomorphic and polymorphic cpDNA fragment patterns are shown in Figure 2. RFLPs were detected in five regions of the leafy spurge cpDNA (Table 2). Seven of the 13 mung bean cpDNA probes hybridized to polymorphic leafy spurge cpDNA fragments. Probes 10 and 11 shared regions of homology and identified the same polymorphic fragments, while probes 7 and 8 and probes 11 and 12 hybridized with overlapping polymorphic fragments (Figure 1). Variation in cpDNA *EcoRI* fragments detected with probes 5, 7 or 8, 9, 10 or 11, and 11 or 12 can be explained by addition/deletion or rearrangement events.

Table 2. Sizes of polymorphic fragments of chloroplast DNA from selected leafy spurge accessions^a.

Accession	Size of fragments hybridized by probe ^b				
	5	7 or 8 ^c	9	10 or 11 ^c	11 or 12 ^c
	kilobase				
Nebraska	2.3, 2.1	11.0	2.3, 1.3	1.4	5.5
Montana	2.3, 2.1	11.0	2.2, 1.3	1.4	5.5
Russia	2.3, 2.1	11.0	2.2, 1.3	1.4	5.5
Italy	2.3, 2.1	6.7	2.2, 1.3	3.9	5.5
Austria	2.2, 2.2	9.7	2.1, 1.5	1.4	5.1

^aTotal DNA was cut with *EcoRI* restriction endonuclease and analyzed by Southern blot hybridization.

^bSee Figure 1 for identification of mung bean probes.

^cProbes detecting overlapping polymorphic fragments.

¹⁰DNA labeling and detection kit. Boehringer Mannheim Biochemicals, Indianapolis, IN.

Figure 2. Examples of Southern blots obtained from total leafy spurge DNA digested with *Eco*RI. Blots were hybridized with cpDNA specific probes from mung beans: (a) probe #1, (b) probe #7, (c) probe #11 (see Figure 1). Lane numbers indicate leafy spurge accessions as follows: 1 = Austria, 2 = Nebraska, 3 = Montana, 4 = Italy, 5 = Russia. CpDNA fragments hybridized by probe #1 were monomorphic, while probes #7 and #11 hybridized to several polymorphic cpDNA fragments of the Austrian and Italian accessions.

The Austria accession of leafy spurge was the most divergent accession evaluated. There were seven polymorphisms between the cpDNA of the Italy and Austria accessions and six polymorphisms between the Austria and Montana, Nebraska, and Russia accessions (Table 3). *Eco*RI-generated fragment patterns of cpDNA of leafy spurge accessions from Montana and Russia were identical (Table 3). CpDNA from the Nebraska leafy spurge accession differed from the Montana and Russia accessions by a single unique fragment detected by probe 9 (Table 2). This fragment was 100 base pairs larger in the Nebraska accession.

Results of this study provided information about RFLPs in the cpDNA of five leafy spurge accessions. Based on structural colinearity and reasonable leafy spurge cpDNA size estimates, the mung bean cpDNA probes were appropriate for characterizing leafy spurge cpDNA. Identification of polymorphic fragments among the 52 unique *Eco*RI cpDNA fragments demonstrated that genetic differences exist among the five leafy spurge accessions evaluated. While morphological, micromolecular chemosystematic, and cytogenetic approaches to measuring genetic relationships within the leafy spurge complex have been largely unsuccessful, RFLP analysis of cpDNA promises to be a valuable technique for establishing the Eurasian origin of leafy spurge in North America.

Information gained from this work facilitates future studies which will include: determination of the mode of plastid

inheritance in leafy spurge, development of a leafy spurge cpDNA map using additional restriction endonucleases, and identification of specific cpDNA probes and restriction endonuclease combinations for rapid assessment of genetic relatedness within and across populations of Eurasian and North American leafy spurge. Chloroplast inheritance is predominantly maternal in higher plants; therefore, cpDNA RFLP analysis will provide a significant amount of information about maternal lineage. Work is currently underway to use the randomly amplified polymorphic DNA technique to examine the variation in nuclear DNA. Establishing these genetic relationships will be essential for developing successful biological control programs. With this information, collection of biocontrol agents (insects and pathogens) could

Table 3. Number of cpDNA polymorphisms detected among leafy spurge accessions.

Accession	Polymorphisms between accession			
	Austria	Italy	Russia	Montana
	no.			
Nebraska	6	3	1	1
Montana	6	2	0	—
Russia	6	2	—	
Italy	7	—		

be directed towards areas in Eurasia where the source of North American leafy spurge is found.

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