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The use of RAPD to detect genetic variation in populations of Liatris aspera Michx.

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THE USE OF RAPD TO DETECT GENETIC VARIATION IN POPULATIONS OF *LIATRIS ASPERA* MICHX.

An Abstract of a Thesis

Submitted

in Partial Fulfillment

of the Requirements for the Degree

Master of Arts

Duane Joseph Kitchen

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ABSTRACT

The tallgrass prairie is one of the most degraded and fragmented ecosystems on earth. Interest in conserving, restoring, and reconstructing tallgrass prairie began early in the 1900s, and has increased dramatically since the 1960s. Early restoration and reconstruction efforts relied primarily on seed collected from prairie remnants near the planting site, but with the increase in prairie planting efforts came a greater demand for large quantities of affordable, viable seed of native prairie species. Following an increase in the use of cultivated varieties (cultivars) of seed, concerns over the loss of genetic integrity of local populations and low genetic variability in prairie plantings have been central to an ongoing debate over the use of cultivars versus locally collected seed.

The Iowa Ecotype Project is an effort to provide seed of native prairie plant species that is readily available, affordable, and is produced in a manner that aims to conserve the genetic integrity of local populations and maximize overall genetic variability in the seed stock. The goal of this research was to use the technique known as random amplification of polymorphic DNA (RAPD) to assess the degree and pattern of distribution of genetic variability of six remnant populations of *Liatris aspera* Michx., one of the species included in the Ecotype Project, and to discuss the results of the analysis as it relates to the goals of the project. Geographic distance and the potential age of populations as determined by the geologic landform on which they occur were both examined as potential factors in explaining the patterns of variation revealed by RAPD.

The results of this study indicate that the majority (78%) of the genetic variability in *L. aspera* occurs within populations while only 22% of the variability revealed by

RAPD is due to differences between populations. There was no correlation between geographic distance and genetic distance (variability) between populations. No statistically significant relationship was found between landform regions in which the populations occur and the patterns of genetic variability observed.

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This Study by: Duane Joseph Kitchen

Entitled: THE USE OF RAPD TO DETECT GENETIC VARIATION IN POPULATIONS OF *LIATRIS ASPERA* MICHX.

has been approved as meeting the thesis requirement for the

Degree of Master of Arts

7/19/99 — Date — Dr. Daryl D. Smith, Chair, Thesis Committee

7/19/99 — Date — Dr. James W. Jurgenson, Thesis Committee Member

15 July 1999 — Date — Dr. Steve L. O'Kane Jr., Thesis Committee Member

8/16/99 — Date — Dr. John W. Somervill, Dean, Graduate College

DEDICATION

This work is dedicated to my father, who has always provided love, inspiration, encouragement, and support, and to my wife, Tina, who has given me unconditional love and support throughout the length of this project.

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There are several people who have provided support to this effort. My major advisor, Dr. Daryl Smith introduced me to the world of the tallgrass prairie, and instilled in me an undying appreciation, respect, curiosity, and love for this vanishing ecosystem. I am grateful for the instruction, advice, and support in the past several years. Dr. James Jurgenson, who acted as committee member and technical supervisor, invited me to work in his lab, despite the fact that I had no experience in molecular biology. During the course of this project I learned many techniques which are certain to be useful throughout my career in science. Many thanks are in order for the opportunity, instruction, and fellowship that he provided. Dr. Steve O'Kane, who is also on my advisory committee, provided excellent advice and instruction on methodology and data analysis for this project, as well as making critical materials available so that the laboratory work could be completed. I truly appreciate the assistance. This work was funded in part by grants to the Iowa Ecotype Project from the Iowa Department of Transportation Iowa Living Roadway Trust Fund and by a GRASP award from the University of Northern Iowa College of Natural Sciences.

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CHAPTER 1

INTRODUCTION

The tallgrass prairie, once the largest continuous ecosystem in North America, is one of the most degraded and fragmented ecosystems in the Western Hemisphere (Chadwick 1995). Prior to Anglo-American settlement of the region, there were approximately 12.5 million hectares of tallgrass prairie in Iowa, of which less than one-tenth of one percent remains today (Samson and Knopf 1994). Although little information is available regarding the loss of prairie plant species, it is well documented that a typical area of tallgrass prairie of 260 hectares (about one square mile) may contain 200-300 species of plants (Steiger 1930, Weaver 1954, Freeman 1998). In contrast to this, the objective of most agricultural practices in the prairie region is mass production of one or a few species in the form of row crops. The conversion of prairie to agricultural land thus represents the loss of an enormous amount of plant diversity. When the thousands of other organisms such as insects, birds, mammals, and microbes that inhabit tallgrass prairie are considered (Risser 1986, Kaufman et al. 1998), the magnitude of the reduction in biodiversity that has resulted from the conversion of prairie to row crop agriculture is even greater. It has long been recognized that the success of agriculture in the United States is largely due to the high fertility of the soils in this region. The high productivity of these soils is the result of enormous accumulations of organic matter during thousands of years of interaction between the biota and environment of the tallgrass prairie (Risser et al. 1981, Ransom et al. 1998). Beyond the obvious economic value of prairie soils, Leopold (1966) stressed that all of the components of prairie are

valuable, and that tallgrass prairie and other natural lands have historic, social, cultural, and intrinsic value as well.

Interest in restoring and reconstructing prairies in Iowa and throughout the tallgrass prairie region began in the 1930s (Curtis 1952; Blewett and Cottam 1984), and has steadily increased since the 1960s (Shirley 1994). One of the largest limitations to prairie restoration and reconstruction efforts has been the inability to obtain sufficient quantities of affordable, viable seed for use in plantings. Historically, high quality, locally collected seed has been rare, and when available, quite expensive (Apfelbaum 1997). The great expense of good quality seed could preclude large-scale restoration efforts, and even with the largest of budgets, lack of seed quantity may have been limiting to restoration efforts. As demand for seed of native species increased, restorationists turned to commercial growers in Kansas and Nebraska who could provide large amounts of viable seed at much lower prices. However, this seed offered by commercial growers was not derived from local sources, rather it was produced from varieties developed at the USDA Soil Conservation Service Plant Materials Center in Manhattan, Kansas for use in rangeland restoration (Smith 1994).

Because these cultivated varieties (cultivars) were intended to increase forage for cattle production, they had been selected from plants that exhibited favorable traits such as high germination rate, vigorous growth, and tolerance to grazing. In some instances, the cultivars were developed from a single plant that exhibited these characteristics. When these 'western cultivars' began to be used in restorations and reconstructions far outside the range of the varieties from which they had originally been derived, concern

arose over the possibility for 'genetic contamination' of local populations that occurred near the site of the restoration (Knapp and Rice 1996). Part of the rationale for insisting on use of locally collected seed is that introduction of foreign germplasm could impact the genetic integrity of native populations and result in the loss of locally adapted genes (Millar and Libby 1989). Plantings utilizing seed other than locally adapted strains could result in plants with reduced competitive ability or perhaps failure of the planting altogether (Knapp and Rice, 1994). The response to this concern was often insistence on the use of only locally collected seed. While this ensured that "alien" genetic material would not be introduced, it confined the area from which seed was collected and potentially limited the genetic variability of collections. In addition, a low initial level of genetic variability could be exacerbated in a small planting by loss of genetic characters through random genetic drift, resulting in even lower genetic variability (Futuyma 1979). The Iowa Ecotype Project has been developed in response to the above concerns regarding the source of seed to be used for prairie plantings in Iowa.

The Iowa Ecotype Project is an effort to provide seed of native prairie plant species that is readily available, affordable, and is produced in a manner that aims to conserve the genetic integrity of local populations and maximize overall genetic variability in the seed stock. An 'ecotype' is a genetically differentiated strain of a species that is adapted to a specific set of environmental conditions, and is restricted to habitats in which those conditions prevail (Smith, 1994). The ecotype concept was first introduced in the 1920s following research conducted with multiple plant species over several years (Turesson 1922). When individual plants originating from different habitat

types were cultivated in a common garden, patterns of dramatic morphological variation were observed. These patterns were consistent within groups of plants from the same habitat type, and did not diminish or disappear during several years of cultivation in the transplant garden. It was concluded that the morphological variation must be a result of genetic variation, since the variation persisted when the plants were cultivated in a different environment. Each group of plants sharing a particular set of character variations was classified as being a particular 'ecotype' (Turesson 1922, 1925). In North America, the work of Clausen, Keck and Hiesey (1940, 1948) and Clausen and Hiesey (1958) in the western United States, and later McMillan (1959*a*, *b*, 1964, 1965*a*, *b*) in the tallgrass prairie region, served to solidify the ecotype concept and further clarify the importance of patterns of genetic variation within plant species.

The primary goal of the Iowa Ecotype Project is to propagate ecotypes of 25-30 species of grasses and forbs while maintaining or enhancing the genetic variability existing in these species. Three ecotype collections for each species are established, based on the division of the state into three zones, each consisting of three latitudinal tiers of counties (Figure 1). The project was originally designed to include more zones, but concerns over marketability of seed in a small area lead to the decision to have only three. Ecotype collections are developed by harvesting seed randomly from remnant populations around the state and increasing the volume of seed by cultivating it in plots located on the University of Northern Iowa campus in Cedar Falls, Iowa and at the USDA Plant Materials Center in Elsberry, Missouri. There is no selection for particular traits during the harvesting or cultivation process, so that the maximum variability is preserved in each

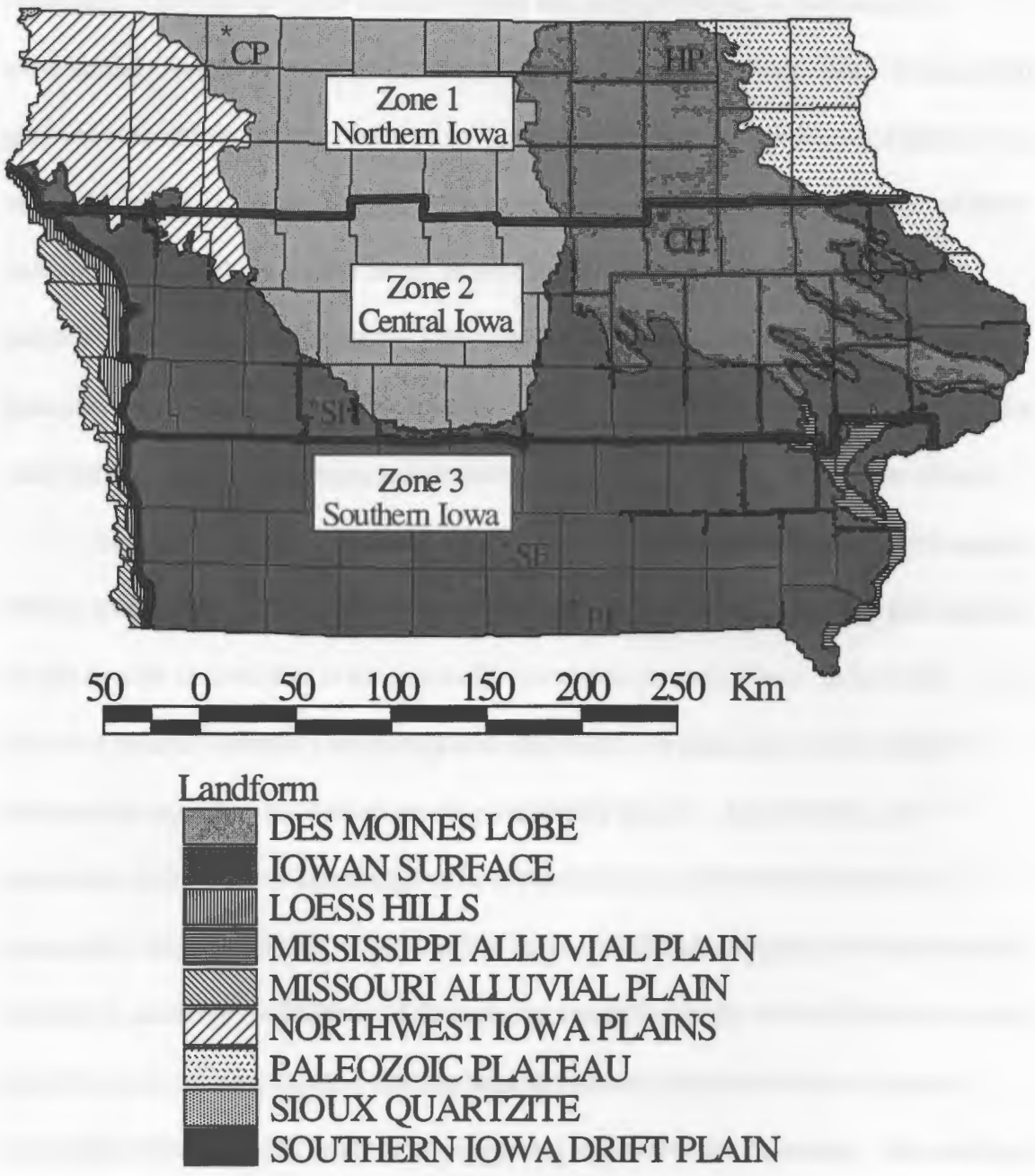

Figure 1. Iowa Ecotype zones, *Liatris aspera* populations, and Iowa landform regions (Prior 1991). The Loess Hills area of western Iowa was excluded from the first phase of the Ecotype Project due to a dramatically different floral composition as compared to the rest of Iowa.(CP = Cayler Prairie, HP = Hayden Prairie, CH = Cedar Hills Sand Prairie, SH = Sheeder Prairie, PJ = Plano-Jerome roadside, SF = Stephen's Forest roadside).

collection. Once an adequate volume of seed has been produced, it is released to commercial growers in the appropriate ecotype zone for further production. Commercial growers cultivate and market the seed as "source identified" Northern Iowa, Central Iowa, or Southern Iowa. Source identified seeds are defined as those which are collected from natural stands, seed production areas, or seed fields where no selection or testing of parent populations has been made. The plants are not genetically modified, and are guaranteed as to source. The seed from the Iowa Ecotype Project is intended for use as a basic mix in roadside plantings, prairie restorations, and prairie reconstruction efforts.

One of the species that has been selected for the Ecotype Project is *Liatris aspera* Michx. (rough blazing star), and it is the existing genetic variation of extant populations of this species in Iowa that is the focus of the research presented here. In order to conserve genetic variability efficiently and effectively it is necessary to first obtain information regarding how much existing variability there is. Additionally, it is imperative to have some knowledge of how the variation is distributed across the geographic range of interest. Once this has been established, sampling strategies can be devised to conserve a maximum of the existing variability in the most efficient and cost-effective manner. One method that has been devised to determine levels of genetic variability relies on direct analysis of the genetic material of the organism. The technique known as random amplification of polymorphic DNA (RAPD) is based on the ability to amplify small portions of the genome of virtually any organism using a genomic DNA template, oligonucleotide primers of arbitrary sequence, and the polymerase chain reaction (PCR) (Mullis and Faloona 1987, Saiki et al. 1988, Williams et al. 1990). The

RAPD method has been shown to be able to detect polymorphisms at both the intra-specific and intra-population levels (Welsh and McClelland 1990, Hedrick 1992). One of the largest advantages of the RAPD technique is that it does not require any prior knowledge of the genome of the organism of interest (Caetano-Anolles et al. 1991, Williams et al 1993). Low-cost, commercially available primers can quickly be screened for usefulness in producing RAPD profiles, and a single primer may produce several polymorphic markers (Hadrys et al. 1992, Newbury and Ford-Lloyd 1993). The RAPD method is technically simpler and far less expensive than other molecular tools, such as restriction fragment length polymorphism (RFLPs) or DNA sequencing, which are used to assess genetic variability between individuals (Parker et al. 1998). Furthermore, both DNA sequencing and RFLPs have the additional requirement of some prior information about the genome under investigation. In a study of genotypic diversity of cocoa, N'Goran et al. (1994) concluded that RAPDs are as effective as RFLPs in detecting genetic variation. Anderson and Fairbanks (1990) suggest that RAPDs are a superior tool for characterizing plant genetic resources (such as seed collections) due to their technical simplicity and low cost relative to RFLPs, and the ability of RAPDs to detect greater polymorphism than isozyme assays. RAPDs have been successfully used to detect genetic variability in a variety of taxa including spruce (van de Ven and McNicol 1995, Bucci and Menozzi 1995), pine (Lu et al. 1995, Plomion et al. 1995), cocoa (Wilde et al. 1992, Russell et al. 1993), corn (Heun and Helentjaris 1993), and forage grasses (Stammers et al. 1995), as well as many other prokaryotic and eukaryotic organisms (Tingey and del Tufo 1993). The goal of this research was to use RAPDs to assess the

degree and pattern of distribution of genetic variability of six remnant populations of *Liatris aspera* in Iowa, and to discuss the results of the analysis as it relates to the current sampling strategy of the Iowa Ecotype Project.

CHAPTER 2

MATERIALS AND METHODS

Site Descriptions

Leaf tissue was collected from 30 plants from populations of *Liatris aspera* at each of a total of nine sites located throughout the state of Iowa. Ultimately, the project was reduced to include only six of the original nine populations, and only 10 individuals from each population, due to time and resource constraints. Only the six populations that were included in the study will be discussed (Figure 1). Below are descriptions of the site where each population was found, including the two-letter codes that are used to refer to each population throughout this paper and the approximate Universal Transverse Mercator (UTM) coordinates for the site (UTM Zone 15, NAD 27). In addition, the geologic landform region that each population occurs in is identified (Prior 1991).

Zone 1 - Northern Iowa

Cayler Prairie State Preserve (CP) (UTM 318173, 4806983). Located approximately five miles west of Iowa Lakeside Laboratory in northwest Dickinson County, this National Natural Landmark preserve is positioned near the western edge of the Des Moines Lobe landform region. This 160 acre site has been managed by the Iowa Department of Natural Resources (DNR) since its dedication in 1971, and is characterized by shallow, rocky soils on ridges interspersed with wet swales, resulting from erosion of the remnant glacial moraine.

Hayden Prairie State Preserve (HP) (UTM 550151, 4809034). This 240 acre black-soil prairie is in Howard County in extreme northern Iowa. With habitats ranging

from mesic to wet, this high quality remnant was preserved in 1948 by the DNR and later named in honor of Iowa prairie researcher Dr. Ada Hayden. The site is located on the Iowan Surface landform, approximately 4.5 miles north of Saratoga along county road V26.

Zone 2 - Central Iowa

Cedar Hills Sand Prairie (CH) (UTM 537440, 4717071). Located in extreme northwestern Black Hawk County just south of Finchford on Butler road, this site also lies on the Iowan Surface. Dedicated in 1985 and managed by the Nature Conservancy, this 35 acre site is characterized by very sandy soils of eolian origin and has habitats ranging from wet prairie fen to dry sand prairie.

Sheeder Prairie State Preserve (SH) (UTM 363399, 4615275). This 25 acre preserve is located on the Southern Iowa Drift Plain about 4 miles west of Guthrie Center in Guthrie County. This mesic prairie remnant was dedicated in 1968 and is managed by the DNR.

Zone 3 - Southern Iowa

Plano-Jerome roadside (PJ) (UTM 496062, 4510146). This small, but species-rich remnant is located just south of state highway 2 near the southern terminus of highway 142 between the towns of Plano and Jerome in Appanoose County. This site in the Southern Iowa Drift Plain region is not officially managed, but there was evidence of haying at the time of tissue collection.

Stephens Forest roadside (SF) (UTM 461520, 4538806). This roadside prairie remnant is about 4 miles south of Lucas on highway 65 in Lucas County, near the entrance to Stephens State Forest. This site also occurs in the Southern Iowa Drift Plain.

Liatris aspera Tissue Collection

Sections of fresh, mature leaf tissue were obtained at random from 30 plants at each site. Care was taken to avoid obviously damaged or potentially infected sections based on visual inspection. Multiple (2-4) samples were taken from each plant by pinching off a 3-4 cm long section of tissue between the lid and opening of a 1.5 ml microcentrifuge tube. The number of sections taken was based primarily on the width of the leaf, so that a total of approximately 400 mg of tissue was obtained from each plant. The tubes had been previously autoclaved and partially filled with silica gel as a desiccant. All sample tubes were packaged in Ziploc® bags and placed on ice until they could be frozen at -75°C.

Isolation of Genomic DNA

Isolation of total genomic DNA for RAPD templates followed a protocol modified from Doyle and Doyle (1987). Approximately 100 mg of tissue from each sample was brushed free of silica gel and placed in a sterile 1.5 ml microcentrifuge tube with about 100 µl of autoclaved washed sea sand (Fisher Scientific, Pittsburgh, PA). A 400 µl volume of hot (60°C) 2X CTAB extraction solution [2% (w/v) hexadecyltrimethylammonium bromide (CTAB) (Sigma-Aldrich Corporation, St. Louis, MO) 100 mM Tris·Cl, pH 8.0, 20 mM EDTA, pH 8.0] containing 0.3% (v/v) β-mercaptoethanol (National Diagnostics, Atlanta, GA) was added and the tissue was

ground thoroughly with a teflon pestle. An additional 350 µl of hot 2X CTAB was added to the tubes followed by incubation for 30 minutes at 60°C. An equal volume of 24:1 (v/v) chloroform:octanol was added and mixed by vortexing. Tubes were centrifuged at 10,000 rpm for 5 minutes in a refrigerated (4°C) centrifuge. The aqueous supernatant was reserved to a sterile 1.5 ml microcentrifuge tube and 2/3 volume of ice-cold isopropanol was added to precipitate the nucleic acids. Tubes were incubated at -20°C for 10 minutes, then spun at 9,000 rpm for 8 minutes to pellet the DNA. The supernatant was discarded, and pellets resuspended in 375 µl TE (Tris EDTA). Once the pellets were dissolved in TE, 1/10 volume of 3M NaOAc and 2 1/2 volumes ice-cold 95% ethanol were added and the tubes were incubated at -20°C for at least one hour to overnight. Following the incubation, tubes were spun at 12,000 rpm for 10 minutes and the supernatant discarded. The DNA pellets were then washed with 250 µl ice-cold 70% ethanol by repeated gentle inversion, followed by centrifugation at 12,000 rpm for 5 minutes. The 70% ethanol was decanted, and the pellets were dried at room temperature in the open tubes. Pellets were then resuspended in 250 µl TE and stored at -75°C. The DNA was then treated with DNAse free RNAse to remove any RNA that was obtained in the CTAB extraction. Templates in 250 µl TE were thawed on ice and 1 µl of RNAse stock (10 mg/ml) was added to each. Tubes were incubated in a 37°C water bath for one hour, after which two successive chloroform extractions were performed by adding an equal volume of 24:1 (v/v) chloroform:octanol and centrifuging for 5 minutes at 10,000 rpm in a refrigerated (4°C) centrifuge. The aqueous phase was reserved to a sterile 1.5 ml microcentrifuge tube following each chloroform extraction. After the second extraction,

1/10 volume of 7 M NH_4OAC and 2 1/2 volumes of ice-cold 95% ethanol were added and the mixture incubated at -75°C for one hour. The DNA was recovered by centrifugation at 10,000 rpm for 10 minutes, discarding of the supernatant, and a single wash with 100 µl ice-cold 70% ethanol by repeated gentle inversion. Finally, samples were spun at 12,000 rpm for 5 minutes, the ethanol decanted, and pellets were dried at room temperature in the open tubes. The dried DNA pellets were resuspended in 250 µl TE and stored at -75°C.

Preparation of Templates for RAPDs

Template stock concentrations were calculated based on A_{260} values (1 O.D. = 50 ng/µl) obtained using a Beckman® Acta™ III Spectrophotometer (Beckman Instruments, Fullerton, CA). Aliquots of each template stock were diluted to a concentration of about 20 ng/µl. Each template dilution was then divided into 20 µl aliquots and stored at -75°C.

Primer Preparation

All primers used in this study are from Operon® oligonucleotide primer Kit A (Operon Technologies, Inc., Alameda, CA). Prior to use, the lyophilized primers were resuspended in ultrapure water to a concentration of 40 µM, and several 25 µl aliquots were made. Working solutions for use in RAPD cocktails were made by diluting an aliquot of the primer stock to 4 µM with ultrapure water immediately prior to use, or a few days in advance. The lyophilized primers and all primer preparations were kept frozen at -75°C until being thawed on ice at the time of use.

DNA Amplification

RAPD amplifications were run in sets of 30 plus one blank. Each set consisted of a single reaction for each of 10 individuals from three populations. Amplifications were performed in a 25 µl volume consisting of 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 0.1% Triton® X-100 (Sigma-Aldrich), 1.5 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP, and dTTP, 0.5 µM primer, 0.75 units of Taq DNA polymerase (Perkin Elmer Corporation, Norwalk, CT), and approximately 20 ng of template DNA. For each set of 30 reactions, a RAPD cocktail was prepared that contained DNA polymerase buffer (Promega Corporation, Madison, WI), dNTPs (Amresco Inc., Solon, OH), primer, and DNA polymerase. A 24 µl aliquot of the cocktail was added to 1 µl of each template solution in a 0.5 ml microcentrifuge tube and overlaid with 25 µl of autoclaved, DNAse, RNAse, and protease free mineral oil. For each cocktail, a blank reaction was also set up in an identical fashion, with the exception of using 1 µl of ultrapure water in place of the template solution. Thermal cycling was performed in a Thermolyne Amplitron® I thermal cycler (BarnsteadlThermolyne Corp., Dubuque, IA) programmed for 40 cycles of one minute at 94°C, two minutes at 35°C, and two minutes at 72°C, followed by a hold at 4°C.

Electrophoresis of Amplification Products

Amplification products were separated by loading 15 µl of each reaction (in 1.5 µl 10X Ficoll/tartrazine (Sigma-Aldrich) loading buffer) onto 1.5% (w/v) agarose gels containing 0.5 µg/ml ethidium bromide. Two molecular weight standards, a *Hin*d III restriction digest of lambda DNA and a *Hin*f I digest of plasmid Bluescript+KS, were

used either separately or together on gels to estimate sizes of amplification products. Gels were run in 1X TAE buffer [40 mM Tris·acetate, 2 mM $Na_2EDTA{\cdot}2H_2O$, pH 8.5] until the tartrazine tracking dye migrated about 6.5 cm. Gels were illuminated with a Chromato-Vue Transilluminator® model TM-36 (UVP, San Gabriel, CA) and images were captured with a BioImaging Technologies (Brookfield, WI) video image capturing system and recorded using NIH Image software v.1.51 (National Institutes of Health, USA) on an Apple Macintosh™ Quadra 840AV™ computer (Figure 2).

Analysis of Amplification Products

The 20 primers from Operon® Kit A were screened for the ability to generate reproducible banding patterns. Only bands that were reproducible were included in the analysis. A band was considered to be reproducible if it appeared in two or more separate amplifications for a particular primer/template combination. Bands were scored as present or absent based on repeated amplifications for each primer/template combination (a total of 60 individuals x 211 amplification products, or 12,660 data points). Gel images were analyzed using GelReader software (v. 2.0.4 National Center for Supercomputing Applications 1991) to assign size values to RAPD fragments. Due to slight differences in the fragment sizes assigned by the GelReader software both within and between gel runs, the molecular weights of RAPD bands presented here represent average values.

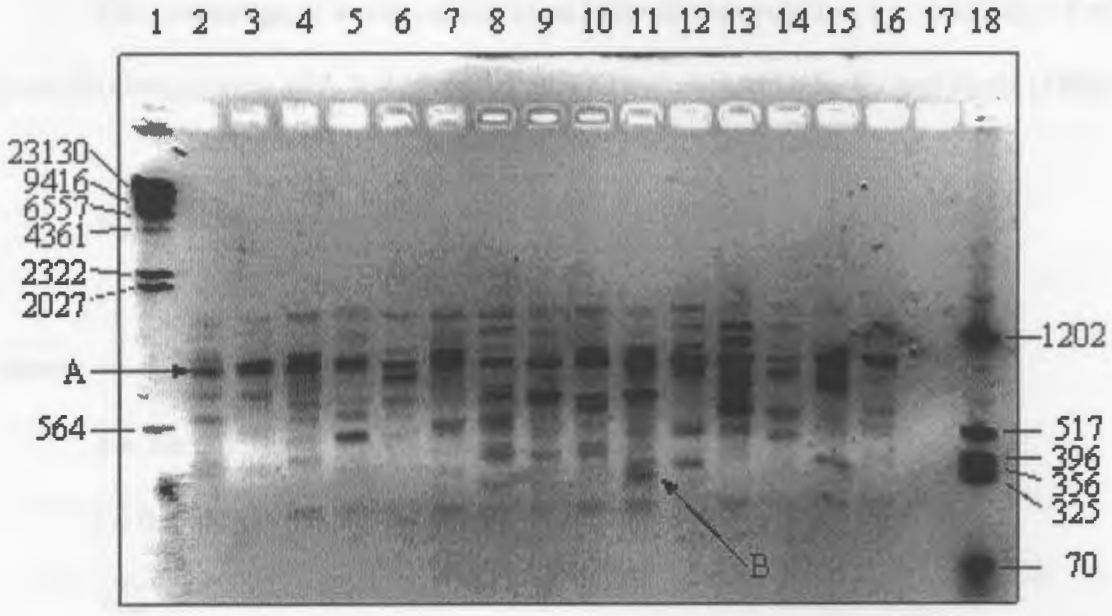

Figure 2. Example of a RAPD gel. Lane 1 is a *Hind*III digest of λ DNA, lane 18 is a *Hinf* I digest of plasmid Bluescript+KS, both as molecular weight standards. Lanes 2-16 contain RAPD products for primer OPA-10 with template DNA from individuals PJ 1-10 and SF 1-5, respectively. Lane 17 is a blank, resulting from a reaction run with ultrapure water used in place of template DNA. Arrow 'A' indicates a band that is monomorphic for all individuals on this gel, while arrow 'B' indicates a band that is clearly polymorphic on this gel. For the purposes of scoring, all visible bands (including faint bands such as those above band 'A' on this gel) were recorded, then comparisons were made between gels from separate runs of the same primer/template combinations made on different dates. After comparisons between all gels for a particular set of RAPD amplifications were made, only those bands that were present on two or more gels were scored as present.

Data Analysis

Population-Specific Bands

The percentage of bands unique to an individual population was calculated for all possible comparisons of 2, 3, 4, 5, and 6 populations according to Yu and Pauls (1993):

$$F_{(n)} = \frac{\sum_{i=0}^{n-2} (n-i) c_{1...n}^{(n-i)}}{\sum_{a=1}^{n} Xa}$$

where:

n = the number of populations being compared,

i = 0...(n-2),

$c_{1...n}^{(n-i)}$ = the number of common bands in all possible comparisons of n populations in (n-i) groups [note (n-i) is not an exponent],

Xa = the total number of bands per population, and

a = 1...n.

Within-Population Genetic Variability

Within-population genetic variability was assessed by making pairwise comparisons of the RAPD profiles of all individuals in each population (total of 10 individuals per population, 45 total comparisons per population) (Kresovich et al. 1992). For each population, genetic similarity of individuals was calculated (Nei 1972, Nei and Li 1979):

$$\text{Similarity } (F) = (2N_{ab})/(N_a + N_b)$$

where,

N_{ab} = number of shared fragments between individuals 'a' and 'b',

N_a = total number of fragments scored for all primers for individual 'a', and

N_b = total number of fragments scored for all primers for individual 'b'.

Within-population F values are reported for each population, and are the mean of all F values for all pairwise comparisons of individuals in that population. The genetic distance (Nei's genetic distance) (D) between two individuals was calculated as (Nei 1972):

$$D = -\ln(F)$$

The degree of within-population variability was determined for each population by calculating the mean value of D for all pairwise comparisons of individuals in the population.

Between-Population Genetic Variability

Between-population genetic similarity (F) and distance (D) were calculated using the same equations as above, except that in the equation for genetic similarity, the variables N_{ab}, N_a, and N_b refer to entire populations rather than individuals (Yu and Pauls 1993). The values of F and D were calculated for all pairwise comparisons of populations (total of 6 populations, 15 pairwise comparisons).

Another index of genetic variability between populations was calculated which takes the frequency of occurrence of each RAPD band in the populations into account (Yu and Pauls 1993). As a measure of similarity, the genetic identity index (I) was calculated:

$$I = 1/N\sum_{i=1}^{N}\frac{2Vi^{(1)} \cdot Vi^{(2)}}{[Vi^{(1)}]^2 + [Vi^{(2)}]^2}$$

where:

N = total number of fragments scored for all primers in the two populations being compared, and

$Vi^{(1)}$ and $Vi^{(2)}$ = the frequency of occurrence of an individual band i in populations 1 and 2, respectively.

The index of genetic distance (IGD) between populations was then calculated as:

$$IGD = -\ln(I)$$

The IGD is an index of the degree of evolutionary divergence between populations.

Apportionment of Diversity

The diversity of RAPD bands was apportioned within and among populations using Shannon's information measure (Lewontin 1972):

$$H_o = -\sum p_i \ln_2 p_i$$

where:

p_i = the frequency of a polymorphic band when present.

H was calculated for two levels of RAPD band frequency, following King and Schaal (1989):

$\overline{H}_{pop}$ = average diversity within populations,

H_{sp} = diversity within species,

$\overline{H}_{pop} / H_{sp}$ = the proportion of diversity within populations, and

$(H_{sp} - \overline{H}_{pop}) / H_{sp}$ = the proportion of diversity among populations.

Principle Components Analysis

A principle components analysis was performed on the RAPD presence/absence data using the PRINCOMP procedure of SAS (v6.12, SAS Institute Inc. 1996). The results of the PCA were used to make a 3-D plot of individuals based on the first three principle components.

Minimum Evolution Tree

A minimum evolution tree was generated using PAUP software (v.4, Swofford 1999) to illustrate the phenetic relationships of all individuals in the analysis based on the RAPD data. An unrooted tree was used because there was no information regarding a potential "origin" population or individual. The tree was generated with Heuristic Search, MAXTREE, and TBR branch swapping options selected.

Significance of Landform Region

To examine whether the landform region on which the populations occur was of importance in determining genetic variability within populations, the six populations were pooled into two groups for a statistical comparison of population-level variability.

variability. Group one consisted of the three populations from the youngest landform regions, CP from the Des Moines Lobe, and HP and CH from the Iowan Surface, while group two consisted of the three populations found on the older Southern Iowa Drift Plain, SH, PJ, and SF. Values of within-population variability ($\overline{H}_{pop}$) were averaged for each of the two groups and an ANOVA (Sokal and Rohlf 1995) was performed to determine if there was a significant difference between the two groups.

CHAPTER 3

RESULTS

Eleven of the 20 primers from Operon® Kit A produced a total of 211 repeatable, polymorphic bands. All 11 primers that were used in the analysis generated bands that detected variability both within and between populations. The number of polymorphic loci detected varied between primers, ranging from 14 for primer OPA-01 to 24 polymorphic loci detected by primer OPA-10. A summary of the primers used and the number of bands generated by each is included in Table 1. A summary of RAPD scores organized by primer is included in the Appendix.

Table 1. The eleven primers from Operon® Kit A that were used to generate reproducible bands in this study. Sequences are 5' to 3', and the number of usable amplification products that each primer produced is listed.

Primer	Sequence (5' to 3')	Number of bands
OPA-01	CAGGCCCTTC	14
OPA-02	TGCCGAGCTG	21
OPA-03	AGTCAGCCAC	19
OPA-04	AATCGGGCTG	22
OPA-05	AGGGGTCTTG	21
OPA-07	GAAACGGGTG	15
OPA-08	GTGACGTAGG	16
OPA-09	GGGTAACGCC	19
OPA-10	GTGATCGCAG	24
OPA-12	TCGGCGATAG	23
OPA-19	CAAACGTCGG	17

In addition, some of the bands generated by the 11 primers were unique to a single population. Overall, the average percentage of bands that were population-specific was only about 14% when any two populations were compared, and less than 1.5% of the bands were population-specific when all six populations were included in the comparison (Table 2).

Table 2. Percentage of population-specific bands in comparisons of 2, 3, 4, 5, or 6 populations of *L. aspera*. Values represent the mean of all possible comparisons of *n* populations.

# of populations being compared	% of population specific bands
2	14.07
3	5.18
4	2.85
5	1.89
6	1.38

A matrix of population similarity values based on the number of shared bands is shown in Table 3. Also included in this table are mean values of within-population similarity based on all possible pairwise comparisons of the 10 individuals sampled from each population. The estimates of similarity ranged from 0.8348 for CH compared to SH, and a maximum of 0.8775 for the comparison of CP to PJ. Estimated similarity (F) between individuals in a single population was much lower than that between populations, with the lowest average similarity of individuals of 0.4745 in population HP, and the greatest average within-population similarity of 0.5322 in population CH. Table

4 includes a matrix of genetic distance (D) values determined by calculating the negative natural log of F for each pair of populations. The pattern of genetic distance relationships between pairs of populations is the exact inverse of the patterns of similarity (e.g., CP and PJ are most similar, and therefore have the lowest genetic distance value).

Table 3. Similarity matrix generated from Nei and Li's (1979) estimate of similarity based on the number of shared bands. Values on the main diagonal are mean values of within-population similarity (F) based on all pairwise comparisons of individuals (n = 10) in that population. Values below the diagonal are between-population similarity (F).

Population	CP	HP	CH	SH	PJ	SF
CP	0.5177					
HP	0.8772	0.4745				
CH	0.8728	0.8554	0.5322			
SH	0.8513	0.8571	0.8348	0.5211		
PJ	0.8775	0.8487	0.8504	0.8639	0.5276	
SF	0.8522	0.8701	0.8537	0.8614	0.8588	0.4811

Table 4. Genetic distance (D) matrix (Nei 1972; Nei and Li 1979).

Population	CP	HP	CH	SH	PJ	SF
CP	0.6691					
HP	0.1310	0.7822				
CH	0.1360	0.1562	0.6438			
SH	0.1610	0.1542	0.1805	0.6595		
PJ	0.1307	0.1641	0.1620	0.1463	0.6504	
SF	0.1600	0.1392	0.1581	0.1491	0.1522	0.7391

A matrix of values of the genetic identity index (I) for comparisons of the six populations is shown in Table 5. This measure of similarity between populations takes both the number and the frequency of occurrence of shared bands into account. The populations that were least similar based on the genetic identity index are CH and SH, with a value of 0.5677, and CP and HP were most similar with I = 0.6583. The rank-order of population similarity changes when this element of genetic structure (frequency of alleles) was included in the analysis (e.g., CP-PJ ranked most similar according to Nei and Li's index, while this pair ranked eighth most similar when the genetic identity index was calculated). Values for the index of genetic distance (IGD) determined by calculating IGD = -ln(I) are presented in Table 6.

Table 5. Genetic identity index (I) (Yu and Pauls 1993) matrix based on a comparison of the number and frequency of occurrence of bands between six populations of *L. aspera*.

Population	CP	HP	CH	SH	PJ	SF
CP	1.0000					
HP	0.6583	1.0000				
CH	0.6322	0.5967	1.0000			
SH	0.5871	0.6105	0.5677	1.0000		
PJ	0.6103	0.5962	0.5895	0.6174	1.0000	
SF	0.5863	0.6290	0.5936	0.6280	0.6130	1.0000

A visual representation of the genetic relatedness of the 60 individuals of *L. aspera* included in the study is presented in a minimum evolution tree (Figure 3) that was generated using PAUP* software (v. 4, Swofford 1999) and RAPD band

Table 6. Index of Genetic Distance (IGD) matrix (Yu and Pauls 1993).

Population	CP	HP	CH	SH	PJ	SF
CP	0.0000					
HP	0.4180	0.0000				
CH	0.4585	0.5164	0.0000			
SH	0.5326	0.4935	0.5662	0.0000		
PJ	0.4938	0.5172	0.5285	0.4823	0.0000	
SF	0.5340	0.4637	0.5216	0.4651	0.4895	0.0000

presence/absence data. Relationships identified by the tree are similar to those described by the genetic identity index (GII) at the population level. Individuals from the CP population are clustered into two separate groups, one each with individuals from populations HP and CH (GII rank #1 and #2 of 15, respectively), individuals from the SH population are grouped with those from the SF population (GII rank #4), and there is one group comprised primarily of individuals from populations PJ and SF (GII rank #6).

A principle components analysis was performed using SAS software (v.6.12, SAS Institute 1996) and the RAPD band data to further evaluate the relationships of the individuals in the study. A 3D plot of the first three principle components (Figure 4) illustrates the nature of the relationships detected by the principle components analysis. This figure corroborates the pattern of individual-level relationships suggested by the minimum evolution tree (e.g., note the clustering of individuals 42,43,44), but allows discrimination of 'distance' between individuals that could not be illustrated in the two-dimensional tree (e.g., individuals 35, 12, and 11 are adjacent in the tree, but the PCA

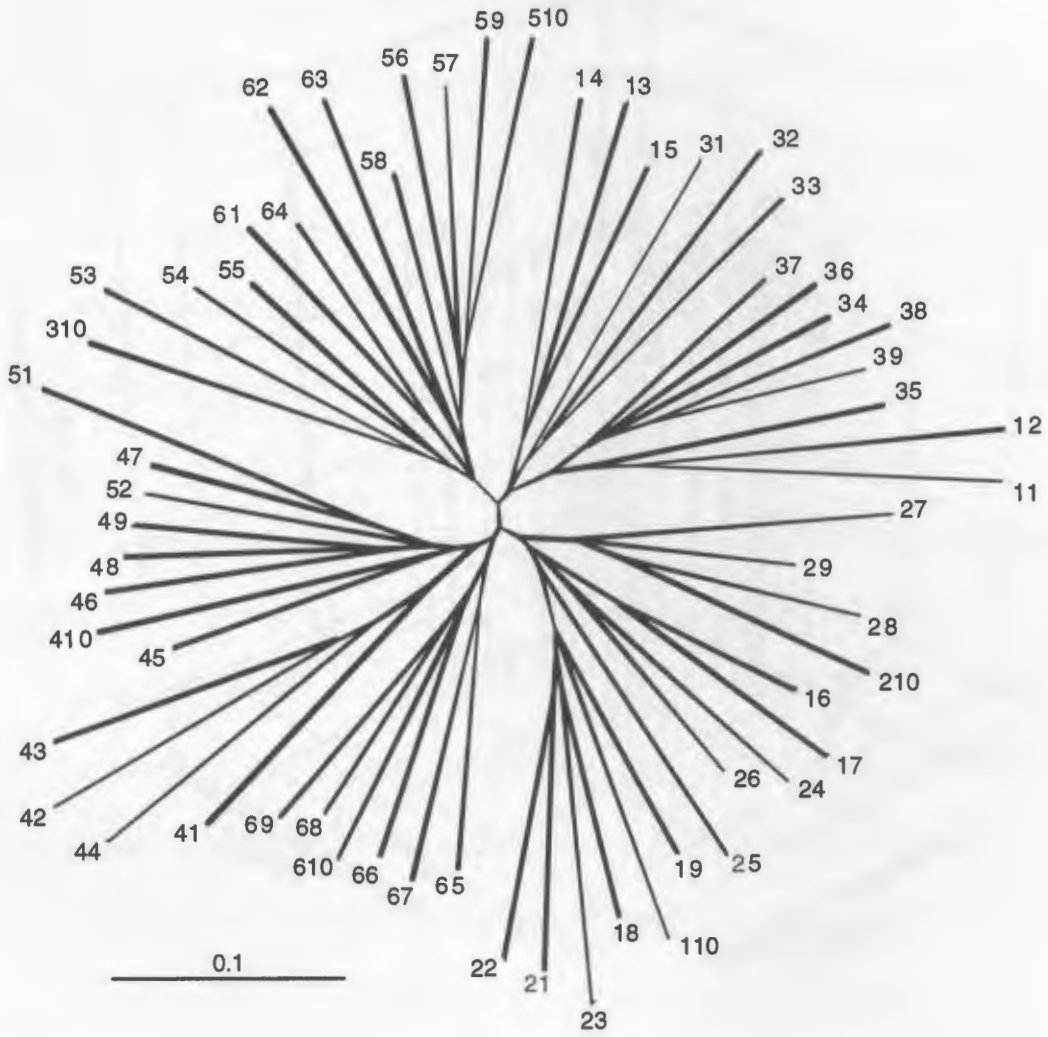

Figure 3. Minimum evolution tree indicating relationships of individuals of *L. aspera* based on RAPD data. Branch lengths indicate number of evolutionary steps necessary for observed variability in RAPD genotype (bar = 0.1 steps). Code numbers of individuals are as follows: first digit refers to population (1 = CP, 2 = HP, 3 = CH, 4 = SH, 5 = PJ, 6 = SF), second (and third) digits refer to the number of the individual from the population (e.g., '510' refers to population 5 (PJ), individual number 10). The tree was generated using RAPD band data and PAUP software to generate an unrooted tree with Heuristic Search, MAXTREE, and TBR branch swapping options selected.

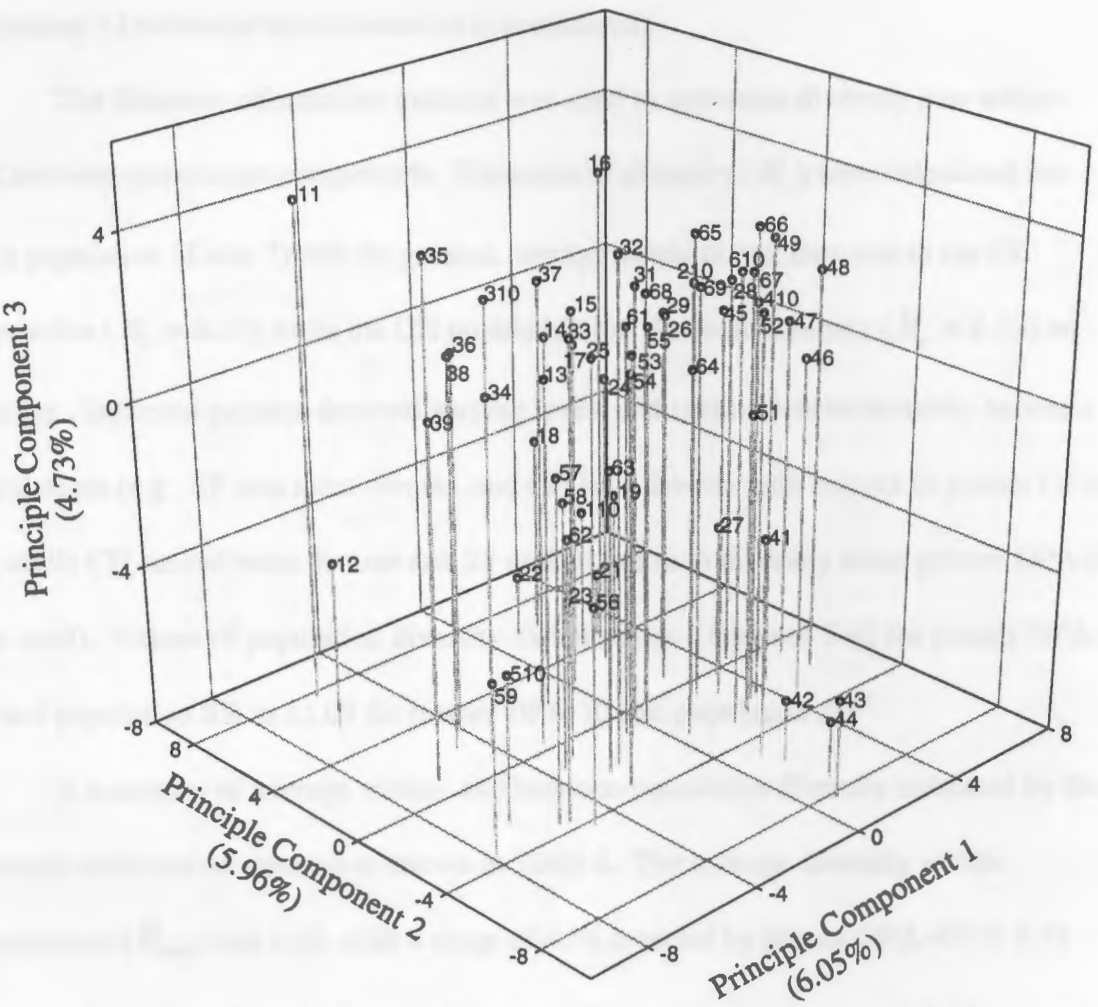

Figure 4. 3D graph of the first three principle components describing the variability between individuals of *L. aspera*. Values in parentheses indicate the proportion of the total variation explained by each component (note: it required 28 principle components to describe 75% of the total variation in the sampled individuals). Code numbers for individuals are as described in Figure 3.

plot clearly shows that individuals 11 and 35 are more similar to one another than to individual 12 when the third dimension is considered).

The Shannon information measure was used to apportion diversity into within- and between-population components. Estimates of diversity (H_o) were calculated for each population (Table 7) and the greatest average diversity was detected in the CP population (H_o = 6.67) while the CH population had the least diversity (H_o = 6.10) on average. Different primers detected varying levels and rank-orders of diversity between populations (e.g., SF was most diverse, and CH least diverse with respect to primer OPA-01, while CH ranked most diverse and SF ranked fourth in diversity when primer OPA-02 was used). Values of population diversity varied across a range of 3.02 for primer OPA-07 and population SH to 11.09 for primer OPA-10 and population SF.

A summary of average within- and between-population diversity indicated by the Shannon information measure is shown in Table 8. The average diversity within populations ($\overline{H}_{pop}$) was 6.35, with a range of 4.16 detected by primer OPA -07 to 9.55 detected by primer OPA-10. Overall diversity (H_{sp}) within *L. aspera* was 8.14, ranging from 5.62 to 11.01 as detected by primers OPA-07 and OPA-10, respectively. The proportion of overall diversity present within populations ($\overline{H}_{pop} / H_{sp}$) ranged from 0.70 (primer OPA-19) to 0.87 (primer OPA-10), and averaged 0.78. The proportion of diversity attributed to variability between populations ($(H_{sp} - \overline{H}_{pop})/ H_{sp}$) ranged from 0.13 to 0.30, with an average of 0.22. The results of the ANOVA performed on average values of $\overline{H}_{pop}$ for the two landform-based groups of populations (F = 0.033, P = 0.864)

indicated that there was no significant difference in average within-population variability for these two groups of populations.

Table 7. Estimates of genetic diversity (H_o) within six populations of *L. aspera* for each of 11 primers.

	Population					
Primer	CP	HP	CH	SH	PJ	SF
OPA-01	3.86	4.77	3.06	5.12	3.30	5.28
OPA-02	7.07	5.33	7.66	7.39	6.25	6.57
OPA-03	6.13	6.46	6.16	6.76	6.84	7.79
OPA-04	7.27	6.39	6.35	7.16	6.56	6.68
OPA-05	8.08	7.24	6.30	5.66	8.24	6.17
OPA-07	3.82	5.59	5.02	3.02	3.54	3.97
OPA-08	6.77	5.43	5.15	4.78	5.07	5.18
OPA-09	7.90	7.01	7.71	7.49	6.38	6.29
OPA-10	7.98	9.70	9.97	9.91	8.65	11.09
OPA-12	7.89	6.72	4.57	7.85	9.02	5.68
OPA-19	6.60	5.09	5.15	4.29	6.78	4.43
Mean	6.67	6.34	6.10	6.31	6.42	6.28

Table 8. Partitioning of genetic diversity (Shannon diversity) within and between populations of *L. aspera* for 11 RAPD primers.

Primer	$\overline{H}_{pop}$	H_{sp}	$\overline{H}_{pop} / H_{sp}$	$(H_{sp} - \overline{H}_{pop}) / H_{sp}$
OPA-01	4.23	5.68	0.74	0.26
OPA-02	6.71	9.19	0.73	0.27
OPA-03	6.69	8.67	0.77	0.23
OPA-04	6.74	8.88	0.76	0.24
OPA-05	6.95	9.05	0.77	0.23
OPA-07	4.16	5.62	0.74	0.26
OPA-08	5.40	6.57	0.82	0.18
OPA-09	7.13	8.41	0.85	0.15
OPA-10	9.55	11.01	0.87	0.13
OPA-12	6.95	8.79	0.79	0.21
OPA-19	5.39	7.69	0.70	0.30
Mean	6.35	8.14	0.78	0.22

CHAPTER 4

DISCUSSION

The RAPD method successfully detected genetic variability both within and between populations of *L. aspera* Michx. in Iowa using 11 decamer primers. Of the 211 polymorphic bands produced by the 11 primers, only 14 were unique to a single population. Similarity values were higher when calculated based only on the number of shared bands (average F = 0.8590) than when both the number and frequency of shared bands was included in the calculation (average I = 0.6077). Overall, the rank-order of similarity of population pairs was different between the two indices of similarity, but the rank-order of similarity of population pairs within an ecotype zone varied little. In Zone 1, the CP-HP pair ranked #2 and #1 (of 15) by Nei and Li's similarity 'F' and the genetic identity index 'I', respectively, while in Zone 2, the rank of CH-SH similarity was #15 by both indices, and in Zone 3, PJ-SF ranked #7 and #6 most similar, respectively. This may indicate that the current zonation for the Ecotype Project may not be effective in conserving "local" genetic integrity in all cases for *L. aspera*, and that the level of genetic variability may differ between collections of *L. aspera* for the three zones. Average similarity (F) of individuals within populations was much lower (average F = 0.5090) than that between populations (average F = 0.8590). Similarly, the Shannon information measure indicated that on average, 78% of the total variability within the sample of L. aspera is found within populations, while an average of only 22% of the genetic variability is attributed to the between-population component. These measures of genetic variability taken together strongly indicate that the greatest amount of variability in the

samples of *L. aspera* that were evaluated is between individuals rather than between populations. This would suggest that with respect to the goal of maximizing genetic variability of seed collections, the effort to sample *L. aspera* in Iowa should be concentrated on maximizing the number of individuals that seed is collected from within populations, and that the total number of populations that are sampled does not necessarily need to be large. The number of novel genetic characters that are likely to be acquired by sampling additional populations is probably very low, as evidenced by the analysis of population-specific bands (Table2).

The minimum evolution tree (Figure 3) and the plot of the principle components (Figure 4) illustrate that while there is some clustering of populations, these groups are not strongly differentiated. When these figures are reviewed in the context of the geographical variation represented by the location of the six populations, there is no clear pattern to the relatedness of the populations (or individuals). When linear distances between pairs of sites were regressed against genetic distance (both D and IGD) values for site-pairs, no relationship was found ($r^2 = 0.04$ and $r^2 < 0.0001$, respectively). This suggests that physical distance between populations of *L. aspera* on a scale of tens to hundreds of kilometers is not a useful criterion for determining a sampling strategy that will maximize genetic variability and maintain local genetic integrity. In fact, the two populations that were closest together (45 Km), PJ and SF, ranked only sixth and seventh most similar (GII and F indices, respectively), and those that were located farthest apart (346 Km), CP and PJ, ranked most similar by Nei and Li's index of similarity (F), and eighth most similar by the GII.

Because the six populations of *L. aspera* included in this study were found in three landform regions that vary greatly in age (Prior 1991), it was of interest to investigate whether the observed patterns of genetic relatedness between populations had any relationship to the landform on which the populations occur. If it is assumed that these populations have been isolated on a geologic time scale, then the age of the surfaces on which they occur could be an important factor contributing to the existing variability among the populations, since genetic differentiation (evolutionary divergence) occurs on a time scale of thousands of years. Population CP occurs on the Des Moines Lobe landform, which was glaciated as recently as 12,000-14,000 years ago. The HP and CH populations are found in the Iowan Surface landform region, and the remaining three populations, SH, PJ, and SF, are in the Southern Iowa Drift Plain. These latter two landforms have similar glacial history, both being free from glacial ice since the Pre-Illinoian glaciers receded some 500,000 years or more ago. An important geological difference between these two landforms is that the Iowan Surface has evidence of large-scale erosion events occurring across this entire region as recently as 16,500 years ago, making this landform region more similar to the Des Moines Lobe than to the Southern Iowa Drift Plain. It is likely that any populations in the youngest regions arose from a founding event where a few individuals (progeny) from a population in an older region became established as habitats became suitable during the period of environmental change as the glaciers receded. Based on this information and the assumption (unfounded though it may be) that the populations in any given region have been genetically isolated on a geologic time scale, one might hypothesize that the greatest amount of divergence

should be found between the populations that occur in the youngest landform regions (the Iowan Surface and the Des Moines Lobe), but that the variability within younger populations might be considerably lower than that in older populations, simply due to the longer period for genetic differentiation to take place within the older populations. Historically, gene flow among populations was probably relatively high as a result of wind-dispersed seeds and pollination by bees and moths. A review of the similarity values computed for the two indices used in this study reveals that the three populations that are potentially the oldest, SH, PJ, and SF, rank fourth through seventh in similarity. This observation provides some support for the above hypothesis, but the two most similar populations, CP and HP, both occur in the Iowan Surface region and are both therefore potentially "young" populations if they arose since the period of intense, widespread erosion in this region only 16,500 years ago. This fact suggests that the landform region in which a population occurs may not be an important factor influencing genetic similarity relationships between these populations. In addition, the results of the ANOVA performed on the $\overline{H}_{pop}$ values for the populations grouped by landform region indicated that there was no significant difference in average within-population variability for these two groups of populations. It appears that the patterns of genetic variation detected by RAPDs in these populations of *L. aspera* cannot be readily explained by simple parameters such as geographic distance between populations or the geological history of the landform regions in which they occur.

The results of this study suggest that the majority of genetic variability of *L. aspera* in Iowa as revealed by RAPD is apportioned within (78%) rather than between

(22%) populations. Because there are few genetic characters that are unique to single populations, the risk of "genetic contamination" resulting from pooling seed collected from a wide range of populations is probably quite low. In order to maximize genetic variability of seed collections of *L. aspera* for the Iowa Ecotype Project, seed should be collected from the maximum number of individuals in any given population, while the addition of single populations to the sampling effort is not likely to add a large amount of variability to the collection. Because the overall level of variability of *L. aspera* is moderate, and the pattern of this variability cannot readily be explained, the current sampling protocol of the Iowa Ecotype Project is probably reasonable with respect to the goals of maximizing variability and conserving local genetic integrity as well as addressing the economic and logistic aspects of collecting, cultivating, and marketing the seed. If the sampling strategy is to be further optimized for *L. aspera* with respect to the goals of the project, more intensive investigations into the mechanisms driving the patterns of genetic variability of this species should be conducted.

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APPENDIX

SUMMARY OF RAPD SCORES

Table 9. Summary of RAPD scores. An 'x' indicates bands scored as present.

	OPA-01													
	1487	1446	1417	1158	967	827	775	730	678	638	606	532	498	407
CP1	x				x			x		x			x	
CP2		x			x			x					x	
CP3		x			x			x					x	
CP4		x			x			x					x	
CP5			x		x			x					x	
CP6			x		x								x	
CP7				x	x								x	
CP8					x		x						x	
CP9		x			x	x				x			x	
CP10		x			x	x			x					
HP1		x			x	x						x	x	
HP2		x			x	x			x				x	
HP3		x			x	x		x		x		x	x	
HP4													x	
HP5	x				x						x			
HP6	x				x								x	
HP7		x			x			x			x			
HP8		x			x		x	x			x		x	
HP9		x			x			x					x	
HP10			x		x		x	x					x	
CH1		x			x							x		
CH2		x			x	x							x	
CH3	x				x				x					
CH4	x				x				x			x		
CH5	x				x						x		x	
CH6	x				x							x		
CH7	x				x				x				x	
CH8	x				x						x			
CH9	x				x							x		
CH10	x				x							x		
SH1		x		x	x			x		x			x	
SH2		x		x	x			x			x		x	
SH3		x		x	x			x					x	
SH4		x			x			x				x	x	x
SH5		x			x				x			x	x	
SH6		x			x		x		x				x	x
SH7		x		x	x					x			x	
SH8			x		x		x		x				x	
SH9			x		x	x							x	
SH10			x		x	x			x				x	
PJ1			x	x	x			x					x	
PJ2			x		x			x					x	
PJ3	x			x	x						x			
PJ4	x				x			x				x		
PJ5	x				x			x				x		
PJ6	x			x	x			x				x		
PJ7	x				x			x				x		
PJ8	x			x	x								x	
PJ9	x				x								x	
PJ10	x				x			x		x		x		
SF1					x	x						x		
SF2	x				x			x				x		
SF3	x			x	x	x				x		x		
SF4	x				x	x			x					
SF5			x		x	x					x		x	
SF6			x	x	x							x		
SF7			x	x	x		x						x	
SF8		x			x	x						x		
SF9		x			x									
SF10		x			x		x					x		

(table continues)

	OPA-02																			
	1553	1271	1153	1084	1037	999	967	936	895	850	797	763	734	684	634	593	546	512	451	403	327
CP1					x				x			x			x	x		x		x	x
CP2				x				x			x	x			x			x	x		
CP3				x				x			x					x		x			
CP4				x				x			x			x		x		x	x	x	
CP5				x				x			x	x				x		x		x	x
CP6					x				x			x						x		x	
CP7							x		x			x			x			x			
CP8					x			x	x		x		x		x			x			
CP9				x				x			x				x			x			
CP10				x		x		x			x			x	x		x				
HP1				x		x		x				x			x			x			
HP2				x				x				x			x			x			
HP3								x			x			x	x			x			
HP4											x										
HP5				x				x				x		x		x					
HP6				x							x										
HP7				x				x			x				x		x				
HP8									x			x				x	x				
HP9				x				x			x					x					
HP10						x			x			x				x		x			
CH1						x				x				x		x	x		x	x	x
CH2					x			x		x			x			x		x		x	x
CH3				x				x	x			x	x		x	x		x	x	x	
CH4							x		x		x		x		x					x	
CH5			x				x		x		x		x			x		x	x	x	x
CH6				x			x		x		x		x		x	x		x			x
CH7							x		x		x		x		x	x					x
CH8				x			x		x		x	x			x	x		x			
CH9				x			x		x			x			x	x		x	x	x	
CH10								x		x		x			x	x		x	x	x	x
SH1									x		x		x		x		x				
SH2	x	x	x	x			x		x		x		x		x	x			x		
SH3	x	x						x	x				x			x	x			x	
SH4	x	x							x		x		x		x	x			x		
SH5							x		x		x	x		x		x	x	x			
SH6								x	x				x	x		x	x			x	
SH7								x	x			x			x	x		x			
SH8									x			x			x	x					
SH9							x		x			x			x	x		x		x	
SH10							x		x		x	x		x		x					
PJ1							x		x				x		x	x		x	x		
PJ2						x										x		x			
PJ3														x				x		x	
PJ4						x					x				x			x			
PJ5											x				x			x			
PJ6						x		x			x				x		x				
PJ7						x		x		x	x			x			x			x	x
PJ8						x		x			x				x			x			
PJ9						x		x			x			x	x			x	x		
PJ10						x		x			x		x				x				
SF1						x		x		x					x			x	x		
SF2												x			x		x		x		
SF3																					
SF4						x		x		x				x	x						
SF5												x				x					
SF6								x		x		x		x							
SF7									x	x				x				x			
SF8									x					x							
SF9					x			x	x				x				x				
SF10								x		x	x		x	x		x	x				

(table continues)

	OPA-03																	
	1184	1123	1045	995	946	894	852	795	750	698	650	601	532	508	466	439	404	380	350
CP1				x			x		x	x		x		x		x			
CP2					x	x				x		x		x		x	x		
CP3				x		x		x		x	x	x		x			x		
CP4									x			x		x					
CP5								x			x		x						
CP6										x		x		x					
CP7							x			x		x		x					
CP8									x		x			x			x		
CP9						x		x	x		x			x			x	x	
CP10				x				x		x	x			x		x			
HP1								x	x		x			x		x	x		
HP2							x		x		x			x			x		
HP3							x		x		x			x		x			
HP4			x			x													
HP5			x						x		x		x				x		
HP6			x			x						x			x				
HP7			x		x		x		x		x			x			x		
HP8			x		x									x				x	
HP9						x			x	x				x		x			
HP10									x	x	x			x		x	x	x	
CH1							x	x		x		x	x	x		x		x	x
CH2					x				x	x	x		x	x			x	x	x
CH3								x		x		x		x					x
CH4							x	x		x		x		x					
CH5														x		x		x	
CH6						x				x		x		x		x	x		
CH7					x					x	x			x		x			
CH8						x				x		x		x				x	
CH9								x		x	x			x		x			
CH10						x		x		x		x		x	x	x	x	x	
SH1						x	x		x		x		x	x			x		
SH2			x		x	x		x		x			x		x				
SH3				x	x		x	x		x			x		x		x		
SH4						x		x			x		x						
SH5				x		x		x			x		x	x			x		
SH6									x		x	x	x			x			
SH7						x			x		x		x			x			
SH8								x	x		x		x			x			
SH9							x		x		x		x			x			
SH10							x	x		x		x		x			x		
PJ1				x	x	x			x		x			x		x	x	x	x
PJ2			x		x	x			x		x		x						
PJ3			x			x	x	x	x		x		x	x	x	x		x	
PJ4		x	x		x	x		x		x			x		x	x		x	x
PJ5			x			x		x			x		x			x	x		
PJ6				x		x			x		x		x		x		x		
PJ7			x			x	x		x		x		x		x		x		
PJ8			x		x	x	x		x	x	x		x		x	x	x		x
PJ9				x		x	x	x	x		x		x		x	x	x		x
PJ10						x	x		x		x	x	x			x	x	x	x
SF1				x		x		x	x		x	x	x			x	x		x
SF2	x	x		x		x	x		x			x	x		x		x	x	x
SF3		x	x		x		x		x		x	x	x		x		x		x
SF4					x				x		x		x	x		x	x		
SF5							x		x		x		x	x		x	x	x	
SF6								x	x					x		x			
SF7					x		x		x				x	x					
SF8					x		x		x					x					
SF9				x	x		x		x					x			x	x	
SF10			x		x		x		x		x			x		x			

(table continues)

	OPA-04																				
	1390	1304	1167	1095	1042	996	942	891	836	803	764	734	690	646	591	546	497	446	403	371	324	276
CP1				x			x			x		x	x		x		x	x	x			
CP2	x		x		x			x		x		x	x		x	x	x		x	x		
CP3						x	x	x			x		x		x	x	x		x			
CP4				x		x	x	x		x		x		x	x	x	x	x	x		x	
CP5								x		x			x		x		x					
CP6							x			x		x	x				x					
CP7								x		x		x	x		x		x				x	
CP8			x				x	x		x		x		x	x		x					
CP9								x		x							x		x			
CP10			x		x		x	x		x			x			x	x		x			
HP1		x	x			x		x		x	x		x			x	x					
HP2		x	x	x				x			x		x			x	x					
HP3		x	x			x		x		x		x		x	x		x		x			
HP4		x		x			x	x									x					
HP5		x	x		x			x		x		x		x			x	x	x			
HP6			x			x		x		x			x		x		x	x	x			
HP7			x			x		x		x		x	x	x		x	x					
HP8						x		x		x		x		x	x	x	x		x			
HP9		x	x	x		x		x		x		x	x		x		x		x			
HP10		x				x		x		x			x		x		x		x			
CH1													x		x		x					
CH2							x		x				x		x		x	x	x	x		
CH3							x					x		x			x					
CH4													x	x	x		x		x			
CH5										x				x	x		x		x			
CH6								x	x			x	x		x		x		x			
CH7							x		x			x	x	x	x		x	x	x			
CH8			x		x		x		x			x			x		x	x	x			
CH9									x			x	x	x	x		x	x	x			
CH10					x		x			x	x			x		x	x		x		x	
SH1							x		x			x		x	x	x	x		x	x	x	x
SH2									x					x			x			x		
SH3									x				x	x	x		x	x		x		
SH4									x		x		x	x		x		x	x	x		
SH5													x		x		x	x	x	x		
SH6										x		x	x		x		x					
SH7								x		x			x	x		x	x			x	x	
SH8											x			x		x	x		x			
SH9										x		x	x			x	x			x		
SH10								x			x		x		x			x				
PJ1					x						x		x			x	x			x		
PJ2											x		x			x		x	x	x		
PJ3								x			x			x			x	x				
PJ4										x							x	x	x			
PJ5								x		x		x		x	x	x	x		x			
PJ6									x		x		x		x		x		x	x		
PJ7										x			x	x	x		x	x		x		
PJ8								x		x		x		x	x		x	x	x	x		
PJ9							x		x		x			x			x		x	x		
PJ10								x	x		x		x	x	x	x	x		x			
SF1			x					x		x			x		x		x	x		x		
SF2								x	x			x			x		x	x	x	x		
SF3						x						x			x	x	x	x				
SF4										x			x		x	x	x		x			
SF5							x				x		x				x	x	x		x	
SF6																x	x	x	x			
SF7								x	x				x			x	x			x		
SF8								x		x				x	x	x	x	x	x			
SF9								x		x			x	x	x			x	x			
SF10								x		x			x	x	x	x			x			

(table continues)

	OPA-05																			
	1408	1142	1063	982	914	872	837	794	739	688	651	608	544	495	437	407	386	354	324	302	274
CP1				x	x		x		x	x			x	x		x			x		x
CP2		x		x	x		x		x			x	x	x		x		x			x
CP3							x		x			x	x	x	x	x				x	
CP4						x	x		x	x	x		x	x	x				x		
CP5									x	x		x	x	x	x						
CP6								x				x	x	x		x					
CP7			x			x		x		x		x	x	x	x	x				x	
CP8					x	x		x			x		x	x	x	x		x		x	
CP9			x					x		x			x	x	x					x	
CP10			x			x		x		x		x		x	x					x	
HP1			x	x		x				x	x			x		x					x
HP2		x	x		x					x	x		x	x	x	x					x
HP3		x		x	x	x		x		x	x			x	x		x				x
HP4			x		x		x	x			x			x							
HP5			x			x				x				x	x		x				
HP6															x						
HP7							x			x		x	x	x			x				
HP8						x		x		x				x	x	x	x				x
HP9						x				x		x		x	x		x				
HP10		x				x		x		x			x	x	x	x	x				
CH1						x				x				x							x
CH2					x		x			x				x	x		x				x
CH3					x			x		x				x	x		x				x
CH4			x		x						x			x	x		x				x
CH5					x			x		x			x			x					x
CH6			x	x		x			x		x			x		x				.	x
CH7			x		x			x			x			x	x		x				x
CH8			x		x		x		x	x				x		x					x
CH9		x	x	x	x				x	x				x	x	x					x
CH10		x	x	x	x			x	x	x			x	x	x	x					
SH1		x		x		x				x		x		x	x		x				
SH2		x		x		x	x			x	x		x	x			x				
SH3											x		x	x	x						x
SH4														x	x						x
SH5				x		x					x			x	x						
SH6								x			x		x	x			x				
SH7							x	x		x		x		x	x						
SH8										x	x		x	x	x		x				
SH9								x		x		x		x	x		x				
SH10								x		x		x		x	x		x				
PJ1				x				x		x			x	x			x				
PJ2			x			x			x	x		x		x							
PJ3	x	x		x			x		x	x		x	x	x			x				
PJ4	x	x				x		x		x			x	x	x		x				
PJ5				x	x			x		x				x			x				
PJ6		x	x		x			x		x		x	x	x	x		x				
PJ7		x	x	x	x			x	x	x			x	x	x		x		x		x
PJ8				x		x				x	x		x	x	x		x				x
PJ9	x	x		x	x		x			x				x	x		x		x		x
PJ10		x		x				x			x			x	x			x		x	
SF1								x			x	x		x	x		x				
SF2		x									x	x		x	x		x				x
SF3		x		x		x				x			x	x	x		x				
SF4										x			x	x	x		x				x
SF5										x				x	x		x	x			
SF6						x				x	x			x	x			x			
SF7			x								x		x	x	x		x				
SF8											x			x	x		x				
SF9				x		x		x			x	x	x	x	x		x				
SF10							x		x		x			x							

(table continues)

	OPA-07														
	1045	974	937	881	836	789	738	700	641	603	569	511	379	358	300
CP1	x	x		x			x			x		x	x		
CP2	x			x			x		x			x	x		
CP3		x		x			x		x	x		x		x	
CP4			x				x	x			x	x	x		
CP5	x	x		x			x		x	x		x	x		
CP6				x			x			x		x	x		
CP7				x			x			x		x	x		
CP8		x		x			x		x			x	x		
CP9		x		x			x		x			x		x	
CP10				x			x		x			x	x		
HP1		x		x			x		x			x	x		
HP2		x		x			x		x			x	x		
HP3		x		x			x		x			x	x		
HP4							x		x			x			
HP5		x		x			x			x		x	x		
HP6			x					x		x		x			
HP7					x			x	x		x	x		x	
HP8					x			x	x		x	x		x	
HP9					x			x		x		x			
HP10			x		x			x		x	x	x		x	
CH1					x				x	x		x		x	
CH2					x			x			x	x			
CH3					x			x	x		x	x		x	x
CH4		x		x				x	x	x		x	x		
CH5				x			x		x	x		x	x	x	
CH6						x	x		x	x		x	x		
CH7					x			x		x		x	x		
CH8					x			x	x	x		x	x		
CH9		x						x	x	x		x	x		
CH10				x	x		x			x		x	x		
SH1			x		x			x	x		x	x			
SH2			x	x				x	x		x	x			
SH3			x	x				x	x		x	x			
SH4					x			x	x	x		x			
SH5					x			x		x		x			
SH6					x			x	x			x			
SH7					x			x		x		x			
SH8					x			x		x	x	x			
SH9				x	x			x		x	x	x	x		
SH10				x	x			x		x	x	x			
PJ1		x			x			x		x		x		x	
PJ2					x			x	x			x	x		
PJ3					x			x	x			x	x	x	
PJ4			x	x				x		x	x	x	x		
PJ5					x			x	x		x	x	x		
PJ6					x			x	x			x	x		
PJ7					x			x	x			x	x		
PJ8				x				x	x			x	x		
PJ9				x				x	x	x		x	x		
PJ10				x				x	x		x	x	x		
SF1					x			x		x		x	x		
SF2				x	x			x	x		x	x	x		
SF3				x	x			x		x		x	x	x	
SF4					x			x				x			
SF5					x			x		x	x	x	x		
SF6					x			x							
SF7					x			x		x		x			
SF8					x							x			
SF9				x			x				x	x			
SF10			x		x			x			x	x			

(table continues)

	OPA-08															
	1263	1170	1080	1028	940	897	841	742	696	607	502	470	440	412	325	286
CP1	x			x			x	x		x		x		x		
CP2		x	x		x	x		x		x	x	x		x		x
CP3		x				x				x	x		x	x		x
CP4			x			x				x	x			x		
CP5		x							x	x	x		x			
CP6						x				x						
CP7				x		x		x		x	x		x		x	
CP8				x		x			x	x	x					
CP9					x				x	x	x					
CP10		x				x				x		x				
HP1			x		x					x		x				
HP2					x		x	x		x	x		x			
HP3				x		x	x	x	x	x			x			
HP4								x								
HP5				x		x	x			x	x	x				
HP6										x			x			
HP7			x				x			x	x					
HP8		x					x		x	x						
HP9										x						
HP10				x		x	x		x	x						
CH1						x				x	x			x		x
CH2										x	x			x		x
CH3		x				x				x	x				x	x
CH4		x	x		x					x				x		x
CH5				x		x		x		x	x	x			x	
CH6			x		x					x	x					x
CH7								x		x	x					
CH8			x		x					x	x		x	x	x	
CH9		x				x				x	x			x		
CH10										x	x			x		
SH1		x								x	x		x			
SH2		x				x				x			x			
SH3		x	x		x					x	x					
SH4		x			x					x	x		x		x	
SH5						x				x	x			x		
SH6							x			x	x		x			
SH7		x				x			x	x	x		x	x		
SH8					x					x		x				
SH9						x				x	x		x	x		
SH10					x			x		x	x			x		
PJ1									x	x	x			x	x	
PJ2									x	x	x					
PJ3										x					x	
PJ4										x					x	
PJ5		x							x		x					
PJ6		x	x							x	x					
PJ7					x			x		x	x					
PJ8			x				x	x		x	x		x			
PJ9		x				x				x	x		x			
PJ10								x		x	x	x				
SF1	x	x			x	x		x		x	x					
SF2		x	x	x		x		x		x	x			x		
SF3		x			x				x	x			x			
SF4					x					x	x					
SF5		x		x		x	x			x	x					
SF6						x				x	x					
SF7		x		x		x				x	x			x		
SF8		x				x		x		x		x		x		
SF9						x		x		x	x		x			
SF10						x				x	x			x		

(table continues)

	OPA-09																	
	1229	1147	1100	1051	990	943	880	846	813	730	679	641	581	544	506	473	438	391	343
CP1			x		x		x		x	x		x	x		x		x		
CP2			x		x		x		x	x		x		x	x			x	
CP3			x			x		x	x			x	x			x	x		
CP4				x	x			x	x		x	x				x		x	
CP5				x				x	x		x	x	x		x				
CP6							x		x	x									
CP7				x	x		x		x	x	x			x	x		x		
CP8	x		x		x		x	x		x	x				x		x		
CP9	x		x		x		x	x		x	x			x			x		
CP10		x			x		x	x		x	x			x			x		
HP1							x	x		x	x			x					
HP2	x				x		x	x		x	x			x					
HP3			x				x	x		x	x			x	x		x		
HP4				x			x			x		x		x					
HP5																			
HP6		x			x		x	x		x		x					x		
HP7	x	x					x		x	x	x					x			
HP8	x		x			x	x		x	x		x		x			x		
HP9			x		x		x		x	x		x		x	x				
HP10			x			x	x		x	x		x			x				
CH1		x	x		x			x	x		x	x	x						
CH2	x			x		x		x	x		x	x	x		x		x		
CH3	x		x			x	x		x	x		x	x		x	x			
CH4					x		x		x	x		x		x			x		
CH5			x		x			x	x	x		x	x	x			x		
CH6			x	x			x		x	x		x		x					
CH7			x	x			x		x	x		x			x		x		
CH8	x				x		x		x	x		x		x	x		x		x
CH9	x	x		x	x		x		x	x		x			x		x		
CH10																			
SH1	x		x				x	x		x		x		x		x			
SH2	x			x	x		x		x	x	x			x					
SH3	x	x		x		x	x		x	x		x			x	x			
SH4	x	x		x			x	x		x	x				x		x	x	
SH5							x		x	x		x							
SH6				x		x		x	x	x		x			x				
SH7								x		x		x		x					
SH8								x	x			x		x	x		x		
SH9			x			x		x	x			x			x				
SH10								x	x			x				x			
PJ1							x	x			x	x			x			x	x
PJ2							x		x					x		x	x		
PJ3				x				x	x				x					x	
PJ4				x			x	x	x			x					x		
PJ5				x		x		x	x					x			x		
PJ6				x			x		x			x							
PJ7							x		x			x					x		x
PJ8				x			x		x	x		x		x	x		x		
PJ9				x	x		x		x			x			x		x		
PJ10				x	x		x		x	x		x			x			x	
SF1			x				x			x		x			x				
SF2		x			x		x	x		x		x		x		x			
SF3			x		x		x	x		x		x					x		
SF4			x				x	x		x		x			x		x		
SF5			x				x		x			x				x			
SF6																			
SF7							x		x	x		x				x			
SF8				x	x			x	x			x			x				
SF9				x		x		x	x			x		x		x			
SF10			x			x	x			x		x		x					

(table continues)

	OPA-10																			
	1631	1522	1449	1371	1286	1209	1123	1049	995	946	906	870	837	793	753	708	656	619	587	550	506
CP1								x		x			x			x		x		x	
CP2						x	x	x		x		x		x				x	x		
CP3							x							x			x			x	
CP4							x	x			x		x		x		x		x		
CP5								x							x		x			x	
CP6							x			x			x		x				x	x	
CP7									x		x		x		x			x		x	
CP8							x	x					x		x		x		x	x	
CP9								x	x					x						x	x
CP10							x	x	x						x		x		x		x
HP1							x	x	x		x			x		x		x			
HP2				x	x		x	x		x		x			x		x		x	x	
HP3		x		x	x		x	x	x			x			x		x		x	x	
HP4																					
HP5		x			x	x	x		x				x		x	x		x	x	x	
HP6							x									x				x	
HP7							x	x	x			x		x		x	x		x		
HP8							x	x		x				x		x		x			
HP9								x	x							x			x		
HP10			x		x		x	x		x			x	x		x	x		x		x
CH1							x				x			x	x		x		x		
CH2							x		x				x		x		x	x		x	x
CH3							x		x		x				x		x	x	x		x
CH4				x			x		x		x		x		x		x		x		x
CH5							x	x		x		x		x				x			
CH6	x					x	x	x		x		x		x		x			x		x
CH7					x		x	x		x		x	x		x		x		x		x
CH8				x		x	x	x		x			x	x	x		x			x	x
CH9				x		x		x		x				x			x		x		
CH10				x	x			x		x				x					x	x	x
SH1			x			x			x	x			x	x		x	x	x	x	x	x
SH2				x		x		x			x		x	x		x	x	x			x
SH3					x	x			x	x				x		x				x	x
SH4				x		x			x		x				x		x		x		x
SH5								x		x			x		x		x				x
SH6							x		x	x				x			x	x			x
SH7							x		x						x		x				
SH8									x		x				x	x		x			
SH9						x	x		x		x			x	x				x	x	x
SH10					x		x		x			x		x		x		x		x	x
PJ1							x		x	x					x		x				
PJ2							x		x				x		x	x					
PJ3								x		x			x	x		x		x	x		x
PJ4									x					x		x	x		x	x	
PJ5							x		x	x				x				x		x	
PJ6							x	x		x				x		x		x	x		
PJ7									x			x		x							
PJ8							x	x	x	x		x		x			x	x		x	x
PJ9					x		x	x	x		x	x		x			x	x	x		x
PJ10					x		x		x	x		x		x		x	x		x	x	x
SF1			x				x	x			x	x		x	x	x			x	x	
SF2		x		x		x		x			x			x			x			x	x
SF3	x			x				x		x					x		x	x	x		x
SF4				x		x	x		x			x		x		x				x	
SF5				x		x		x									x		x		x
SF6									x		x			x		x		x			x
SF7				x	x			x		x			x			x			x		x
SF8					x	x		x	x	x		x			x			x			
SF9									x		x				x	x		x	x		
SF10																					

(table continues)

	OPA-10		
	467	435	395
CP1			
CP2	x		
CP3	x		
CP4	x		
CP5		x	
CP6			
CP7			
CP8	x		
CP9			
CP10			
HP1	x		
HP2	x		
HP3	x		
HP4			
HP5			
HP6	x		
HP7			
HP8			
HP9			
HP10		x	
CH1	x	x	
CH2		x	
CH3	x		x
CH4	x		
CH5	x		
CH6			
CH7			x
CH8			x
CH9	x		x
CH10		x	
SH1	x		
SH2	x		x
SH3	x		
SH4		x	
SH5			
SH6			
SH7	x		
SH8			
SH9			
SH10	x	x	
PJ1	x		x
PJ2	x		x
PJ3		x	
PJ4			
PJ5			
PJ6			x
PJ7	x	x	x
PJ8	x	x	
PJ9	x		
PJ10	x		
SF1			
SF2	x		
SF3			
SF4			x
SF5			
SF6			
SF7		x	
SF8			
SF9	x		
SF10			

(table continues)

	OPA-12																			
	2163	1474	1417	1339	1295	1177	1135	1047	983	928	861	823	744	697	648	609	535	487	461	399	368
CP1			x		x	x			x	x						x		x			x
CP2			x		x	x			x			x	x			x		x	x	x	
CP3			x		x		x			x				x		x		x			
CP4									x			x				x		x			x
CP5							x		x						x	x		x		x	x
CP6																			x		
CP7																x		x			
CP8																x		x		x	
CP9										x			x			x		x			
CP10		x			x				x					x		x	x	x	x		
HP1		x				x						x	x	x				x		x	
HP2		x													x	x		x		x	
HP3		x			x				x	x			x		x		x	x		x	
HP4	x	x		x		x							x					x			
HP5																x		x		x	
HP6																			x		
HP7								x					x		x			x		x	
HP8																			x		
HP9																		x			
HP10																x		x		x	
CH1													x			x		x			
CH2			x		x		x									x	x	x			
CH3		x												x		x		x			
CH4						x										x		x		x	
CH5																x		x			
CH6																x		x			
CH7																		x			
CH8																x		x		x	
CH9						x										x		x		x	
CH10					x	x			x	x						x	x				
SH1							x								x			x			
SH2			x		x		x		x		x					x		x			
SH3			x		x		x	x			x			x		x		x		x	
SH4			x		x		x		x	x	x		x	x		x		x			
SH5			x			x															
SH6			x		x		x			x		x				x			x		
SH7							x												x		x
SH8					x				x				x						x		x
SH9			x		x	x													x		
SH10						x		x		x			x			x			x		
PJ1				x	x		x		x					x				x			x
PJ2																			x	x	
PJ3							x		x			x				x	x				
PJ4																				x	
PJ5																x					x
PJ6		x		x		x		x		x	x		x		x		x			x	
PJ7															x	x		x		x	
PJ8																x		x		x	
PJ9		x		x		x		x		x		x	x			x		x		x	
PJ10		x		x		x		x	x			x		x		x		x		x	
SF1																					
SF2																x		x			
SF3			x	x		x							x			x		x		x	
SF4					x															x	
SF5			x		x													x			x
SF6									x										x		
SF7														x				x		x	
SF8										x								x		x	
SF9																			x		
SF10		x		x																	

(table continues)

	OPA-12	
	329	235
CP1		x
CP2	x	
CP3		
CP4		
CP5		
CP6		
CP7		
CP8		
CP9		
CP10		
HP1		
HP2		
HP3		
HP4		
HP5		
HP6		
HP7		
HP8		
HP9		
HP10		
CH1		
CH2		
CH3		
CH4		
CH5		
CH6		
CH7		
CH8		
CH9		
CH10		
SH1		
SH2		
SH3		
SH4		
SH5		
SH6		
SH7		
SH8		
SH9		
SH10		
PJ1		
PJ2		
PJ3		
PJ4		
PJ5		
PJ6		
PJ7		
PJ8		
PJ9		
PJ10	x	
SF1		
SF2		
SF3		
SF4		
SF5		
SF6		
SF7		
SF8		
SF9		
SF10		

(table continues)

	OPA-19																
	1350	1243	1138	1040	998	935	889	849	796	729	671	627	575	547	475	452	380
CP1				x							x		x		x		x
CP2				x					x		x		x		x		
CP3					x						x		x		x		x
CP4												x	x		x		x
CP5							x						x		x		
CP6								x						x			
CP7				x				x		x	x	x		x		x	
CP8				x		x	x			x		x		x	x	x	
CP9					x							x		x		x	
CP10				x				x			x			x		x	
HP1					x		x		x	x		x		x		x	x
HP2				x		x		x				x		x		x	
HP3							x		x	x		x		x		x	
HP4												x					
HP5								x				x		x	x		
HP6														x			
HP7							x					x		x		x	
HP8														x		x	
HP9					x		x							x			
HP10				x			x				x	x		x		x	
CH1					x		x							x		x	x
CH2	x			x		x		x					x		x		x
CH3				x		x			x		x		x		x		
CH4						x		x					x		x		x
CH5						x							x		x		x
CH6							x						x				
CH7				x		x							x		x		
CH8					x		x						x		x		x
CH9						x					x		x		x		x
CH10													x				
SH1									x		x		x			x	
SH2							x		x	x		x		x		x	
SH3			x		x		x		x			x		x		x	
SH4			x							x	x			x		x	
SH5				x			x	x		x		x		x		x	
SH6					x		x		x		x	x		x		x	
SH7				x			x		x			x		x		x	
SH8					x		x		x			x		x		x	
SH9					x		x		x					x		x	
SH10							x			x		x		x		x	
PJ1		x		x	x		x		x	x		x		x		x	x
PJ2							x									x	
PJ3					x	x			x				x		x		
PJ4					x						x		x		x		x
PJ5							x						x		x		
PJ6				x		x		x			x		x		x		x
PJ7			x			x			x		x		x		x		x
PJ8													x		x		
PJ9			x	x		x			x		x		x		x		x
PJ10					x	x		x			x		x		x		x
SF1					x		x						x		x		x
SF2		x			x			x			x		x		x		x
SF3					x			x	x		x		x		x		
SF4			x		x		x			x	x		x		x		
SF5													x				
SF6								x	x	x			x		x		
SF7		x					x		x				x		x		
SF8													x		x		
SF9									x				x		x		
SF10					x		x		x				x		x		