

DISSERTATION

GENETIC VARIATION

AMONG INLAND AND COASTAL POPULATIONS

OF *DISTICHLIS SPICATA* SENSU LATO (POACEAE)

IN THE WESTERN UNITED STATES

Submitted by

Judith Eileen Harrington

Department of Soil and Crop Sciences

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

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Fort Collins, Colorado

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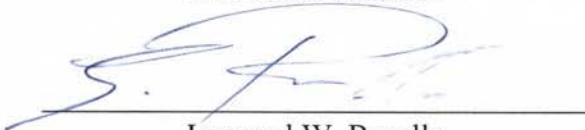
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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JUDITH EILEEN HARRINGTON ENTITLED GENETIC VARIATION AMONG INLAND AND COASTAL POPULATIONS OF *DISTICHLIS SPICATA* SENSU LATO (POACEAE) IN THE WESTERN UNITED STATES BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work



William C. Black IV



Leonard W. Panella



Sarah M. Ward



Advisor: Mark A. Brick



Department Head: Gary A. Peterson

ABSTRACT OF DISSERTATION

GENETIC VARIATION

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IN THE WESTERN UNITED STATES

The taxonomic status of the North American endemic grass *Distichlis spicata* subsp. *stricta* has been in flux for more than a century. *Distichlis spicata* hosts the larval stage of a federally endangered butterfly and is being investigated for use in restoration and recreation, so the relationship between the species and its subspecies merits clarification. Although the subspecies *stricta* was once recognized as a species (*Distichlis stricta*), most current treatments either consider it an inland subspecies within *Distichlis spicata* or decline to recognize it at all. Two recent studies did not find genetic or morphological evidence differentiating the subspecies *stricta* from *Distichlis spicata*. Genetic variation among 13 coastal and inland populations of *Distichlis spicata* sensu lato was characterized using chromosome counts, chloroplast DNA segments, microsatellite alleles, RAPD bands, and DNA C-values. Plants grown in a common garden were evaluated for date of first flowering. The results suggest the existence of two

genetically segregated lineages that differ for chromosome number, molecular sequences in cpDNA and nuclear DNA, DNA C-value, and flowering time. One lineage has a somatic chromosome number of $2n = 40$ and encompasses plants from the West Coast and several inland locations in Nevada, Utah, and southern New Mexico. The other lineage has a somatic chromosome number of $2n = 38$ and consists of plants distributed only inland among the populations surveyed. Genetic distances among populations were closer within each lineage than between the two lineages, even when different lineages occurred in geographic proximity. The 38-chromosome lineage should be recognized as a distinct species corresponding to the previously recognized *Distichlis stricta*. The 40-chromosome lineage is *Distichlis spicata*.

Judith Eileen Harrington
Department of Soil and Crop Sciences
Colorado State University
Fort Collins, Colorado 80523
Fall 2010

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I thank my advisor, Dr. Mark Brick, for being willing to take on a non-traditional student with a full-time job who wanted to do a crazy, self-financed project in an obscure wild species. Dr. Brick's broad spectrum of interests and his genuine desire to help students with statistical problems were much appreciated, as was his guidance through the treacherous waters of graduate school. Dr. William Black IV was always available, friendly, and helpful. He offered good suggestions, guidance, and encouragement, and he embodied what an academic researcher should be – widely-informed, analytical, sensible, and modest. Dr. Black is made of awesome. I thank Dr. Lee Panella for his good advice on the manuscript and for encouraging Scott and me to collect *Distichlis*. Without his backing we would not have added the Honey Lake and Goose Lake locations to the project. To all involved, my sincere thanks.

DEDICATION

To my dear mother

She bore her misfortunes in private and expressed in public her appreciation for her blessings. Her best face and her greatest reserves of patience, politeness, generosity, and service were reserved for those she loved most. In keeping with her values, after retirement she volunteered in the public schools, teaching disadvantaged children to read, write, and do math. Her name is not preserved on library shelves or public monuments, but her influence is profound.

Rest in peace

Yvonne Annie Harrington (née Robinson)

26 August 1921 – 6 August 2010

and

To my beloved Jeune Fils

He lives forever in my memory. Time cannot diminish the pain of his loss.

Speak to me through the wind, little brush-tail.

5 June 1977 – 5 September 1995

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Chapter 1: Introduction

Morphological and molecular characterization of plant taxa can provide information to plant taxonomists, plant breeders, and conservationists about the scope of variation and the possible phylogenetic relationships of taxa that are of interest for economic or ecological reasons. Taxonomists use such information in making decisions about correct hierarchical placement. Breeders consider the potential for developing improved varieties by selection from natural variation and by sexual transference of desirable traits. Conservationists evaluate the level of diversity when deciding where to direct limited resources for conservation. Morphological variation has been the most accessible evidence until recent decades and remains the principal evidence upon which many plant taxonomic decisions are based. However molecular methods are being increasingly employed to elucidate relationships that have not received satisfactory resolution over decades of investigation by morphological means.

One such relationship involves the C₄ grass *Distichlis spicata* (L.) Greene and *D. spicata* subsp. *stricta* (Torr.) Thorne (Thorne, 1978) which has been classified previously as a separate species, *D. stricta* (Torr.) Rydberg (Rydberg, 1905) and as a variety (Beetle, 1943, 1955) of *D. spicata*. The species/subspecies/variety *stricta* usually is described as occurring inland and is given the common name inland or desert saltgrass in contrast to the coastal or seashore saltgrass that occurs along coasts in North and South America. Clayton et al. (2006) did not recognize the *stricta* taxon at any level. Barkworth (2003)

declined to recognize it, but later (Barkworth, 2010) amended an electronic version of her grass manual to accept the taxon *stricta* as a subspecies of *D. spicata* based on preliminary research results (Harrington et al., 2009). Questions remain about the ability of the current classifications to interpret the variation in *D. spicata* observed over western North America.

The terms “subspecies” and “variety” in botany have been used ambiguously, inconsistently, and confusingly for 200 years (Benson, 1962; Stuessy, 2009). A subspecies is often understood as an intermediate rank between a species and a variety. However different botanical factions favor the use of one term or the other. No attempt will be made here to address the dispute or to regularize the usage of the two terms. Recognition as either a subspecies or a variety is recognition of differences at a subspecific level, in contrast to no recognition of differences and to recognition of differences at the species level.

In this dissertation, the term *D. spicata* sensu lato will be used to indicate the group comprising all plants classified as *D. spicata* plus all plants classified in the taxon *stricta* regardless of the rank at which the taxon is recognized. When research by others is described, the names *D. spicata* and *D. stricta* follow the usage in the research report.

This study characterized morphological, cytological, molecular, and phenological variation in western United States collections of *D. spicata* sensu lato in order to determine whether evidence exists to support the classification of *D. stricta* as a distinct species.

Uses for Distichlis

The genus *Distichlis* is known by the common name ‘saltgrass’ because it has the ability to tolerate high soil salinity. Because *D. spicata* tolerates soil salinity, it has been investigated for potential applications in salt-affected soils and under saline irrigation conditions. Potential uses include revegetation of saline soils, forage production in saline soils, and turf that can be irrigated with saline water.

The salt tolerance of saltgrass is of interest because salt-affected soils are a world-wide problem. Salt impacts approximately 955 million hectares worldwide, or 7.3% of total land area, of which nearly 16 million hectares are located in the United States (Szabolcs, 1989; Ghassemi et al., 1995). In addition, saline water is used for irrigation in areas where high-quality water is unavailable or where its use is legally restricted. For example, some areas of the United States restrict the use of potable water for irrigating turfgrass (California State Water Resources Control Board, 1993; Arizona Department of Water Resources, 1995, 2003; Southern Nevada Water Authority, 2003). Under these circumstances, often saline sources of water must be used (Marcum, 1999).

Salinity tolerance

Marcum (1999) evaluated salinity tolerance of *D. spicata* in interspecies and intraspecies trials. In the interspecies trials, salinity tolerance was measured in seven Chloridoid grasses, sideoats grama [*Bouteloua curtipendula* (Michx.) Torr.], buffalograss [*Buchloë dactyloides* (Nutt.) Engelm.], Bermuda grass [*Cynodon dactylon* (L.) Pers.], Zoysia grass (*Zoysia japonica* Steud.), saltgrass (*D. spicata*), alkali sacaton [*Sporobolus airoides* (Torr.) Torr.], and sand dropseed [*S. cryptandrus* (Torr.) A. Gray]. The traits

measured were leaf firing (chlorotic area due to salt damage), root length, root dry weight, concentration of glycinebetaine in leaves, levels of Na^+ and Cl^- in leaves, and secretion of Na^+ and Cl^- from leaves. Plants were grown in hydroponic solution in which the NaCl concentration of the solution was raised over time from an initial level of 50 mM to a concentration of 600 mM NaCl. Species that tolerated high salinity exhibited less than 5% leaf firing while salt-sensitive species had up to 100% leaf firing. Salt-tolerant species had longer root length in salt solution than the low-salt solution while salt-sensitive species had shorter roots in salt solution than in the low-salt solution. Salt-tolerant species had less than 250 mM Na^+ and Cl^- in leaf sap, in contrast to 1350 mM Na^+ and 3000 mM Cl^- in leaf sap of salt-sensitive species. Salt-tolerant species excreted more than 10 mg of Na^+ and Cl^- g^{-1} leaf dry matter (DM) week^{-1} from leaves, compared to 1 mg g^{-1} leaf DM week^{-1} for salt-sensitive species. Glycinebetaine, a chemical constituent believed to contribute to osmotic adjustment of cells under salt stress, was present in concentrations up to 60 mM in leaves of salt-tolerant species in salt solution, but less than 20 mM in leaves of salt-sensitive species. *Distichlis spicata* was the most salt-tolerant of the species studied. In the intraspecies trials, Marcum et al. (2007) compared an accession of *D. spicata* collected at a marine estuary near San Diego, California, six *D. spicata* accessions collected from inland areas of the United States, and the *D. spicata* cultivar ‘NyPa Turf’ (United States Patent and Trademark Office, 1994). Plants were grown in hydroponic solution with five levels of NaCl ranging in electrical conductivity from 0 to 88 deciSiemens (dS) m^{-1} . Plants were evaluated for percent green leaf area, rooting depth, dry weight of roots and shoots, excretion of Na^+ and K^+ from leaves, and concentration of Na^+ and Cl^- in leaf sap. The San Diego accession was superior to the others for most traits

under salt stress. In the highest salinity treatment, the San Diego accession had 84% green leaf area, roots twice as long, shoot dry weight of 52%, and root dry weight of 137% compared to the no-salt solution. It also had the lowest concentrations of sodium and chloride ions in leaf sap. The San Diego accession excreted more Na^+ than the other accessions at a salinity level of 44 dS m^{-1} .

Enberg and Wu (1995) grew inland and coastal plants in sand irrigated with 17.8 and 36.8 dS m^{-1} solutions of either NaCl or Na_2SO_4 . Both coastal and inland plants showed reduced dry weight accumulation in salt solution compared to plants grown in no-salt solutions. Coastal plants showed significantly less reduction in growth than inland plants at $36.8 \text{ dS m}^{-1} \text{ NaCl}$ and at $17.8 \text{ dS m}^{-1} \text{ Na}_2\text{SO}_4$. Differences in growth reduction at $17.8 \text{ dS m}^{-1} \text{ NaCl}$ and $36.8 \text{ dS m}^{-1} \text{ Na}_2\text{SO}_4$ were not significant because of large variability in the coastal plants. Wrona and Epstein (1982) found that plants collected at a coastal location near Bodega, California, tolerated higher salinity than plants collected at the inland location near Putah, California. They grew plants in solutions of fresh water and in water adjusted to 0.8 and 2.0 times the salinity of seawater. (Seawater has approximately 35 grams of various salts per liter.) Sodium ions and chloride ions accumulated in the roots of inland plants grown at salinity twice that of seawater and inland plants showed more salt damage in their aerial parts. The species commonly called saltgrass shows high tolerance to salt in controlled experiments. Salt tolerance appears to vary within the species, with coastal accessions often rated as more salt-tolerant than inland accessions.

Revegetation and ground cover

Salt-tolerant species may form an important part of a reclamation strategy for a site with saline soil that has been invaded by non-native species. *Distichlis spicata* has been proposed as a revegetation species in New Mexico on xeric lands cleared of saltcedar (*Tamarix* spp.) infestations (Lair and Wynn, 2002; Taylor and McDaniel, 2004). Saltcedar can re-establish and other exotic species can invade if cleared sites are revegetated with species incapable of thriving in adverse conditions such as high soil salinity. *Distichlis* spp. has also been used in wetland-restoration efforts (Howard, 2003).

Because *Distichlis* has high salt tolerance, it is used as a ground cover in areas with saline soils. Mota Urbina (1979) tested *D. spicata* as a ground cover to control dust arising from the former Lake Texcoco near Mexico City where drainage projects resulted in soils devoid of vegetation and high in salt content. Plants grown for five months in pots of Lake Texcoco soil were irrigated with water at electrical conductivities from 30 dS m⁻¹ to 150 dS m⁻¹. Number and height of shoots decreased as salinity increased. Plants survived at salinity levels of 30, 50 and 70 dS m⁻¹ but died at salinity levels of 90, 110, 130 and 150 dS m⁻¹.

Westover (1928) proposed *Distichlis* for use as golf course turfgrass along the coasts and in alkaline areas of the western United States. Interest in *Distichlis* as a turfgrass has been recently revived because of pressure to reduce the use of limited water resources on golf courses and other landscape areas (Marcum et al., 2007). Kopec and Marcum (2001) conducted field evaluations in Tucson, Arizona, of 21 accessions of *Distichlis* sp. for growth habit and maintenance of green color under conditions of frequent mowing and infrequent irrigation with saline water. They identified five

accessions that had good green color and high shoot density at 38°C while receiving only two irrigations in late spring. Fraser and Anderson (1980) compared twenty accessions of *D. stricta* with cultivars of western wheatgrass (*Agropyron smithii* Rydb.), yellow bluestem (*Bothriochloa ischaemum* Keng.), blue grama [*Bouteloua gracilis* (Willd. ex H.B.K.) Lag. ex Griffiths], and buffalograss for tolerance to traffic and for growth under limited irrigation. Traffic was applied by driving a vehicle over plots of each species. Levels of traffic were none, moderate, and severe. One set of plots was irrigated when it showed water stress and the other set of plots was watered approximately half as often to provide two levels of irrigation. All species exhibited reductions in percentage cover as irrigation decreased and traffic increased. During three years of trials, the best accession of *D. stricta* under adequate irrigation maintained 80% cover with no traffic and 50% cover with severe traffic. The same accession under inadequate irrigation maintained 70% cover with no traffic and 40% cover with severe traffic. These reports suggest that *D. stricta* can maintain ground cover under low soil moisture and high traffic. These characteristics are valuable traits to sustain ground cover and reduce water use in high-traffic areas of golf courses.

Forage production in saline soils

Forage crops grown under saline conditions must maintain high DM production. In order to explore the economic feasibility of seawater agriculture, Glenn and O'Leary (1985) evaluated the DM productivity of *D. palmeri* (Vasey) Fassett and eight other salt-tolerant species, including five species or subspecies of *Atriplex*, irrigated with seawater at Puerto Peñasco, Mexico. The DM yield of *D. palmeri* was 1.364 kg m⁻² yr⁻¹ for one

cutting, intermediate between *Atriplex lentiformis* (Torr.) S. Wats. ($1.794 \text{ kg m}^{-2} \text{ yr}^{-1}$, two cuttings) and *Atriplex canescens* subsp. *canescens* (Pursh) Nutt. ($0.303 \text{ kg m}^{-2} \text{ yr}^{-1}$, two cuttings). Pasternak et al. (1993) evaluated *D. spicata*, Rhodes grass (*Chloris gayana* Kunth), Bermuda grass, Kallar grass [*Leptochloa fusca* (L.) Kunth], seashore paspalum (*Paspalum vaginatum* Swartz), and alfalfa (*Medicago sativa* L.) for DM production. Plants were irrigated with fresh water at 1.2 dS m^{-1} or saline water at 9.5 dS m^{-1} at the Negev Experimental Station for Desert Agriculture in Israel. Levels of irrigation were similar but not identical for the two salinity levels. Fresh water was applied at $6,000 \text{ m}^3 \text{ ha}^{-1}$, $8,500 \text{ m}^3 \text{ ha}^{-1}$, $12,000 \text{ m}^3 \text{ ha}^{-1}$, and $18,000 \text{ m}^3 \text{ ha}^{-1}$. Saline water was applied at $4,500 \text{ m}^3 \text{ ha}^{-1}$, $7,500 \text{ m}^3 \text{ ha}^{-1}$, $11,000 \text{ m}^3 \text{ ha}^{-1}$, and $18,000 \text{ m}^3 \text{ ha}^{-1}$. Under saline irrigation, *D. spicata* had DM yield that ranged from 1.5 to 3.75 kg m^{-2} , while the other forage yields ranged from 0.25 to 3.5 kg m^{-2} . Under fresh water irrigation, *D. spicata* had intermediate DM yield compared to the other forage grasses. At the low irrigation level of fresh water, alfalfa DM yield was 1 kg m^{-2} followed by *D. spicata* (0.75 kg m^{-2}). At the high irrigation level of fresh water, Rhodes grass 5 kg m^{-2} DM yield followed by *D. spicata* (3 kg m^{-2}) and alfalfa (2.5 kg m^{-2}). These results indicated that *D. spicata* can be a productive forage grass under saline irrigation.

A forage species must also be palatable and nutritious. The palatability of *Distichlis* has been evaluated in Argentina, where South American species of *Distichlis* are common in the lowland plains. Brizuela et al. (1990) studied the diet of grazing steers in Buenos Aires Province by sampling intake through an opening in the esophagus of each animal. The natural vegetation of the study area was not reported in Brizuela et al. (1990), but a later report (Hidalgo et al., 2002) gives the principal forage plants in the

region as beardgrass, *Bothriochloa laguroides* (D.C.) Herter, sedge, *Carex phalaroides* Kunth., rush, *Juncus imbricatus* Laharpe, saltgrass, *D. spicata* (L.) Greene, and a relative of barley, *Hordeum stenostachys* Godron. Brizuela et al. (1990) found that *D. spicata* and *D. scoparia*, two species naturally present in the lowland pampas of Argentina, constituted from 8% to 24% of the dry weight intake by grazing cattle during the summer months, when these species compose a significant portion of the pasture. Cattle did not consume *Distichlis* species during spring, fall or winter months. Brizuela et al. (1990) concluded that *Distichlis* species were valuable summer forage on native range. However Lencoff et al. (1977) and Cauh  p   and Fern  ndez Grecco (1981) reported that *Distichlis* species were seldom consumed or were rejected by cattle when encountered. Hidalgo et al. (1998) evaluated the digestibility and crude protein content of *D. spicata*, *D. scoparia*, and other species commonly found in the lowland pampas of Argentina. Warm-season grasses included in the study were *Bothriochloa laguroides* (D.C.) Herter, *Paspalum dilatatum* Poiret, and *Sporobolus indicus* (L.) R. Brown. Cool-season grasses were *Lolium multiflorum* Lamarck and *Stipa formicarum* Delile. Dry matter digestibility (DMD) of leaves at flowering was 51 to 70% in other grasses but only 37% in *Distichlis*. Early in the grazing season the crude protein (CP) content of *Distichlis* leaves was 12.6% compared to 12.1 to 18.8% for other grasses. During flowering, the CP content of *Distichlis* leaves was lower (9.6%) than that of cool-season grasses (13.9 to 17.3%) but similar to other warm-season grasses (8.1 to 11.8%). Overall, *Distichlis* had low DMD and CP compared to other grasses commonly found in the lowland pampas. Bustan et al. (2005) evaluated forage quality in 48 *D. spicata* samples. Coastal genotypes were collected in Connecticut, Delaware, Georgia, and Alabama. Inland genotypes were

collected in Utah, Chile, and Argentina. Samples from California included both coastal and inland types. Coastal accessions had higher leafiness (a composite score of visual ratings for four traits) than inland accessions (7.32 versus 6.40), while inland accessions had higher CP than coastal accessions (12.7 versus 11%). DM production, ash, fiber, metabolizable energy, and organic matter digestibility were not different between coastal and inland accessions. These reports suggest that *Distichlis* is lower in quality than some common forage species.

Ecological importance of Distichlis

Species in the genus *Distichlis* are found in salt marshes and salt flats of coastal and inland alkaline and saline areas (Quattrochi, 2006; Shadow, 2007). *Distichlis* species are an important component of many salt marsh ecosystems (Seliskar and Gallagher, 2000). For example, *D. spicata* is the second most common plant species in saline marshes in coastal Louisiana, constituting 14% of the cover (Chabreck, 1972). In North America, *D. spicata* is an important colonizer in marshes recovering from physical disturbance (Bertness, 1991; Allison, 1995, 1996). It has been used as a pioneering species for restoration of coastal wetlands (Shadow, 2007). *Distichlis spicata* provides food and shelter for wild animals such as insects, birds, and small mammals (Bertness et al., 1987; Barnett and Crewz, 1990; Thorn and Zwank, 1993; Weller, 1994). Three endangered animal species in the United States are known to rely on *Distichlis* species. The endangered Florida salt marsh vole (*Microtus pennsylvanicus dukecampbelli* Woods, Post and Kilpatrick) depends almost exclusively on *D. spicata* for habitat (Woods et al., 1982; Woods, 1992; United States Fish and Wildlife Service, 1997). The endangered salt

marsh harvest mouse (*Reithrodontomys raviventris* Dixon) in northern California uses *D. spicata* for shelter (United States Fish and Wildlife Service, 2010a, 2010b). The endangered Carson wandering skipper (*Pseudocopaeodes eunus obscurus* Austin and Emmel) lays its eggs exclusively on plants of *D. spicata* sensu lato and the larvae feed on these plants (Austin and Emmel, 1998; Sanford, 2006).

Taxonomy, distribution, and morphology of the genus Distichlis

The genus *Distichlis* is a member of the family Poaceae (the grasses), subfamily Chloridoideae (C₄ grasses of warm, semi-arid regions such as prairies), tribe Eragrostideae, subtribe Monanthochloinae. Within the subtribe Monanthochloinae, Clayton and Renvoize (1992) recognized seven genera: *Aeluropus* Trin., *Swallenia* Soderst. & Decker, *Distichlis* Raf., *Reederochloa* Soderst. & Decker, *Allolepis* Soderst. & Decker, *Monanthochloë* Engelm., and *Jouvea* Fourn. Peterson et al. (2007) listed the placement of *Allolepis*, *Jouvea*, and *Swallenia* as uncertain, potentially reducing the number of genera in the subtribe to four. Bell and Columbus (2008) proposed renaming *Reederochloa* and *Monanthochloë* as members of the genus *Distichlis* based on molecular and morphological analysis. Such renaming would reduce the number of genera in the subtribe Monanthochloinae by two without removing any species. Peterson et al. (2010) listed *Aeluropus* in a separate subtribe, the Aeluropodinae, further reducing the number to one genus in the subtribe Monanthochloinae if all of these proposals for reclassification are accepted. Whether broadly or narrowly interpreted, the Monanthochloinae occur in xeric and/or salty and alkaline habitats and have distichous (two-ranked, occurring on opposite sides of the culm) leaves, vigorous rhizomes and/or

stolons, and seven to eleven nerves (prominent veins or ribs) on a thick-textured lemma (the lower bract surrounding the grass floret) (Clayton and Renvoize, 1986; Watson and Dallwitz, 1994, Peterson et al., 1997). Most members of the subtribe Monanthochloinae are endemic to the New World. However, the genus *Aeluropus* is found in Europe and Asia, and the species *D. distichophylla* (Labill.) Fassett, which is recognized by some but not all taxonomists, occurs only in Australia. The genera *Distichlis*, *Reederochloa*, *Allolepis*, *Monanthochloë* and *Jouvea* are dioecious, while *Aeluropus* and *Swallenia* have hermaphroditic flowers.

The genus *Distichlis* is often considered to be most closely related to the genera *Monanthochloë* and *Reederochloa*. A report of a putative hybrid between members of the genera *Distichlis* and *Monanthochloë* (Stephensen, 1971) suggests that these two genera may be able to hybridize in the wild. Bell and Columbus (2008) examined phylogenetic relationships in the subfamily Chloridoideae using a nuclear DNA sequence comprising Internal Transcribed Spacer 1, the 5.8 S gene, and Internal Transcribed Spacer 2 of ribosomal DNA, and also two chloroplast DNA sequences, the *trnL-F* and the *ndhF* regions. Bell and Columbus (2008) found that the genera *Distichlis*, *Monanthochloë*, and *Reederochloa* were strongly supported as a monophyletic group, that is, a group containing all of the descendants and only the descendants of a single ancestor in the evolutionary history of the subfamily Chloridoideae.

The genus *Distichlis* includes species distributed over the New World and one species in Australia. The currently accepted species number varies from six to ten, depending on the botanical authority, with an additional twelve infraspecific taxa. Species names with naming authorities, infraspecific taxa if they exist, and countries

from which specimens have been recorded are given in Table 1-1. Among several commonly cited authorities in the field of grass taxonomy who have classified *Distichlis* are Peterson (2000), who wrote the *Distichlis* section for the Catalogue of New World Grasses; Clayton et al. (2006), who developed a flora for the Royal Botanic Garden; the USDA-ARS National Genetic Resources Program (1994), which developed the Germplasm Resources Information Network; and Quattrocchi (2006), who edited the CRC World Dictionary of Grasses. The species *D. australis*, *D. distichophylla*, *D. humilis*, *D. laxiflora*, *D. palmeri*, *D. scoparia*, and *D. spicata* have been accepted by two or more of these four authorities. The species *D. ammobia*, *D. dentata*, and *D. hirsuta* are accepted by only one authority. *Distichlis scoparia* and *D. spicata* have subspecific taxa that are accepted by some authorities but not all.

D. spicata sensu lato has the widest distribution, found throughout North America and the western half of South America. *Distichlis ammobia*, *D. australis*, *D. hirsuta*, *D. humilis*, *D. laxiflora*, and *D. scoparia* are found in the southern cone of South America. *Distichlis palmeri* is found only in Mexico, while *D. distichophylla* is found only in Australia and *D. dentata* is found only in the United States. Only *D. spicata* sensu lato has been evaluated at a molecular level (Ram et al., 2004; Bell and Columbus, 2008).

The distributions of four *Distichlis* species in South America are shown in Figure 1-1. These maps were generated by the Missouri Botanical Garden's Tropicos web site (Missouri Botanical Garden, 2008) using species accepted by Clayton et al. (2006) and specimens for which latitude and longitude data are available. In many cases, the information for a specimen does not include geospatial data; therefore many specimens are not represented on the maps and the extent of overlap of the South American species

Table 1-1. Recognized species of the genus *Distichlis*, with infraspecific taxa, accepting authorities, and distributions.

Species name and authority	Infraspecific taxa	Accepting authorities				Distribution
		CNWG ¹	RBG ²	GRIN ³	CRC ⁴	
<i>Distichlis ammobia</i> Phil.	None				√	Chile
<i>Distichlis australis</i> (Speg.) Villamil		√	√		√	Argentina
<i>Distichlis dentata</i> Rydb.					√	USA
<i>Distichlis distichophylla</i> (Labill.) Fassett			√		√	Australia
<i>Distichlis hirsuta</i> Phil.					√	Chile
<i>Distichlis humilis</i> Phil.		√	√		√	Argentina, Bolivia, Chile, Peru
<i>Distichlis laxiflora</i> Hack.		√	√		√	Argentina
<i>Distichlis palmeri</i> (Vasey) Fassett ⁵		√	√	√	√	Mexico
<i>Distichlis scoparia</i> (Nees ex Kunth) Arechav. ⁶		√	√	√	√	Argentina, Chile, Uruguay
	var. <i>scoparia</i> ⁷	√			√	Argentina, Chile, Uruguay
	var. <i>erinacea</i> (Beetle) Nicora ⁸	√			√	Argentina

¹CNWG -- Catalogue of New World Grasses, Soreng et al. (2000)

²RBG -- Royal Botanic Gardens, Kew, GrassBase, Clayton et al. (2006)

³GRIN -- USDA-ARS Germplasm Resources Information Network (USDA-ARS National Genetic Resources Program (1994)

⁴CRC -- CRC World Dictionary of Grasses, Quattrocchi (2006)

⁵GRIN, CRC add: ex I.M. Johnst.

⁶GRIN and CRC list authority as (Kunth) Arechav.

⁷CRC lists authority as (Kunth) Arechav.

⁸CRC adds (Kunth) Arechav before the variety name

Table 1-1 cont.

Species name and authority	Intraspecific taxa	Accepting authorities				Distribution
		CNWG ¹	RBG ²	GRIN ³	CRC ⁴	
<i>Distichlis spicata</i> (L.) Greene		√	√	√	√	Canada, USA, Belize, Guatemala, Mexico, Argentina, Bolivia, Chile, Ecuador, Peru, Venezuela, Bahama Archipelago, Cuba, Dominican Republic, Virgin Islands, Australia, Caribbean, Cayman Islands, Cuba, Uruguay
	var. <i>andina</i> Beetle	√			√	Bolivia
	var. <i>borealis</i> (J. Presl) Beetle	√			√	USA: Oregon, Washington
	var. <i>divaricata</i> Beetle	√			√	USA: California; Belize, Mexico, Bahama Archipelago
	var. <i>mendocina</i> (Phil.) Hack.	√			√	Argentina, Chile, Uruguay
	var. <i>mexicana</i> Beetle	√			√	Mexico
	var. <i>nana</i> Beetle	√			√	USA: California
	subsp. <i>spicata</i>	√			√	Bolivia, Canada, Caribbean, Chile, Mexico, USA, Uruguay
	var. <i>spicata</i>	√		√	√	USA: Missouri; Mexico, Argentina, Chile
	var. <i>stolonifera</i> Beetle	√			√	USA: California, Oregon
	subsp. <i>stricta</i> (Torr.) Thorne	√				USA: Oklahoma
	var. <i>stricta</i> (Torr.) Scribn.			√	√	

Figure 1-1. South American distributions of *Distichlis humilis* (A), *Distichlis laxiflora* (B), *Distichlis scoparia* (C), and *Distichlis spicata* (D).

A. *D. humilis*



B. *D. laxiflora*



C. *D. scoparia*



D. *D. spicata*



cannot be fully evaluated. As shown by the maps, the southern cone of South America has the greatest diversity of species. *Distichlis spicata* sensu lato, the most widely distributed species, occurs there along with *D. scoparia*, *D. laxiflora*, and *D. humilis*. These four species have overlapping geographic distributions within Argentina. *Distichlis spicata* has been reported in Argentina, Bolivia, Chile, Peru, Uruguay, and Venezuela. *Distichlis scoparia* has been reported in Argentina, Chile, and Uruguay while *D. laxiflora* has been reported only in Argentina. *Distichlis humilis* has been reported in Argentina, Bolivia, Chile, and Peru. Geospatial data are lacking for *D. australis* so it is not represented in Figure 1-1, although it has been reported to occur in Argentina. *Distichlis ammobia* and *D. hirsuta* have been reported in Chile but are not shown in Figure 1-1 because they are not accepted by the botanical authority (Clayton et al., 2006) followed by the developer of the Tropicos web site (Missouri Botanical Garden, 2008).

The distributions of *Distichlis* species in Mexico and Central America are shown in Figure 1-2. These maps were generated by the Missouri Botanical Garden's Tropicos web site (Missouri Botanical Garden, 2008) using specimens for which latitude and longitude data are available. In many cases, the information for a specimen does not include geospatial data; therefore many specimens are not represented on the maps. *Distichlis spicata* sensu lato occurs throughout Mexico, Belize, Guatemala, and Ecuador. *Distichlis palmeri* occurs in northwestern Mexico.

The distribution of *D. spicata* sensu lato in North America and the Caribbean is shown in Figure 1-3. These maps were generated by the Missouri Botanical Garden's Tropicos web site (Missouri Botanical Garden, 2008) using species accepted by Clayton

Figure 1-2. Mexican and Central American distributions of *Distichlis palmeri* (A) and *Distichlis spicata* (B).

A. *D. palmeri*

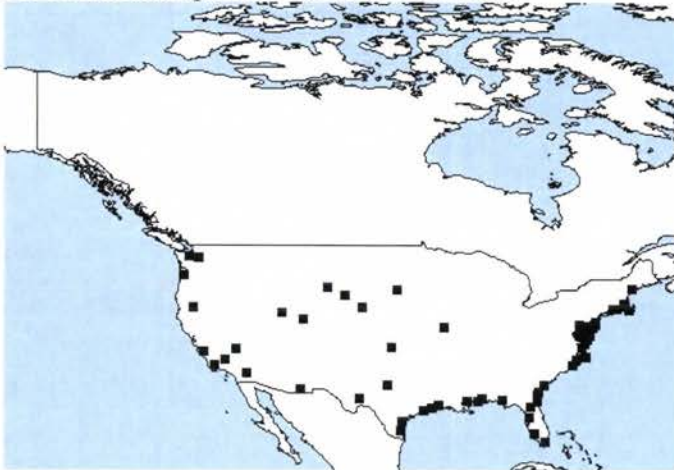


B. *D. spicata*



Figure 1-3. Distribution of *Distichlis spicata* in North America (A) and the Caribbean (B).

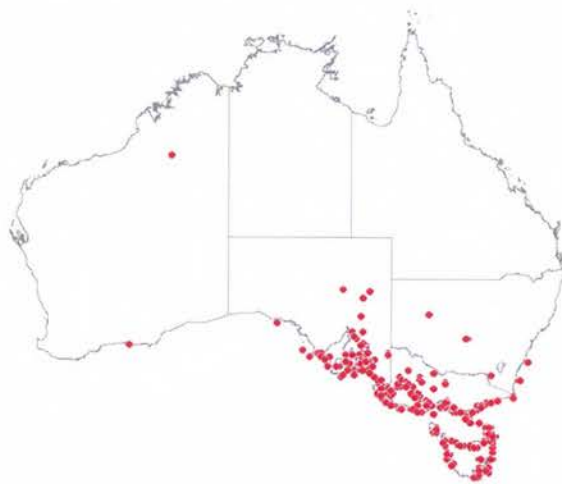
A. *Distichlis spicata* in North America



B. *Distichlis spicata* in the Caribbean



Figure 1-4. Distribution of *Distichlis distichophylla* in Australia



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et al. (2006) and specimens for which latitude and longitude data are available. In many cases, the information for a specimen does not include geospatial data; therefore many specimens are not represented on the maps. *Distichlis dentata* has been reported in the United States but is not shown in Figure 1-3 because it is not accepted by the botanical authority (Clayton et al., 2006) followed by the developer of the Tropicos web site (Missouri Botanical Garden, 2008). *Distichlis spicata* sensu lato occurs in Canada, United States, Bahama Archipelago, Cuba, Dominican Republic, Virgin Islands, and the Cayman Islands.

The distribution of *D. distichophylla* is shown in Figure 1-4. This map was generated using the web site Australia's Virtual Herbarium (Council of Heads of Australasian Herbaria, 2009). Specimen data is reproduced with permission of the Council of Heads of Australasian Herbaria Inc. *Distichlis distichophylla* is restricted to Australia and is the only Old World member of the genus. It is not recognized by all authorities as a separate species, being considered by Peterson (2000) as *D. spicata*.

In summary, the genus consists minimally of one species distributed throughout the New World, four species endemic to the southern cone of South America, and one species endemic to Mexico. There are four disputed species: two endemic to South America, one endemic to the United States, and one endemic to Australia.

Clayton et al. (2006) listed morphological traits for seven species of *Distichlis* (Table 1-2). The traits included growth habit, inflorescence, fertile spikelets, glumes, male florets, and female florets. After an identification was narrowed to *Distichlis*, further identification was based on the presence or absence of rhizomes, the form of the

Table 1-2. Selected morphological traits of *Distichlis* species, from Clayton et al., 2006.

species	habit	inflorescence	fertile spikelets	glumes	male florets	female florets
<i>Distichlis australis</i>	Rhizomes elongated. Culms 2-5 cm. Ligule a ciliolate membrane. Leaf blades conduplicate, 0.1-0.5 cm long, 0.5-1 mm wide.	A few spikelets.	Spikelets having 1-2 fertile florets, spikelets elliptic, 5 mm long.	Lower glume lanceolate, 3.5-4 mm long, 1-keeled, 3-veined, apex obtuse. Upper glume ovate, 3.5-4 mm long, 1-keeled, 5-7-veined, apex obtuse.	Anthers 1.8 mm long, lemma 7-9-veined.	Lemma 3.5-4 mm long, 5-11-veined. Palea keels winged. Spikelets 5 mm long with 1-2 fertile florets.
<i>Distichlis distichophylla</i>	Rhizomes elongated. Culms 20-30 cm. Ligule a fringe of hairs. Leaf blades flat or involute, 2-4 mm wide.	Two to 10 racemes closely spaced along a central axis, in a head, racemes 1-2 cm long, central axis 2.5-5 cm long.	Spikelets having 6-14 fertile florets, spikelets ovate, 10-20 mm long, 3-5 mm wide.	Lower glume ovate, 3-4.5 mm long, 1-keeled, 3-5-veined, apex acute. Upper glume ovate, 3.5-5 mm long, 1-keeled, 7-9-veined, apex acute.	Anthers 2.5-3 mm long.	Lemma 3-6.5 mm long, 12-veined. Spikelets 10-20 mm long with 6-14 fertile florets.
<i>Distichlis humilis</i>	Rhizomes elongated. Culms 2-5 cm. Ligule a ciliolate membrane. Leaf blades conduplicate, 7-15 cm long, 1 mm wide.	A few solitary spikelets or one raceme 1-1.5 cm long.	Spikelets having 5-7 fertile florets, spikelets ovate, 7-9 mm long.	Lower glume ovate, 3 mm long, 1-keeled, 5-veined, apex acute. Upper glume ovate, 3-4 mm long, 1-keeled, 5-veined, apex acute.	Spikelets 6-18 mm long.	Lemma 3-4 mm long, 9-veined. Palea keels winged. Spikelets 7-9 mm long with 5-7 fertile florets.
<i>Distichlis laxiflora</i>	Rhizomes absent. Culms 20-30 cm. Ligule a ciliolate membrane. Leaf blades convolute, 6-11 cm long, 1-2 mm wide.	A few racemes closely spaced along a central axis, with 9-25 fertile spikelets, racemes 1-3 cm long, central axis 3-7 cm long.	Spikelets having 5 fertile florets, spikelets oblong, 8-9 mm long, 2 mm wide.	Lower glume lanceolate, 3-4 mm long, 1-keeled, 3-5-veined, apex acute. Upper glume ovate, 3-4 mm long, 1-keeled, 3-5-veined, apex acute.	Anthers 2-2.5 mm long, lemma 4 mm long, 9-veined. Spikelets 9-10 mm long with 5-7 florets.	Lemma 5 mm long, 11-veined. Palea keels winged. Spikelets 8-9 mm long with 5 fertile florets.

species	habit	inflorescence	fertile spikelets	glumes	male florets	female florets
<i>Distichlis palmeri</i>	Rhizomes elongated. Culms 25-60 cm. Ligule a ciliate membrane. Leaf blades flat or involute, 4-12 cm long, 3-5 mm wide.	A panicle 6-20 cm long.	Spikelets having 7-9 fertile florets, spikelets lanceolate, 25-30 mm long.	Lower glume lanceolate, 6-10 mm long, 1-keeled, 3-veined, apex acute. Upper glume ovate, 8-15 mm long, 1-keeled, 3-veined, apex acute.	Spikelets 15-20 mm long.	Lemma 8-15 mm long, 5-11-veined. Palea keels winged. Spikelets 25-30 mm long with 7-9 fertile florets.
<i>Distichlis scoparia</i>	Rhizomes absent. Culms 10-20 cm. Ligule a ciliate membrane. Leaf blades aciculate, conduplicate, 1-6 cm long, 0.5-1 mm wide.	A single raceme, 1.5-3 cm long.	Spikelets having 5-9 fertile florets, spikelets oblong, 8-15 mm long.	Lower glume lanceolate, 2.5-5 mm long, 1-keeled, 1-5-veined, apex acute. Upper glume ovate, 4.5-6 mm long, 1-keeled, 5-7-veined, apex acute.	Spikelets 6-18 mm long.	Lemma 5-9 mm long, 7-9-veined. Palea keels winged. Spikelets 8-15 mm long with 5-9 fertile florets.
<i>Distichlis spicata</i>	Rhizomes elongated. Culms 10-60 cm. Ligule a ciliate membrane. Leaf blades involute, 2-8 (or even 20) cm long, 1-4 mm wide.	Two to 10 racemes closely spaced along a central axis, in a head, racemes 1-2 cm long, central axis 2.5-8 cm long.	Spikelets having 5-15 fertile florets, spikelets ovate, 6-18 (or even 28) mm long.	Lower glume ovate, 0.9 times length of upper glume, 1-keeled, 3-9-veined, apex acute. Upper glume ovate, 1-keeled, 3-9-veined, apex acute.	Anthers 2 mm long. Lemma 5-11-veined. Spikelets 6-18 mm long with 5-20 florets.	Lemma 3-6 mm long, 5-11-veined. Palea keels winged. Spikelets 6-18 (or even 28) mm long with 5-15 fertile florets.

ligule, the length of the culm, the length of the leaf blades, the form of the inflorescence, the length of the spikelets, the number of fertile florets within a spikelet, and the number of veins on the lemma.

A dichotomous key based on selected morphological traits as listed by Clayton et al. (2006) is presented in Table 1-3. No examination of South American species was done for the present project, so the key depends on information provided by Clayton et al. (2006). At each step in the key, contrasting traits are presented that lead to a subsequent number in the key for consideration of an additional set of contrasting traits or to identification of a species. There are six steps in the key. Step 1 distinguishes *D. distichophylla* from all other *Distichlis* species by its distinctive ligule, which consists of a fringe of hairs rather than a ciliolate membrane. The identification to *D. distichophylla* can be confirmed by the 12-veined lemma of the female floret if flowers are present. If the specimen in question does not fit the description of *D. distichophylla*, step 1 directs the user to step 2, which directs the user to step 3 for plants lacking rhizomes or to step 4 for plants with rhizomes. Step 3 distinguishes *D. laxiflora* from *D. scoparia* on the basis of culm height, leaf blade morphology, number of racemes in the inflorescence, glume length, and number of veins in the lemma of the female floret. Step 4 directs the user to step 5 for plants with culms shorter than 5 cm or to step 6 for plants with culms longer than 5 cm. Step 5 distinguishes *D. australis* from *D. humilis* on the basis of leaf size. Step 6 distinguishes *D. palmeri* from *D. spicata* on the basis of inflorescence morphology and contrasts between the male and female spikelets. Definitions of the botanical terms in the key are provided facing Table 1-3.

Table 1-3. Dichotomous key to the species of the genus *Distichlis*.

1. Ligule a fringe of hairs; female floret lemma 12-veined	<i>D. distichophylla</i>
	[Australia]
1. Ligule a ciliolate membrane	2
2. Rhizomes absent	3
2. Rhizomes present	4
3. Culms 20-30 cm; leaf blades convolute, 6-11 cm long, 1-2 cm wide; inflorescence a few racemes; upper glume 3-4 cm long; female floret lemma 11-veined	<i>D. laxiflora</i>
	[Argentina]
3. Culms 10-20 cm; leaf blades aciculate, conduplicate, 1-6 cm long, 0.5-1 cm wide; inflorescence a single raceme; upper glume 4.5-6 cm long; female floret lemma 7-9-veined	<i>D. scoparia</i>
	[Argentina, Chile, Uruguay]
4. Culms 2-5 cm	5
4. Culms longer than 5 cm	6
5. Leaf blades 0.1-0.5 cm long, 0.5-1 mm wide	<i>D. australis</i>
	[Argentina]
5. Leaf blades 7-15 cm long, 1 mm wide	<i>D. humilis</i>
	[Argentina, Bolivia, Chile, Peru]
6. Inflorescence a panicle 6-20 cm long; male inflorescence spikelets 15-20 mm long; female inflorescence lemma 8-15 mm long; female spikelets 25-30 mm long with 7-9 fertile florets	<i>D. palmeri</i>
	[Mexico]
6. Inflorescence 2-10 racemes 1-2 cm long closely spaced along a central axis 2.5-8 cm long; male inflorescence spikelets 6-18 mm long; female inflorescence lemma 3-6 mm long; female spikelets 6-18 mm long with 5-15 fertile florets	<i>D. spicata</i>
	[North and South America]

Definitions:

aciculate – needle-shaped

ciliolate – having a marginal fringe of minute hairs

blade – the upper, broad part of the leaf that does not enclose the culm

conduplicate – folded together lengthwise with the upper surface within

convolute – rolled up longitudinally with parts in an overlapping arrangement like shingles on a roof

culm – the grass stalk or stem composed of rolled leaf sheaths

floret – an individual flower in the grass spikelet, which often consists of several florets

glume – one of the paired bracts at the base of a grass spikelet below the series of florets.

There is a lower glume paired with an upper glume immediately above the lower glume.

inflorescence – the flowering axis, which may consist of multiple individual flowers

lemma – the lower of the two bracts that enclose a grass floret, often overlapping the palea to partially surround it

ligule – a membranous appendage on the inner surface of the leaf at the junction where the leaf sheath joins the leaf blade in grasses

palea – the upper of the two bracts that enclose a grass floret, often partially surrounded by the lemma

panicle – a branched inflorescence consisting of racemes attached to a central axis

raceme – a unbranched inflorescence with pedicellate flowers (a pedicel is a tiny stalk joining the flower to the central axis of the raceme)

sheath – the leaf base that is rolled to enclose the culm

vein – a vascular bundle visible externally

Bell and Columbus (2008) developed a different morphological key based strongly on leaf blades and either stolons or rhizomes. According to this key, *D. scoparia* and *D. laxiflora* have rhizomes. This conflicts with the list of traits presented by Clayton et al. (2006), in which *D. scoparia* and *D. laxiflora* lack rhizomes.

Taxonomic status of D. spicata and D. stricta in North America

Two taxa, *Uniola spicata* L. and *U. stricta* Torrey, were independently transferred from the genus *Uniola* to the genus *Distichlis* and have been variously separated, combined, and subordinated over the past 100 years. *Uniola spicata*, named by Linnaeus (1753) from specimens collected on the coasts of North America, was transferred to *Distichlis* by Greene (1887) during his work in California. *Uniola stricta* was named by Torrey (1824) from specimens collected in 1820 by Edwin James, probably along Ute Creek in New Mexico. James's route has been retraced (Goodman and Lawson, 1995) and the type specimen (James, 1820) lists an approximate collection site along that route. *Uniola stricta* was transferred to *Distichlis* by Scribner (1894) as a variety of *D. spicata*. *Uniola stricta* was independently transferred by Rydberg (1905) as the distinct species *D. stricta*.

Beetle revised the North American members of *D. spicata* sensu lato (1943) and later the entire genus *Distichlis* (1955). He applied the name *D. spicata* var. *stricta* to the most widely distributed taxon, occurring from Canada to southern South America. According to Beetle's classification, the variety *stricta* is synonymous with the species *D. spicata* that was previously and subsequently recognized in other treatments of *Distichlis*. However, most compilers list *D. spicata* as the more widespread species and limit *D.*

spicata var./subsp. *stricta* to a North American inland distribution. Thorne (1978) changed the taxonomic rank of *stricta* from variety to subspecies in his revision.

None of the recent treatments has been universally accepted in modern botanical compilations covering U.S. states, regions, or the world. Authors differ as to the taxonomic rank and distribution of the taxon *stricta* and as to the sequence of naming authorities (Table 1-4). Rydberg, Scribner, Beetle, and Thorne variously receive credit for revising Torrey's original identification. Hitchcock (1951) described *D. spicata* (L.) Greene as the coastal type and *D. stricta* (Torr.) Rydberg as the inland type in the United States. Kearney and Peebles (1960) reported *D. stricta* (Torr.) Rydberg, but not *D. spicata* (L.) Greene, in Arizona. Harrington (1964) reported *D. stricta* (Torr.) Rydberg in Colorado. Munz (1970) reported *D. spicata* (L.) Greene var. *stolonifera* Beetle along the U.S. Pacific Coast, var. *nana* Beetle in southern California, var. *divaricata* Beetle in the southwestern U.S. deserts, and var. *stricta* (Torr.) Beetle inland from California eastward to Texas and northward through Kansas and the Dakotas to Saskatchewan, Canada. Weber (1976) accepted *D. spicata* (L.) Greene var. *stricta* (Torr.) Beetle in his flora of Colorado. Cronquist et al. (1977) recognized *D. spicata* (L.) Greene var. *spicata* as the coastal variety and var. *stricta* (Torr.) Scribn. as the inland variety. Gould (1983) reported *D. spicata* (L.) Greene var. *spicata* with a coastal distribution (Atlantic, Pacific, and Gulf) and *D. spicata* var. *stricta* (Torr.) Beetle with an inland distribution from Montana, Iowa, and Texas westward to the Pacific Coast. Sutherland (1986) reported *D. spicata* (L.) Greene var. *stricta* (Torr.) Beetle distributed from the Great Plains westward to the Pacific Coast and also in South America. Hallsten et al. (1987) accepted *D. spicata* var. *stricta* (A. Gray) Beetle in Wyoming. Smith (1993) accepted only *D. spicata* (L.) Greene

Table 1-4. Levels of recognition for the taxon *stricta*.

Level of taxon <i>stricta</i>	Scope of flora	Distribution of <i>stricta</i>	Author, Year	Naming authorities credited	Comments
species	---	---	Torrey, 1824		Original identification as <i>Uniola</i> , found in New Mexico
variety	---	---	Scribner, 1894	(Torr.)	Transferred from <i>Uniola</i>
species	Colorado	inland western U.S.	Rydberg, 1905	(Torr.)	Transferred from <i>Uniola</i>
species	United States	inland western North America	Hitchcock, 1951	(Torr.) Rydberg	
variety	range of <i>Distichlis</i>	western North America, Bolivia, Peru, Chile, Argentina	Beetle, 1955	(Torr.)	
species	Arizona	inland western North America	Kearny & Peebles, 1960	(Torr.) Rydberg	
species	Colorado	inland western North America	Harrington, 1964	(Torr.) Rydberg	
variety	California	California and inland western U.S.	Munz, 1970	(Torr.) Beetle	
variety	Colorado	given only for Colorado	Weber, 1976	(Torr.) Beetle	Later recognized as a species
variety	intermountain United States	inland western North America	Cronquist et al., 1977	(Torr.) Scribn.	
subspecies	southern California	inland western North America	Thorne, 1978	(Torr.)	
variety	United States	inland western U.S.	Gould, 1983	(Torr.) Beetle	

Level of taxon <i>stricta</i>	Scope of flora	Distribution of <i>stricta</i>	Author, Year	Naming authorities credited	Comments
variety	plains United States	western U.S., South America	Sutherland, 1986	(Torr.) Beetle	
variety	Wyoming	given only for Wyoming	Hallsten et al., 1987	(A. Gray) Beetle	
not recognized	California	---	Smith, 1993	---	
species	Colorado	given only for Colorado	Weber & Wittmann, 1996	(Torr.) Rydberg	
subspecies	New World	western North America, Argentina, Bolivia, Chile, Ecuador, Peru, Uruguay	Peterson, 2000	(Torr.) Thorne	
subspecies	Colorado	Colorado	Rubright, 2000	(Torr.) Rydberg	
not recognized	United States	---	Barkworth, 2003	---	Later recognized as a subspecies
not recognized	world	---	Clayton, 2006	---	
variety	world	western North America, Bolivia, Peru, Uruguay, Argentina	Quattrocchi, 2006	(Torr.) Scribner	
species	Montana	given only for Montana	Lavin & Seibert, 2009	(Torr.) Rydberg	
subspecies	United States	inland western U.S.	Barkworth, 2010	(Torr.) Thorne	

and listed var. *stricta* (Torr.) Beetle as an unrecognized name in his treatment of grasses for a California flora. Weber and Wittmann (1996) reversed Weber's 1976 publication and revived *D. stricta* (Torr.) Rydberg as the taxon present in Colorado. Peterson (2000) recognized *D. spicata* subsp. *stricta* (Torr.) Thorne distributed in both North and South America, while Rubright (2000) recognized *D. spicata* subsp. *stricta* (Torr.) Rydberg in Colorado. Quattrocchi (2006) and the U.S. Germplasm Resources Information Network (USDA, 1994) accepted *D. spicata* var. *stricta* (Torr.) Scribner. Lavin and Seibert (2009) continued to reference the inland version of saltgrass as the species *D. stricta*, rather than as a subspecies or variety of *D. spicata*. Barkworth (2003) and Clayton et al. (2006) declined to recognize the taxon *stricta* at any level. Barkworth (2010) later amended an electronic version of her grass manual to accept the taxon as *D. spicata* subsp. *stricta* (Torr.) Thorne. Compilations that cover an area encompassing both taxa typically describe morphological traits that distinguish between the two, while compilations that report only one or the other taxon provide only a description distinguishing the reported taxon from other species of grasses.

Morphological traits have been the basis for proposing or opposing classifications of *Distichlis*. However, many morphologic traits overlap (Bell and Columbus, 2008), including a few that are used in dichotomous keys to distinguish *D. spicata* from *D. stricta* (Table 1-5). For example, in his key Beetle (1943) described *D. spicata* as having 5 to 9 florets and *D. spicata* var. *stricta* as having 3 to 14 florets. The entire range of this trait in *D. spicata* is thus encompassed by the range of *D. spicata* var. *stricta*. Hitchcock (1971) described both sexes of *D. spicata* as having 5 to 9 florets in the inflorescence and *D. strica* as having 8 to 15 florets in male plants and 7 to 9 florets in female plants.

Table 1-5. Direct comparison of traits in *D. spicata* and *D. stricta*. Table continued on overleaf.

Source	Trait	<i>D. spicata</i>	<i>D. stricta</i>	Trait Overlap	Comments
Fassett	culm height	1.5-4 dm	1-5.5 dm	√	
Fassett	leaf blade angle	spreading or ascending	strongly ascending or somewhat spreading	√	Munz gives the opposite for this trait, <i>spicata</i> erect and <i>stricta</i> erect or decumbent.
Fassett	leaf blade length	5-15 cm	2-15 cm	√	
Fassett	leaf blade	--	often pubescent on adaxial surface		
Fassett	leaf edge	smooth, rarely serrated at tip	strongly serrate, but obscurely serrate in WA, OR, CA	√	
Fassett	leaf tip	blunt or oblique	pointed		
Fassett	hairs at mouth of sheath	rarely, sparse tuft	often, copious tuft		
Fassett	panicle	compact	open		
Fassett	spikelet #	10-20	4-10 in some interior plants, usually 16-24	√	Munz gives 6-20 for males, 8-35 for females of <i>spicata</i>
Fassett	spikelet length	5-10 mm, rarely 14 mm	9 or 12-25 mm	√	
Fassett	floret #	4-9, rarely 12	6-18	√	
Fassett	lower glume	rarely 0.4 mm, usually 2-3.5 mm	3.2-7.8 mm	√	
Fassett	upper glume	2.5-4 mm	rarely 2.1 mm, usually 3-7 mm	√	
Fassett	lemma	3.5 mm, rarely 3.6 mm, female with slight hyaline margin	3.2-5 mm for one variety, usually 4 or 4.5-7.8 mm, female with distinct hyaline margin	√	

Table 5. Direct comparison of traits in *D. spicata* and *D. stricta*. Table continued on overleaf.

Source	Trait	<i>D. spicata</i>	<i>D. stricta</i>	Trait Overlap	Comments
Fassett	culm height	1.5-4 dm	1-5.5 dm	√	
Fassett	leaf blade angle	spreading or ascending	strongly ascending or somewhat spreading	√	Munz gives the opposite for this trait, <i>spicata</i> erect and <i>stricta</i> erect or decumbent.
Fassett	leaf blade length	5-15 cm	2-15 cm	√	
Fassett	leaf blade	--	often pubescent on adaxial surface		
Fassett	leaf edge	smooth, rarely serrated at tip	strongly serrate, but obscurely serrate in WA, OR, CA	√	
Fassett	leaf tip	blunt or oblique	pointed		
Fassett	hairs at mouth of sheath	rarely, sparse tuft	often, copious tuft		
Fassett	panicle	compact	open		
Fassett	spikelet #	10-20	4-10 in some interior plants, usually 16-24	√	Munz gives 6-20 for males, 8-35 for females of <i>spicata</i>
Fassett	spikelet length	5-10 mm, rarely 14 mm	9 or 12-25 mm	√	
Fassett	floret #	4-9, rarely 12	6-18	√	
Fassett	lower glume	rarely 0.4 mm, usually 2-3.5 mm	3.2-7.8 mm	√	
Fassett	upper glume	2.5-4 mm	rarely 2.1 mm, usually 3-7 mm	√	
Fassett	lemma	3.5 mm, rarely 3.6 mm, female with slight hyaline margin	3.2-5 mm for one variety, usually 4 or 4.5-7.8 mm, female with distinct hyaline margin	√	

Source	Trait	<i>D. spicata</i>	<i>D. stricta</i>	Trait Overlap	Comments
Munz	leaf blade angle	erect	erect or decumbent	√	Fassett gives the opposite for this trait, <i>spicata</i> spreading or ascending and <i>stricta</i> strongly ascending or somewhat spreading.
Munz	panicle	congested, pedicels not visible	not congested, pedicels visible		A distinguishing trait in the key.
Munz	panicle length	male: -- female: 1.5-5 cm	2-7 cm	√	
Munz	spikelet #	male: 6-20 female: 8-35	--		
Munz	spikelet length	male: -- female: 1 cm	2-7 cm		
Munz	spikelet width	male: -- female: 4 mm	4-6 or 4-7 mm	√	
Munz	floret #	male: 7-10 female: 5-9	3-14 or 5-20	√	A distinguishing trait in the key.
Munz	lower glume length	male: 3-3.5 mm female: 2.5 mm	2-3 mm	√	
Munz	upper glume length	male: 3-3.5 mm female: 3.5 mm	3-4 mm	√	
Munz	lemma length	male: 3.5 mm female: 5 mm	3.5-6 mm	√	
Munz	palea	male: female: broad winged	--		Fassett and Hitchcock give the opposite for this trait, <i>stricta</i> broadly winged.

According to Hitchcock's description, the numbers of florets overlap at 7, 8, and 9. In addition to problems of overlap, there is occasional disagreement among taxonomists about which taxon has which trait, for example, which taxon has more upright leaf blades or has wings on the palea. Thus, questions persist about the correct taxonomic divisions. In an attempt to resolve the taxonomy of *D. spicata* sensu lato with morphological traits at a different scale than floret number, López Soto et al. (2009) studied leaf anatomy. They concluded that extreme variations in internal leaf structure were responses to differing environments, not genetic differences that would suggest the existence of two species, subspecies, or varieties.

Beyond morphology

Cytological and molecular methods may be helpful to illuminate differences between plants from different geographic regions. A cytological approach taken by Reid (2001) showed that a previously unknown 38-chromosome race of *D. spicata* sensu lato was found in California, Nevada, Idaho, Wyoming, Colorado, Kansas, and Nebraska. Plants having the previously published 40-chromosome number (Stebbins and Löve, 1941; Bowden, 1960; Rahn, 1961; Reeder, 1967, 1977) were found in California, Arizona, Utah, and Florida. Ram et al. (2004) combined molecular and morphological approaches, using molecular markers and morphological traits to compare 37 *Distichlis* accessions from coastal and inland areas of North and South America. Ram et al. used canopy growth vigor, shoot direction, ramet density, leaf color, angle between the leaf and the shoot, leaf length, leaf width, and leaf texture as morphological traits. The molecular markers for the study were 70 RAPD bands generated by PCR using 20 decamer primers.

Neither the molecular nor the morphological approach provided a clear distinction between plants of coastal versus inland origin. The correlation between genetic and geographic distance was weak ($r = 0.19$). Ram et al. (2004) concluded that the absence of major differences among plants for the morphological and molecular traits studied supported classification as a single species. However, inland locations can have plants belonging to either the 38- or the 40-chromosome race (Reid, 2001), presenting the possibility that differing chromosome numbers, rather than inland or coastal location, should be the basis for classifying accessions of *D. spicata* sensu lato in North America.

Cytological and molecular characterization

Cytogenetics has been an important component of grass taxonomy since Avdulov (1931) showed that chromosome number is useful in grass systematics. Chromosome number is relatively conserved in comparison with the more variable molecular sequences of DNA that constitute the chromosomes. In many taxa, “the chromosome number is an important landmark to distinguish monophyletic clades” (Guerra, 2008). Chromosome numbers in Poaceae continue to be a subject of study (Hilu, 2004). Changes in chromosome number are believed to be important mechanisms in the evolution of plants (Guerra, 2008; Luo et al., 2009).

The amount of DNA in nuclear chromosomes, irrespective of the sequence, has been investigated as a trait indicating taxonomic relationships. The C-value, expressed as picograms (pg) of DNA in the $1n$ genome (or 2C-value for the $2n$ genome) is “a character of fundamental biological significance” (Bennett et al., 1982) and is reported to be fairly constant within a species in plants (Ohri and Khoshoo, 1986). Some reports of

inconstancy within species have been traced to faulty technique or to the presence of B chromosomes, aneuploidy, or major mutations involving loss or duplication of chromosome segments, and some reports of inconstancy have not been confirmed by subsequent research (Ohri and Khoshoo, 1986). Some reports of inconstancy within species show clinal patterns, changing with latitude or with moisture (Price et al., 1981; Ceccarelli et al., 1991). However, reports of constancy far outnumber reports of inconstancy (Ohri and Khoshoo, 1986). The species within a genus can have different C-values. The genus *Phaseolus*, for example, has a 2.6-pg group containing four species and a 3.6-pg group containing four species (Ayonoadu, 1974). In the genus *Lathyrus*, 21 species cluster into five discontinuous groups with regular intervals of 3.5 pg between the 2C-values of the groups (Narayan, 1982).

Nuclear coding and non-coding regions have also been used to characterize genetic relationships among plants. Mori et al. (1995) used restriction fragment length polymorphisms (RFLP) in nuclear DNA to estimate the genetic distance between *Triticum dicoccoides* Körn and *T. araraticum* Jakubz. The RFLP technique can also be used in chloroplast coding regions to explore phylogenetic relationships at high taxonomic levels, but non-coding regions are more informative at low taxonomic levels (Soltis and Soltis, 1998). The first report of intraspecific chloroplast variation was published in 1979 for *Nicotiana debneyi* Domin using a single restriction enzyme (Soltis et al, 1992). Since then, chloroplast variation has been used to support subspecies recognition (Tan and Thomson, 1990), demonstrate introgression (Palmer et al. 1983; Kemble, 1987; Duvall and Doebley, 1990), and suggest multiple origins involving

different maternal parents (Ogihara and Tsunewaki, 1982, 1988; Murai and Tsunewaki, 1986; Soltis and Soltis, 1989; Soltis et al., 1989; Furnier et al., 1990; Wolf et al., 1990).

Chloroplast DNA has also been used to investigate auto- and allopolyploidy. Ogihara and Tsunewaki (1988) used chloroplast DNA sequences to support a single origin for most polyploid descendants of diploid species in the genera *Triticum* and *Aegilops*. However they postulated that *Ae. triuncialis* L. resulted from reciprocal crosses involving *Ae. caudata* L. and *Ae. umbellulata* Zhuk. Individuals of *Ae. triuncialis* have chloroplast sequences specific to each of these two postulated parents, suggesting that each served as the maternal parent in a cross. Soltis and Soltis (1989) used restriction site variation in chloroplast DNA to identify the maternal parents in two naturally occurring allotetraploid species in the genus *Tragopogon*. Ownbey (1950) had previously used floral and vegetative traits such as leaf length, size and shape of the inflorescence, and color of the ligule (the strap-shaped corolla of the ray flower in the family Asteraceae) to show that the allotetraploid *T. mirus* Ownbey was derived from diploids *T. dubius* Scop. and *T. porrifolius* L. while the allotetraploid *T. miscellus* Ownbey was derived from *T. dubius* and *T. pratensis* L. Using RFLP analysis, Soltis and Soltis (1989) identified *T. porrifolius* as the maternal parent for *T. mirus*, and *T. pratensis* as the maternal parent for *T. miscellus*, with *T. dubius* serving as the paternal parent for both allotetraploids. Erickson et al. (1983) and Palmer et al. (1983) used RFLP analysis of chloroplast DNA to identify the diploid maternal parent species of allotetraploids in Brassica. Chloroplast DNA is widely used tool for investigating polyploidy in plants.

Analysis of cytoplasm-transmitted DNA has been used to elucidate genetic relationships in animals as well as in plants. The North America flicker, a bird in the

genus *Colaptes*, is commonly divided into three groups on the basis of plumage and geographic distribution. The red-shafted flicker, *C. cafer* Gmelin, occurs west of the Rocky Mountains. The yellow-shafted flicker, *C. auratus* L., occurs east of the Rocky Mountains. The gilded flicker, *C. chrysoides* Malherbe, occurs in the desert southwest. Although once considered separate species, they were reclassified as *C. auratus* subspecies *cafer*, *auratus*, and *chrysoides* in the 1980s (American Ornithologists' Union, 1983) because frequent hybridization along the margins of their ranges suggested that they were all members of a single species under Mayr's biological species concept (Mayr, 1942). *Colaptes chrysoides* was restored to the status of a separate species in 1998 (American Ornithologists' Union, 1998) based on restriction site variability in mitochondrial DNA. In another example of the use of cytoplasm-transmitted DNA to elucidate genetic relationships in animals, Degner et al. (2007) used mitochondrial DNA sequences in the mouse *Peromyscus polionotus niveiventris* Chapman to support subspecies designation. The use of mitochondrial DNA is problematic in plants because plant mitochondrial DNA rearranges very rapidly at the gene-order level and mutates very slowly at the nucleotide level (Palmer, 1992). Rapid rearrangement prevents the establishment of a consistent overall framework within which homologous restriction sites can be identified, while slow mutation prevents the accumulation of sufficient point mutations to be useful for phylogenetic analysis at the generic and specific levels. Nevertheless, evaluation of cytoplasmic DNA continues to be a method of choice for describing genetic relationships among closely related taxa (Duangjial et al., 2006; Aoki et al., 2006), with chloroplast DNA the molecule of choice in plant systematics (Palmer, 1992).

Microsatellites, or simple sequence repeats (SSR), are tandem repeats of short 2- to-5 bp sequences that are useful as genetic markers because there are numerous SSR loci dispersed through the nuclear genome and each locus can be highly polymorphic for number of repeats. Polymorphism at a locus is produced by slip-strand mispairing during DNA replication (Eisen, 1999). The template strand and the nascent strand become temporarily separated and then rejoin in a misaligned configuration, with one or more repeats of one strand looping out from the duplex configuration. Because each repeat motif is identical to neighboring repeat motifs, mistakes in re-alignment are common and the mutation rate at microsatellite loci is higher than at other loci in the same genome (Eisen, 1999). Microsatellite markers typically are co-dominant, revealing the allelic state at equivalent loci on homologous chromosomes, although so-called null alleles that do not amplify due to variation in the primer annealing sites lead to ambiguous interpretations. Randomly amplified polymorphic DNA (RAPD) (Williams et al., 1990; Welsh and McClelland, 1990) bands are segments of DNA flanked by inverted repeat sequences that are plentiful in the genome. RAPD markers typically act as dominant markers that are visible as the presence or absence of bands. The absence of a band is unambiguous; however the presence of a band can indicate either a homozygous state or a heterozygous state at the locus. Microsatellites and RAPD markers are not highly conserved, so they are not useful for constructing higher-level phylogenies. However they are useful for applications at generic and specific levels. Both can be used to study mating systems, diversity, parentage, and geographic variation, while RAPD markers also are useful for studying hybrid zones and species boundaries (Dowling et al., 1996).

Research objectives

The objectives of the research were to:

- Determine whether collections of *D. spicata* sensu lato from different geographic areas of the western United States differ for phenotypic and/or molecular traits.
- Determine whether molecular variation between inland and coastal *D. spicata* sensu lato collections from the western United States is correlated with chromosome number and/or geographic distribution.
- Determine whether the RAPD marker reported by Eppley (1998) could identify gender in collections of *D. spicata* sensu lato from the western United States.
- Evaluate the taxonomic validity of *D. spicata* var. *stricta* as a species distinct from *D. spicata*.

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**Chapter 2: Genetic variation among inland and coastal populations
of *Distichlis spicata* sensu lato (Poaceae) in the western United States**

ABSTRACT

The taxonomic status of the North American endemic grass *Distichlis spicata* subsp. *stricta* has been in flux for more than a century. *Distichlis spicata* hosts the larval stage of a federally endangered butterfly and is being investigated for use in restoration and recreation. Therefore the relationship between the species and its subspecies merits clarification. Although the subspecies was once recognized as a separate species (*D. stricta*), most current treatments either consider it an inland subspecies/variety within *D. spicata* or decline to recognize it at all. Two recent studies did not find genetic or morphological evidence differentiating the subspecies *stricta* within *D. spicata*. In this study, genetic variation in 13 coastal and inland populations of *D. spicata* was characterized using chromosome counts, chloroplast DNA segments, microsatellite alleles, RAPD bands, and DNA C-values. Plants from coastal and inland populations were grown in a common garden and evaluated for date of first flowering. The results suggest the existence of two genetically distinct lineages that differ for chromosome number, molecular sequences in cpDNA and nuclear DNA, DNA C-value, and flowering time. One lineage has a somatic chromosome number of $2n = 40$ and encompasses plants from the West Coast and several inland locations in Nevada, Utah, and southern New Mexico. The other lineage has a somatic chromosome number of $2n = 38$ and consists of plants distributed only inland among the populations surveyed. Genetic distances among

populations were closer within each lineage than between the two lineages, even when different lineages occurred in geographic proximity. The 38-chromosome lineage is a distinct species corresponding to the previously recognized *Distichlis stricta*. The 40-chromosome lineage is *Distichlis spicata*.

Key words: chloroplast DNA; *Distichlis*; *Distichlis spicata*; *Distichlis stricta*; hybrid speciation; microsatellites; phylogenetic relationships; Poaceae; polyploids; saltgrass

The genus *Distichlis* Raf. is composed of dioecious, rhizomatous, salt-tolerant, C₄ grasses. The genus includes six to nine species (Peterson et al., 2001; Quattrocchi, 2006) distributed mostly over North and South America. These species include *D. australis* (Speg.) Villamil, *D. humilis* Phil., *D. laxiflora* Hack., and *D. scoparia* (Kunth) Arechav in South America, *D. palmeri* (Vasey) Fassett ex I.M. Johnst. in Mexico, and *D. spicata* (L.) Greene in both North and South America. *Distichlis distichophylla* (Labill.) Fassett, endemic to Australia, has received only limited acceptance because of suggestions (Beetle, 1955; Peterson, 2000) that it is *D. spicata*. A paleologic species, *D. africana* Dugas and Retallack, has been claimed in Africa based on fossil leaves found in Kenya, but this is not extant. An additional New World endemic taxon is sometimes recognized, at various taxonomic levels, bearing the specific epithet or subspecific/variety name *stricta*.

The taxonomic status of *D. spicata* subsp. *stricta* (Torr.) Thorne has been in flux for more than a century. The grass was originally classified by Torrey (1824) as *Uniola*

stricta. A related species, *U. spicata* L. (Linnaeus, 1753), was transferred to the genus *Distichlis* by Greene (1887). Subsequently Scribner (1894) transferred *U. stricta* to the genus *Distichlis* as a variety, *D. spicata* var. *stricta*. Rydberg (1905), independently of Scribner, also transferred *U. stricta* into the genus *Distichlis*, but as a distinct species, *D. stricta*.

Beetle revised the North American members of *Distichlis* in 1943 and later (1955) revised the entire genus. He considered *D. spicata* var. *stricta* to be widely distributed, from Canada across the western United States and down through South America, as well as in Australia. The distribution described by Beetle would make the variety *stricta* synonymous with the species *D. spicata* recognized in most other treatments of *Distichlis*. However, most botanists list *D. spicata* as the more widespread species and limit *D. spicata* subsp./var. *stricta* to a North American inland distribution. Many of the earlier taxonomists who worked on *Distichlis* considered the taxon *stricta* to be a variety, but Thorne (1978) changed the taxonomic rank of *stricta* from variety to subspecies in his revision.

None of the treatments by Scribner, Rydberg, Beetle, or Thorne has been universally accepted in subsequent major botanical compilations. Experts differ about the existence, taxonomic level, distribution, and authority name associated with the taxon *stricta*. Hitchcock (1951) listed *D. spicata* (L.) Greene as the coastal type and *D. stricta* (Torr.) Rydberg as the inland type of saltgrass in the United States. Kearney and Peebles (1960) listed *D. stricta* (Torr.) Rydberg, but not *D. spicata* (L.) Greene, in Arizona. Munz (1970) listed *D. spicata* (L.) Greene var. *stolonifera* Beetle along the Pacific Coast, var. *nana* Beetle in southern California, var. *divaricata* Beetle in the southwestern U.S.

deserts, and var. *stricta* (Torr.) Beetle distributed inland from California eastward to Texas and northward through Kansas and the Dakotas to Saskatchewan, Canada. Weber's (1976) flora of Colorado lists *D. spicata* (L.) Greene var. *stricta* (Torr.) Beetle. Cronquist et al. (1977) recognized *D. spicata* (L.) Greene var. *spicata* as coastal and var. *stricta* (Torr.) Scribn. as the inland variety. Gould (1983) listed *D. spicata* (L.) Greene var. *spicata* with a coastal distribution (Atlantic, Pacific, and Gulf) and *D. spicata* var. *stricta* (Torr.) Beetle with an inland distribution from Montana, Iowa, and Texas westward to the Pacific Coast. Sutherland (1986), treating *Distichlis* for The Great Plains Flora Association (1986) listed *D. spicata* (L.) Greene var. *stricta* (Torr.) Beetle distributed from the Great Plains westward to the Pacific Coast and, in accordance with Beetle (1955), also in South America. Smith (1993), treating *Distichlis* for The Jepson Manual (Hickman, 1993), accepted only *D. spicata* (L.) Greene and did not recognize var. *stricta* (Torr.) Beetle. Weber and Wittmann (1996) reversed Weber's 1976 nomenclature by listing the species that occurs in Colorado as *D. stricta* (Torr.) Rydberg. Peterson (2000), treating *Distichlis* in Soreng et al. (2000 and subsequent), recognized *D. spicata* subsp. *stricta* (Torr.) Thorne. Barkworth (2003) declined to accept *stricta* as a species, a subspecies, or a variety, on the grounds that it was morphologically indistinguishable from *D. spicata*, but later (Barkworth, 2010) accepted it as a subspecies after reviewing the molecular data presented here.

Recent studies have not resolved the question of whether *stricta* exists and at what taxonomic level. Ram et al. (2004) examined both molecular and morphological data from a survey of North American *D. spicata* collected from coastal and inland locations. They concluded that gene flow among populations of *D. spicata* was widespread and that

there was no genetic basis for distinguishing between inland and coastal varieties. López Soto et al. (2009) concluded that extreme variations in internal leaf structure in herbarium samples labeled *D. spicata* and *D. stricta* reflected responses to differing environments, not genetic differences that would imply the existence of two species, subspecies, or varieties. In an examination of the Chloridoid grasses, Bell and Columbus (2008) did not recognize *stricta* as a taxon at any rank within *Distichlis*. However, they noted that *D. spicata* was polyphyletic according to molecular analysis of nuclear and chloroplast sequences. The polyphyly of *D. spicata* in the analysis by Bell and Columbus (2008) leads one to question whether *D. spicata* sensu lato, encompassing both coastal and inland accessions, encompasses distinct phylogenetic lineages corresponding to the two previously recognized species *D. spicata* and *D. stricta*.

The Carson wandering skipper, an endangered butterfly, lays its eggs on *D. spicata* (U.S. Fish and Wildlife Service, 2007). Clarification of the taxonomy is a starting point for determining whether genetic variation in *D. spicata* s.l. is significant for conservation efforts associated with the skipper. Recovery efforts should consider whether both *D. spicata* and *D. spicata* subsp. *stricta* occur at the locations of the four remaining skipper populations and whether the skipper recognizes a difference between the species and its subspecies.

Because of its tolerance to soil salinity, *D. spicata* s.l. has been investigated as a potential grass for recreation areas irrigated with reused water that is high in salts (Marcum et al., 2007). Information about genetic variation in *D. spicata* s.l. would also help breeders develop commercial cultivars. High tolerance to salt has also been a motivating factor to utilize *D. spicata* for projects to reclaim riparian land invaded by

tamarisk (*Tamarix* sp.) (Lair and Wynn, 2002). Information about possible genetic variation in *D. spicata* s.l. over the western United States could impact choices about where to obtain source material for these reclamation projects.

In light of the differences of opinion about the existence and classification of the taxon *stricta* within *Distichlis*, this study examined cytological and molecular variation in samples of *Distichlis spicata* s.l. from 13 sites in western North America, including coastal and inland locations. The characters studied were chromosome number, chloroplast haplotype using PCR-RFLP of three intergenic segments, microsatellite variation at four loci, RAPD variation using two primers, and DNA C-values. A cross was made between two plants having different chromosome numbers and the progeny were evaluated for chromosome number. Samples from four collection sites were grown in a common garden and evaluated for date of first flowering.

MATERIALS AND METHODS

Origin of plant material—Collection sites of *Distichlis spicata* s.l. (L.) Greene are listed in Table 2-1. Plants from San Francisco Bay, California, were collected as seeds at the northern end of San Francisco Bay by employees of Freshwater Farms in Eureka, California, and were sold as *D. spicata*. Plants from Washoe County, Nevada, were collected as seeds in the Great Smoke Desert and were sold as “inland saltgrass” by Comstock Seed in Gardnerville, Nevada. Plants from Great Salt Lake, Utah, were collected as seeds on the eastern shore of the lake and were sold as “inland saltgrass” by Granite Seed in Lehi, Utah. Seeds were germinated following the scarification method described in Harrington (2000). Seedlings were grown in individual pots. Plants from

Table 2-1. Sampling sites in North America for two cytoraces of *Distichlis spicata* s.l.

Site	Approx. latitude, longitude, elevation	Environment	Abbreviation in figures and tables
San Francisco Bay, California, USA	N 38.0 W 122.5 0 m	bay on the Pacific Coast	SFB, Sfby
Smoke Creek Desert, Nevada, USA	N 40.4 W 119.7 1,200 m	dry plain	Washoe, Wash
Great Salt Lake, Utah, USA	N 41.0 W 112.5 1,300 m	lake	G8, Gate
Gray's Harbor, Washington, USA	N 47.0 W 124.2 0 m	bay on the Pacific Coast	Seat
near Las Cruces, New Mexico, USA	N 32.5 W 107.4 1,200 m	dry	Cruc, TomCru
near Truth or Consequences, New Mexico, USA	N 32.9 W 107.4 1,200 m	dry	TorC, TomToC
near Albuquerque, New Mexico, USA	N 34.7 W 107.3 1,600 m	dry	Albu, TomAlb
near Rock River, Wyoming, USA	N 41.7 W 106.0 2,100 m	mountainous	RRiv
Antero Reservoir, Colorado, USA	N 39.0 W 105.9 2,750 m	seasonally dry high reservoir	Antero, Antr
Pawnee National Grasslands, Colorado, USA	N 40.8 W 104.7 1,500 m	dry plain	Pawnee, Pawn
near Wray, Colorado, USA	N 40.1 W 102.2 1,100 m	dry plain	Wray
Goose Lake, California, USA	N 41.8 W 120.5 1,400 m	seasonally dry lake	Goos
Honey Lake, California, USA	N 40.2 W 120.2 1,200 m	seasonally dry lake	Hony

Honey Lake, California, Goose Lake, California, and Pawnee Grasslands, Colorado, were collected as vegetative rhizomes by the author and Scott Reid. Plants from Wray, Colorado, Antero Reservoir, Colorado, and Rock River, Wyoming, and Seattle (Gray's Harbor), Washington, were collected by Scott Reid. Plants from Albuquerque, New Mexico, Truth or Consequences, New Mexico, and Las Cruces, New Mexico, were collected by Tom Creegan. The plant labeled Archery 5 was collected by the author in Fort Collins, Colorado. The plant from Florida was bought as a vegetative sample of *D. spicata* from Horticultural Systems in Parrish, Florida, USA. Single rhizomes were grown in individual pots. Vegetative samples of *D. littoralis* (Englem.) Bell & Columbus (formerly *Monanthochloë littoralis* Engelm.) and *D. eludens* (Soderstrom & Decker) Bell & Columbus (formerly *Reederochloa eludens* Soderstrom & Decker) were provided by Hester Bell, Rancho Santa Ana Botanic Gardens, Claremont, California, USA. Seeds collected at the Honey Lake and Goose Lake sites are deposited with the USDA-ARS National Plant Germplasm System (NPGS) and found in the Genetic Resources Information Network Database under the Plant Introduction numbers W6 27715 and W6 27719.

Chromosome examination--Root tips were collected from pots in the greenhouse and treated as described in Reid (2001) using colchicine (Matheson Coleman & Bell, Gardena, California, USA) acetocarmine (various manufacturers), and Macerozyme R-10 and "Onozuka" R-10 (Yakult Honsha Company, Tokyo, Japan). Chromosome spreads were made using the squash method of Reid (2001) and were examined under a 100X oil objective on a Zeiss Photomicroscope II (Carl Zeiss AG, Oberkochen, Germany)

equipped with phase contrast. Spreads were photographed on Kodak Technical Pan or Kodak T-Max 100 film (Eastman Kodak Company, Rochester, New York, USA).

Molecular methods--DNA was extracted from fresh leaves using the CTAB method modified from Doyle and Doyle (1987) to achieve a volume suitable for 1.5 mL microfuge tubes. DNA was quantified by spectrophotometer and diluted to 10 ng/ μ L.

Chloroplast segments were amplified using primer pairs published by Saltonstall (2001) and then digested with restriction enzymes. The following primer pairs (Integrated DNA Technologies, Inc., Coralville, Iowa, USA) and restriction enzymes (Fermentas International, Burlington, Canada) were used: trnH(GUG) and psbA digested with BsuRI (recognition site GGCC) at 37 °C, trnS(GCU) and psbD digested with Hin6I (recognition site GCGC) at 37 °C, rps16F and trnQ digested with TaqI (recognition site TCGA) at 65 °C, and rps16F and trnQ digested with TruII (recognition site TTAA) at 65 °C. Chloroplast segments were amplified in a cocktail consisting of 1X reaction buffer containing 15 mM MgCl₂, 0.5 mM additional MgCl₂, 0.2 mM dNTPs (Sigma-Aldrich Corporation, St. Louis, Missouri, USA), 0.4 μ M primer 1, and 0.4 μ M primer 2. One unit of Sigma JumpStart REDTaq and 20 ng of DNA were added to each 25 μ L reaction. The thermocycler (MJ Research PTC-200, MJ Research, Inc., Waltham, Massachusetts, USA, or Bio-Rad MyCycler, Bio-Rad Laboratories, Hercules, California, USA) profile was as given in Saltonstall (2001) except that the annealing temperature was 57 °C for the rps16F and trnQ primer pair and 59 °C for the trnS and psbD primer pair. The amplified segments were digested overnight using 5 μ L of the PCR product and the manufacturer's recommended buffer for each enzyme in a restriction reaction with a total volume of 15 μ L, covered with 10 μ L of Chill-Out liquid wax (Bio-Rad).

Primers (Integrated DNA Technologies, Inc., Coralville, Iowa, USA) for microsatellites were taken from Tsyusko et al. (2007). The primer pairs used were Dis 1 and EF193006, Dis 3 and EF193007, Dis 10 and EF193012, and Dis 22 and EF193017. Amplification was done using the same cocktail as described above for chloroplast segments except that primer concentrations were 0.2 μ M. The thermocycler profile was as given in Tsyusko et al. 2007, except that denaturation was done at 94 °C; 11 cycles of touchdown were used, in which early cycles used a higher-than-desired annealing temperature and the temperature was decreased 1 °C each cycle until the desired annealing temperature was reached; 25 cycles were run at the desired annealing temperature; and there was a final extension at 72 °C for 5 minutes.

RAPD primers (Integrated DNA Technologies, Inc., Coralville, Iowa, USA) were OPF13 (5'-GGCTGCAGAA-3') and OPD16 (5'-AGGGCGTAAG-3') from the sets developed by Operon Biotechnologies (Huntsville, Alabama, USA). DNA fragments were amplified in a cocktail consisting of 1X reaction buffer containing 15 mM MgCl₂, 0.5 mM additional MgCl₂, 0.25 mM dNTPs (Sigma), 1.2 μ M primer, with 1 unit of Sigma JumpStart REDTaq and 10 ng of DNA per 25 μ L reaction. The thermocycler profiles were as described in Levitan and Grosberg (1993) except that the annealing temperature for OPD16 was 40 °C.

Separation of DNA products--Unrestricted chloroplast segments were loaded on 1.6% agarose gels using Amplisize (Bio-Rad) DNA size marker to check that each set of primers amplified only one band in each DNA sample and that bands in all samples appeared to be the same size before restriction. Restricted chloroplast segments, microsatellite PCR products, and RAPD PCR products were separated by polyacrylamide

gel electrophoresis in 5% denaturing gels using 40% 19:1 acrylamide-bisacrylamide (Bio-Rad), 7.5M urea, and tris-borate-EDTA buffer. Samples were mixed 1:1 with formamide loading buffer and loaded onto gels made in Sequi-Gen (Bio-Rad) sequencing gel rigs. DNA size markers were 10 bp DNA Ladder (Invitrogen Corporation, Carlsbad, California, USA), individual 400 bp, 500 bp, 600 bp NoLimits bands (Fermentas) and/or GeneScan 600 (Applied Biosystems, Foster City, California, USA). Because multiple gels were needed to accommodate the number of samples, duplicate DNA samples were used across gels as additional references to adjust for variation from gel to gel. After loading, gels for chloroplast segments and RAPD fragments were run at constant 80 Watts for 2.5 hours. Samples for each microsatellite primer pair were loaded on a single gel in three waves, with 40 to 60 minutes of run time between the first and second waves of samples, 40 to 60 minutes of run time between the second and third waves, and 75 to 90 minutes of run time after the third wave of samples. DNA was visualized with silver staining (Bassam et al., 1991). Gels were allowed to dry at room temperature overnight and scanned on a Microtek (Microtek International Inc., Hsinchu, Taiwan) flatbed 12 X 17 inch scanner. Digital scans were scored using Kodak 1D to detect bands and estimate band sizes.

Software analyses--The RAPD tree was created with RAPDDIST (Black and Antolin, 1997) with 250 bootstrapping runs resolved with the CONSENSE program in PHYLIP 3.69 (Felsenstein, 2009). The chloroplast tree was created with PAUP*4.0 beta 10 (Swofford, 2002). The microsatellite tree was created in NTSYSpc 2.11f (Rohlf, 2002) using genetic distances among populations generated by GenAlEx 6.2 (Peakall and Smouse, 2006). The Mantel test was done in GenAlEx 6.2 using genetic distances

generated by GenAEx 6.2 and geographic distances obtained from a distance calculator (NOAA, 2005). The program STRUCTURE Version 2.3.3 (Pritchard et al., 2000, 2009) was used to analyze a combined data set consisting of chromosome numbers, chloroplast restriction sites, and microsatellite profiles. The burn-in length and the run length were each 10,000 for all k from 1 to 15, two more k than the 13 sampling locations. The optimal value of k was determined using the Δk procedure from Evanno et al. (2005).

Field experiment—Asexually propagated clones of 100 accessions each from San Francisco Bay, California; Washoe County, Nevada; Great Salt Lake, Utah; and Pawnee Grasslands, Colorado, were planted in the summer of 2003 on 5-ft centers in a common garden at Fort Collins, Colorado, USA. The experiment was replicated twice. Entries were completely randomized in each replication. Plants were observed weekly from early spring (25 April 2004, 9 May 2005) through late summer (20 September 2004, 24 August 2005). Date of first flowering was recorded.

Hybrid progeny—Archery 5, a 38-chromosome plant collected in Fort Collins, Colorado, USA, was fertilized with pollen from Washoe 16, a 40-chromosome plant collected in Washoe County, Nevada, USA. Ten seeds were harvested and germinated. The chromosome numbers of these progeny were counted as described above.

DNA C-values—Leaves from 22 plants representing four 38-chromosome populations and four 40-chromosome populations were sent for flow cytometry analysis at Benaroya Research Institute at Virginia Mason University, Seattle, Washington, USA, where 2C-values were measured for nuclei stained with propidium iodide. Results were grouped according to chromosome number and group mean 2C-values in picograms (pg) were compared using a t-test.

RESULTS

Chromosome numbers in accessions—Most plants in the Seattle, San Francisco Bay, Washoe, Great Salt Lake, Truth or Consequences, and Las Cruces populations had somatic chromosome number $2n = 40$ (Fig. 2-1). Twelve plants out of 76 counted from these populations had $2n = 41$ or $2n = 42$. These were considered to be aneuploids. Most plants from the Pawnee, Wray, Antero, Rock River, Albuquerque, Goose Lake, and Honey Lake populations had somatic chromosome number $2n = 38$ (Fig. 2-1). Four plants out of 108 counted from these populations had $2n = 39$ or $2n = 41$. These were considered to be aneuploids. An additional 13 plants in these populations had $2n = 56$, $2n = 57$, or $2n = 74$. These were considered to be hexaploids and octaploids. The ten progeny from the cross of Archery 5, a 38-chromosome plant, and Washoe 16, a 40-chromosome plant, showed variable chromosome numbers from $2n = 38$ to $2n = 40$. The two *D. littoralis* plants had chromosome numbers of $2n = 40$. A few accessions from the San Francisco Bay, Rock River, Pawnee, and Honey Lake populations had small, dot-like, dark bodies that were present in every squash in addition to the 38 or 40 chromosomes of normal size. These small bodies were interpreted as B chromosomes. B chromosomes are extra, dispensable chromosomes, variable in number and smaller than the chromosomes typical for a species (Swanson et al., 1981; Appels et al., 1998). A summary of the results for each population is shown in Table 2-2. Results for individual samples are shown in Appendix 1.

Chloroplast variation—An unrooted tree based on restriction sites in the chloroplast genome revealed two main groups among accessions (Fig. 2-2). One group

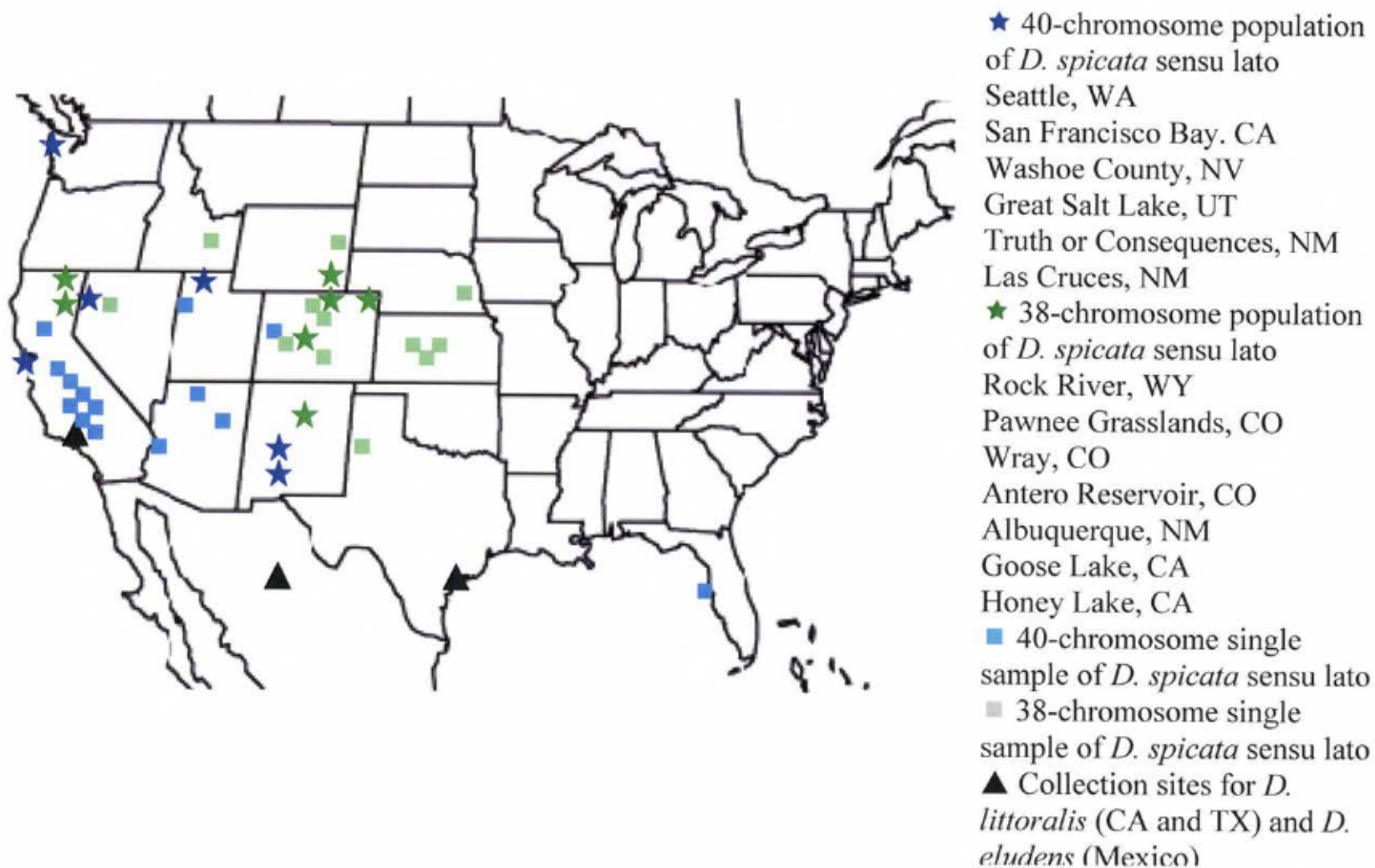


Fig. 2-1. Map of chromosome numbers at collection sites for populations and single samples of *Distichlis* in North America.

Table 2-2. Summary of somatic chromosome numbers in accessions of *Distichlis spicata* s.l. in the western United States.

	Somatic chromosome number									
	38	39	40	41	42	56	57	ca. 74	multi- ple num- bers	not det. *
Population										
San Francisco Bay, CA			18		1					1
Washoe County, NV			15	1						4
Great Salt Lake, UT			14	2	1					3
Rock River, WY	10									0
Antero Reservoir, CO	17									3
Wray, CO	15						1			4
Pawnee Grassland, CO	19					2	4	5		0
Honey Lake, CA	13	1		1						0
Goose Lake, CA	12			2			1			0
Albuquerque, NM	5									0
Truth or Consequences, NM			3		3				1	1
Las Cruces, NM			10	1	3					0
Seattle, WA			3							5
Archery5 X Washoe16	2	4	2						1	2

* not determined

was distinguished by the lack of a restriction site for Hin6I in the trnS-psbD segment, with one exception discussed below, while the other group had a single restriction site for Hin6I that produced two fragments of size 480 bp and 365 bp from the trnS-psbD segment. The red line in Fig. 2-2 separates the “no Hin6I site” group on the left from the “Hin6I site” group on the right. The populations from Seattle, San Francisco, Washoe, Great Salt Lake, Truth or Consequences, and Las Cruces and the single sample from Florida lacked the Hin6I restriction site. The populations from Pawnee, Wray, Rock River, Antero, Albuquerque, Goose Lake, and Honey Lake and the samples of *D. littoralis* and *D. eludens* had the Hin6I restriction site.

Seattle 07008 differed from the other samples in two ways. The trnS-psbD segment for this sample was 865 bp long, in contrast to 860 bp in all other samples, and there was a Hin6I restriction site that produced fragments of 525 bp and 335 bp, in contrast to fragments of 480 bp and 365 bp in all other samples with a Hin6I restriction site.

A table showing presence/absence of all restriction sites is given in Appendix 2.

Microsatellite variation—All populations contained multiple alleles at all four microsatellite loci. Null alleles were found at some loci. The assumed microsatellite profile for each sample is given in Appendix 3. Nine of the 13 populations had at least one private allele (Table 2-3), i.e., an allele found only in that population. Four of the six 40-chromosome populations had private alleles. Five of the seven 38-chromosome populations had private alleles. Not counting alleles that were private to a single population, 12 alleles were found exclusively in the 40-chromosome group while 15 alleles were found exclusively in the 38-chromosome group (Table 2-4).

Table 2-3. Microsatellite private alleles, found in single populations of *Distichlis spicata* s.l. collected in the western United States.

Population	Locus	Allele	Frequency
Washoe	1	258	0.050
Washoe	1	260	0.150
Washoe	2	206	0.025
Truth or Consequences	1	252	0.063
Seattle	1	270	0.125
Antero	1	214	0.150
Pawnee	1	210	0.017
Rock River	4	174	0.100
Wray	3	168	0.050
Honey Lake	4	152	0.200
Honey Lake	4	194	0.167
Goose Lake	3	202	0.133
Goose Lake	4	202	0.067

Table 2-4. Alleles found only in the 38- or the 40-chromosome group, excluding alleles that are private to a single population, in *Distichlis spicata* s.l. collected in the western United States.

Chromosome group	Locus code ^a	Allele	Freq.	No. of pops	Population code ^b	Chromosome group	Locus code ^a	Allele	Freq.	No. of pops	Population code ^b
40	1	218	0.017	2	SF, TC	38	1	null	0.383	7	found in all pops
40	1	221	0.017	3	SF, SL, CR	38	1	233	0.074	3	An, Wr, Go
40	1	225	0.017	2	SF, TC						
40	1	238	0.078	5	SE, SF, SL, TC, CR						
40	1	244	0.028	2	TC, CR						
40	1	246	0.011	2	TC, CR						
40	1	254	0.033	2	WA, SL						
40	1	256	0.044	2	WA, CR						
40	2	214	0.017	2	SB, TC	38	2	216	0.017	2	Pa, Wr
40	2	224	0.033	2	SE, WA						
40	3	180	0.189	4	SB, SL, TC, CR	38	3	186	0.052	4	Pa, Wr, Go, Ho
40	3	183	0.039	3	SF, SL, CR	38	3	188	0.183	4	Pa, Rr, Go, Ho
						38	3	190	0.187	7	found in all pops
						38	3	198	0.061	2	Pa, Wr
						38	4	null	0.043	3	An, Pa, Wr
						38	4	150	0.100	4	An, Pa, Rr, Wr
						38	4	178	0.191	6	Al, Go, Ho, Wr, Rr, Pa
						38	4	182	0.030	2	Rr, Ho
						38	4	186	0.013	2	Go, Ho
						38	4	190	0.009	2	Go, Ho
						38	4	198	0.017	2	Go, Ho
						38	4	206	0.026	2	Go, Al

^aLocus code: 1 = Dis 1 primer pair, 2 = Dis 3 primer pair, 3 = Dis 10 primer pair, 4 = Dis 22 primer pair. See Materials and Methods.

^bPopulation code: SE = Seattle, SF = San Francisco Bay, SL = Great Salt Lake, WA = Washoe County, TC = Truth or Consequences, CR = Las Cruces, Rr = Rock River, Pa = Pawnee, An = Antero, Wr = Wray, Go = Goose Lake, Ho = Honey Lake, Al = Albuquerque.

The Florida sample had several alleles not found in the other 40-chromosome plants, along with alleles that were specific to the 40-chromosome lineage or were found in both lineages (Table 2-5). None of the alleles in the Florida sample was specific to the 38-chromosome lineage. The *D. littoralis* and *D. eludens* samples were null for three of the microsatellite loci (Table 2-5). At locus 4, *D. eludens* had one allele not found in the *D. spicata* lineages and one allele found in both lineages. At locus 4 the two *D. littoralis* samples each had two different alleles, for a total of four, that were not found in the *D. spicata* populations or in the Florida or *D. eludens* samples.

An unrooted tree (Fig. 2-3) using Nei's (1972) genetic distance between populations shows closely clustered collections from Seattle, Washington; San Francisco Bay, California; Washoe County, Nevada; Great Salt Lake, Utah; Truth or Consequences, New Mexico; and Las Cruces, New Mexico. Collections from Rock River, Wyoming; Pawnee Grasslands, Colorado; Wray, Colorado; Antero Reservoir, Colorado; Albuquerque, New Mexico; Goose Lake, California; and Honey Lake, California, are more distantly spaced than the collections from Seattle, San Francisco Bay, Washoe County, Great Salt Lake, Truth or Consequences, and Las Cruces.

Genetic distance (linearized F_{st} , Slatkin, 1993) between populations was correlated with geographic distance (Fig. 2-4) ($p = 0.05$) in the Mantel test. The regressions within chromosome lineages ($R^2 = 0.2781$ within the 38-chromosome lineage, $R^2 = 0.1477$ within the 40-chromosome lineage) indicated a higher correlation within than across chromosome lineages ($R^2 = 0.0002$ for 38-chromosome populations paired with 40-chromosome populations).

Table 2-5. Microsatellite bands in accessions of *D. spicata* from Florida, *D. littoralis* from California and Texas, and *D. eludens* from Mexico.

Accession ID	Chromosome number	Locus	Allele	Comments
Florida	40	1	230	Present in both <i>D. spicata</i> lineages
		1	200	Present only in this accession
		2	224	Present in the 40-chromosome lineage
		2	220	Present only in this accession
		3	184	Present in both <i>D. spicata</i> lineages
		3	165	Present only in this accession
		4	170	Present in both <i>D. spicata</i> lineages
		4	166	Present in both <i>D. spicata</i> lineages
Monan CA	40	4	221	Present only in this accession
		4	110	Present only in this accession
Monan TX	40	4	220	Present only in this accession
		4	109	Present only in this accession
Reeder	38	4	160	Present only in this accession
		4	158	Present in both <i>D. spicata</i> lineages

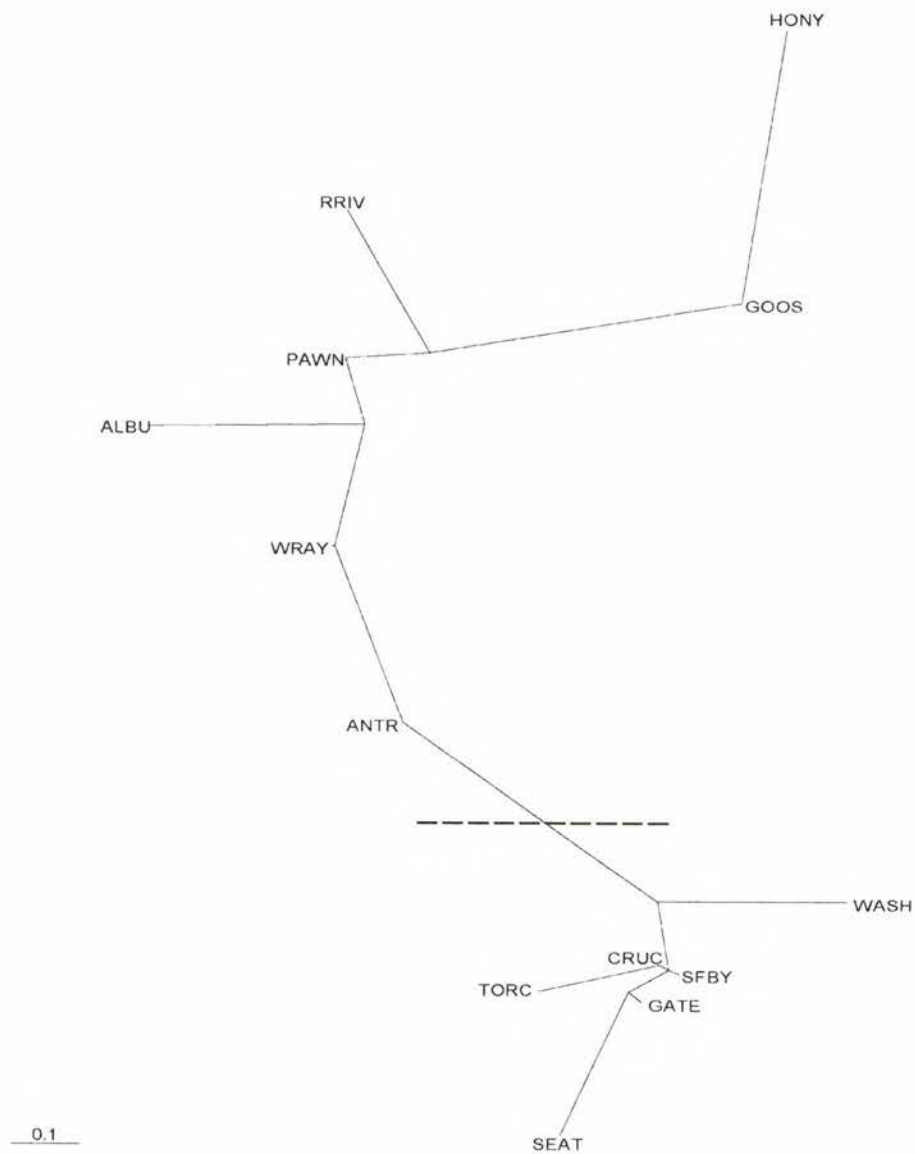


Fig. 2-3. Unrooted tree diagramming Nei's (1972) genetic distance between populations of *Distichlis spicata* s.l. in the western United States. Populations above the dashed line have 38 chromosomes. Populations below the dashed line have 40 chromosomes.

**Linearized Fst vs. Geographic Distance
for All Pairwise Combinations of *Distichlis* Populations**

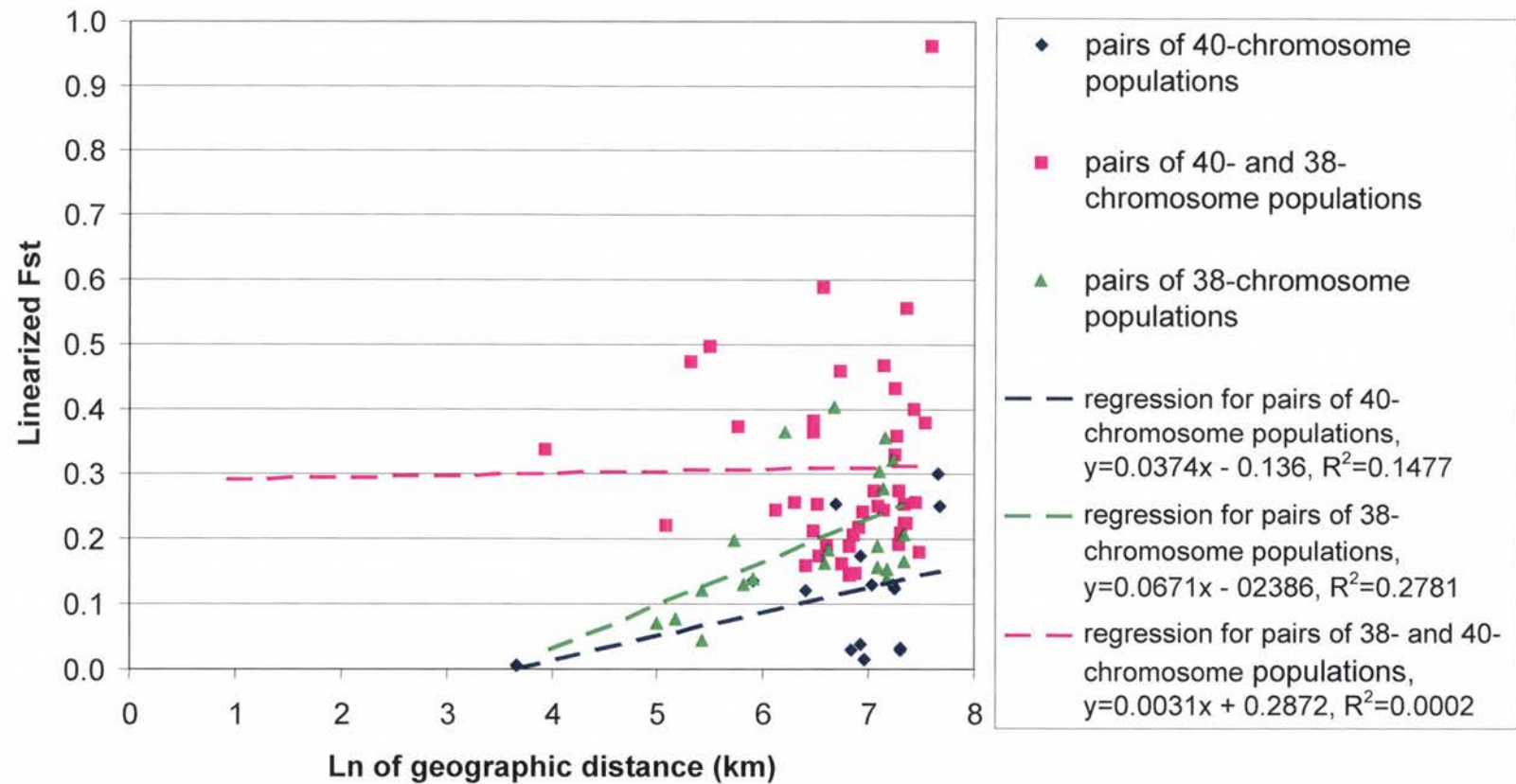


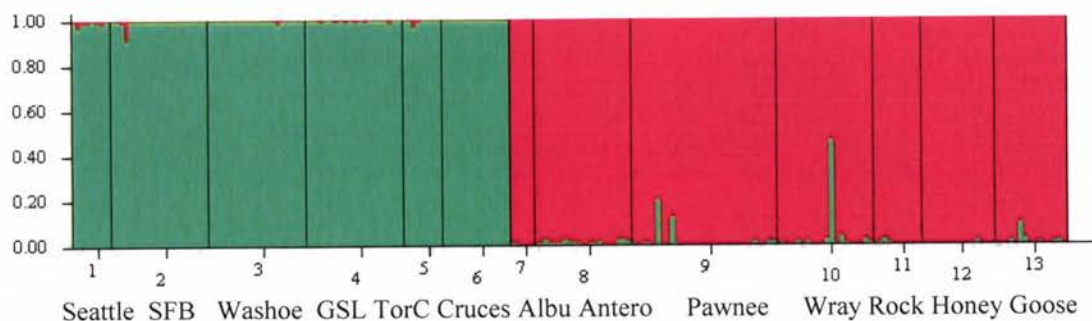
Fig. 2-4. Linearized Fst (Slatkin, 1993) vs. ln of geographic distance for pairs of populations of *Distichlis spicata* s.l. collected in the western United States.

Most gels lanes showed a maximum of two microsatellite alleles per plant, but nineteen plants had three alleles at one or more loci.

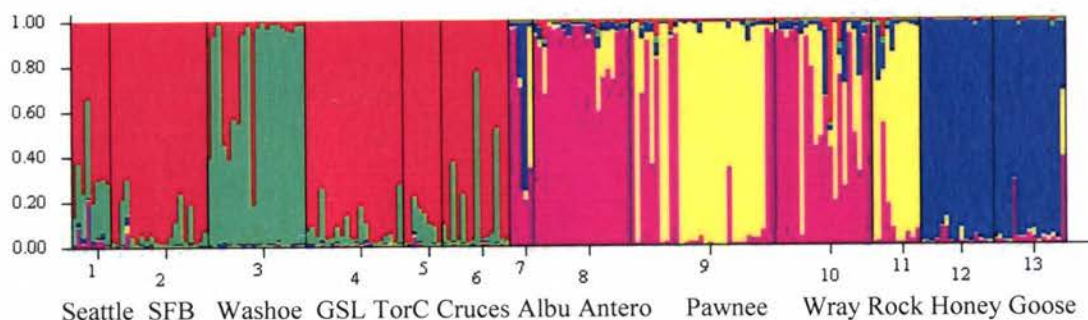
Combined data set for chromosome numbers, chloroplast sequences, and microsatellite alleles--The program STRUCTURE 2.3.3 (Pritchard et al., 2000, 2009) combined with the cluster-estimating statistic Δk (Evanno et al., 2005) identified two main groups and five subgroups within the 13 collection locations (Fig. 2-5). The two main groups were the 40-chromosome populations and the 38-chromosome populations. The 40-chromosome group was divided into two subgroups, with San Francisco Bay/Salt Lake/Truth or Consequences/Las Cruces as one subgroup and Washoe as the other subgroup. The 38-chromosome group was divided into three subgroups, with Albuquerque/Antero/Wray as one subgroup, Pawnee/Rock River as a second subgroup, and Honey Lake/Goose Lake as a third subgroup.

RAPDs—Forty-one RAPD fragments amplified in 68 accessions by OPD16 caused plants to cluster into two groups (Fig. 2-6). One group contained samples from San Francisco Bay, Washoe, and Great Salt Lake, all 40-chromosome populations. The other group contained samples from Pawnee, Wray, Antero, and Rock River, all 38-chromosome populations. RAPD OPF13 amplified a band at about 450 bp (Eppley, 1998) (Fig. 2-7) in female plants that had 40 chromosomes, but not in female plants that had 38 chromosomes.

DNA C-values—Mean 2C-values were about 13% higher for 38-chromosome plants (1.526 pg) than for 40-chromosome plants (1.349 pg) ($p = 0.000000054$) (Fig. 2-8).

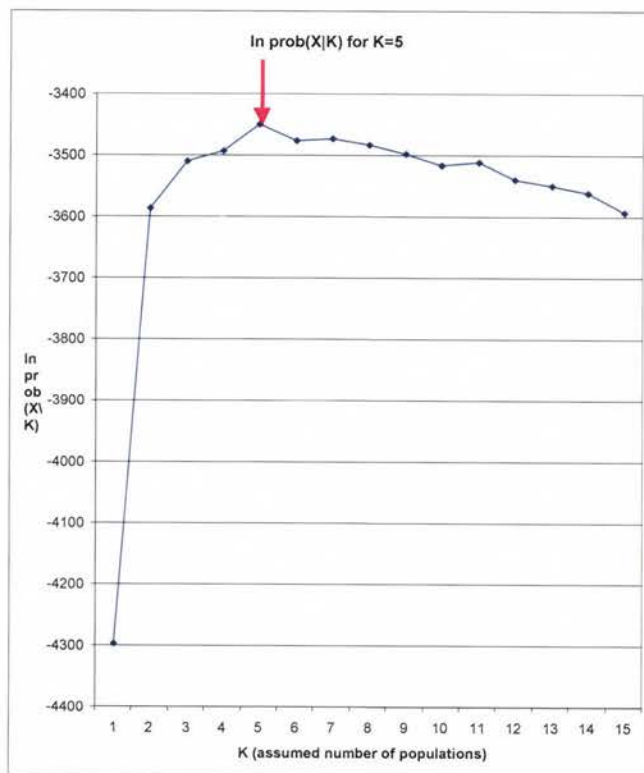
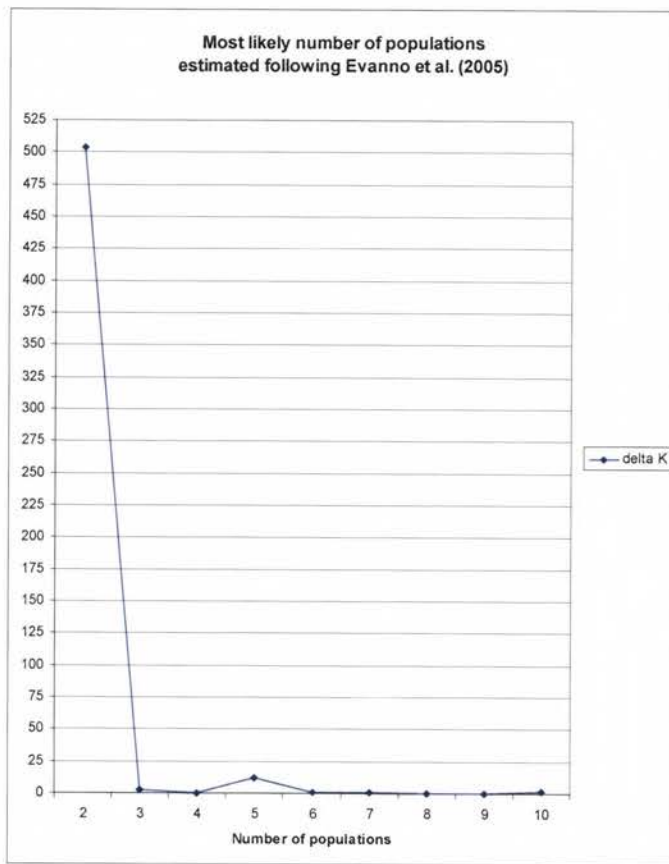


K = 2



K = 5

Fig. 2-5. Inferred population cluster diagram using the software program Structure (Pritchard et al., 2000; Pritchard et al., 2009) with a combined data set of chromosome number, chloroplast haplotype, and microsatellite alleles found in *Distichlis spicata* sensu lato samples from 13 locations in the western U.S. The locations are (1) Seattle, Washington, (2) San Francisco Bay, California, (3) Washoe County, Nevada, (4) Great Salt Lake, Utah, (5) Truth or Consequences, New Mexico, (6) Las Cruces, New Mexico, (7) Albuquerque, New Mexico, (8) Antero Reservoir, Colorado, (9) Pawnee Grassland, Colorado, (10) Wray, Colorado, (11) Rock River, Wyoming, (12) Honey Lake, California, and (13) Goose Lake, California. Two main groups are estimated by the cluster-estimating statistic developed by Evanno et al. (2005) shown at top on the facing page. Five groups are estimated by the method described in Structure, graph shown at bottom on the facing page.



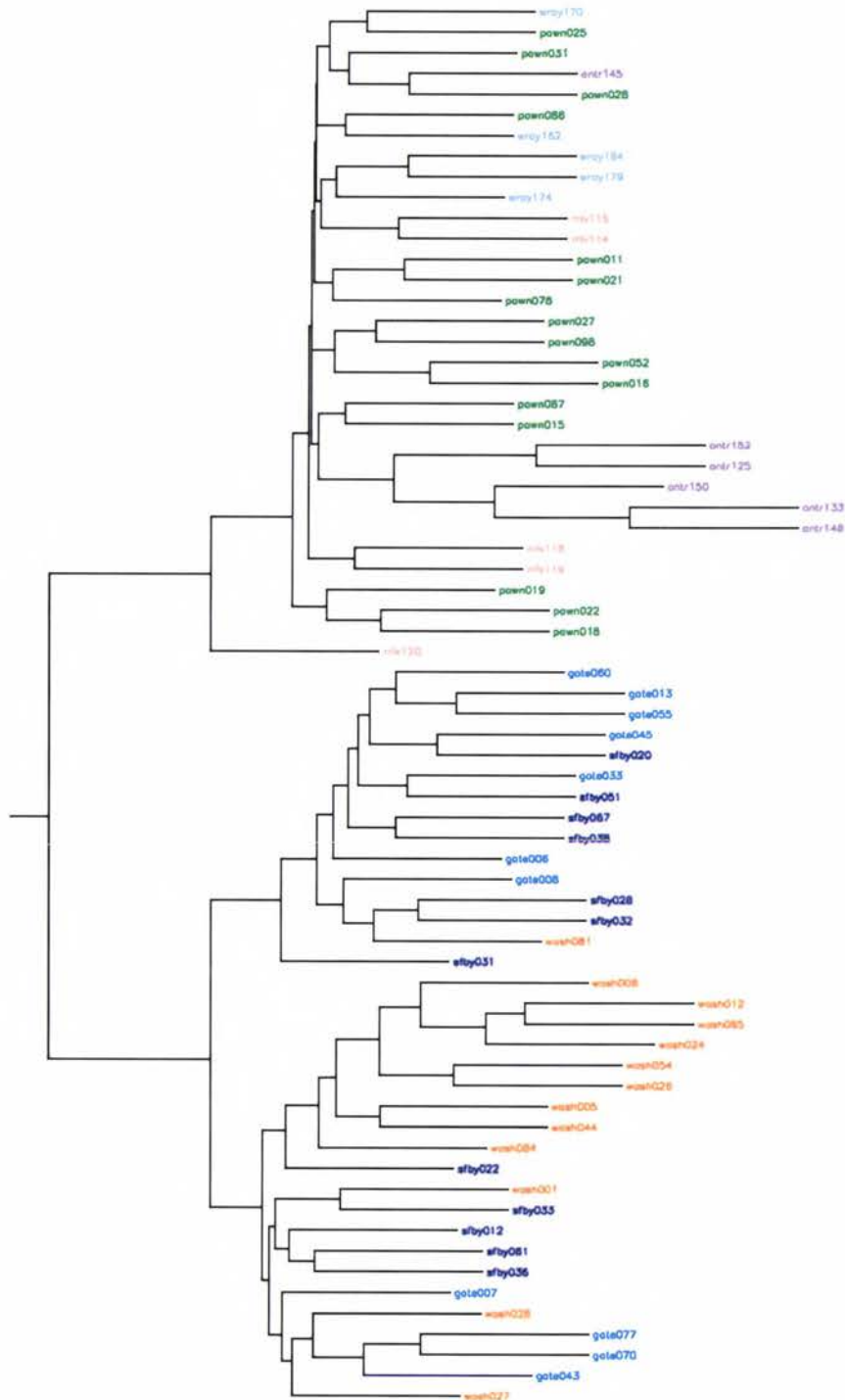


Fig. 2-6. Clustering diagram based on 41 bands amplified by RAPD primer OPD16. The top branch leads to 38-chromosome plants with branch support of 96%. The bottom branch leads to 40-chromosome plants with branch support of 97%.

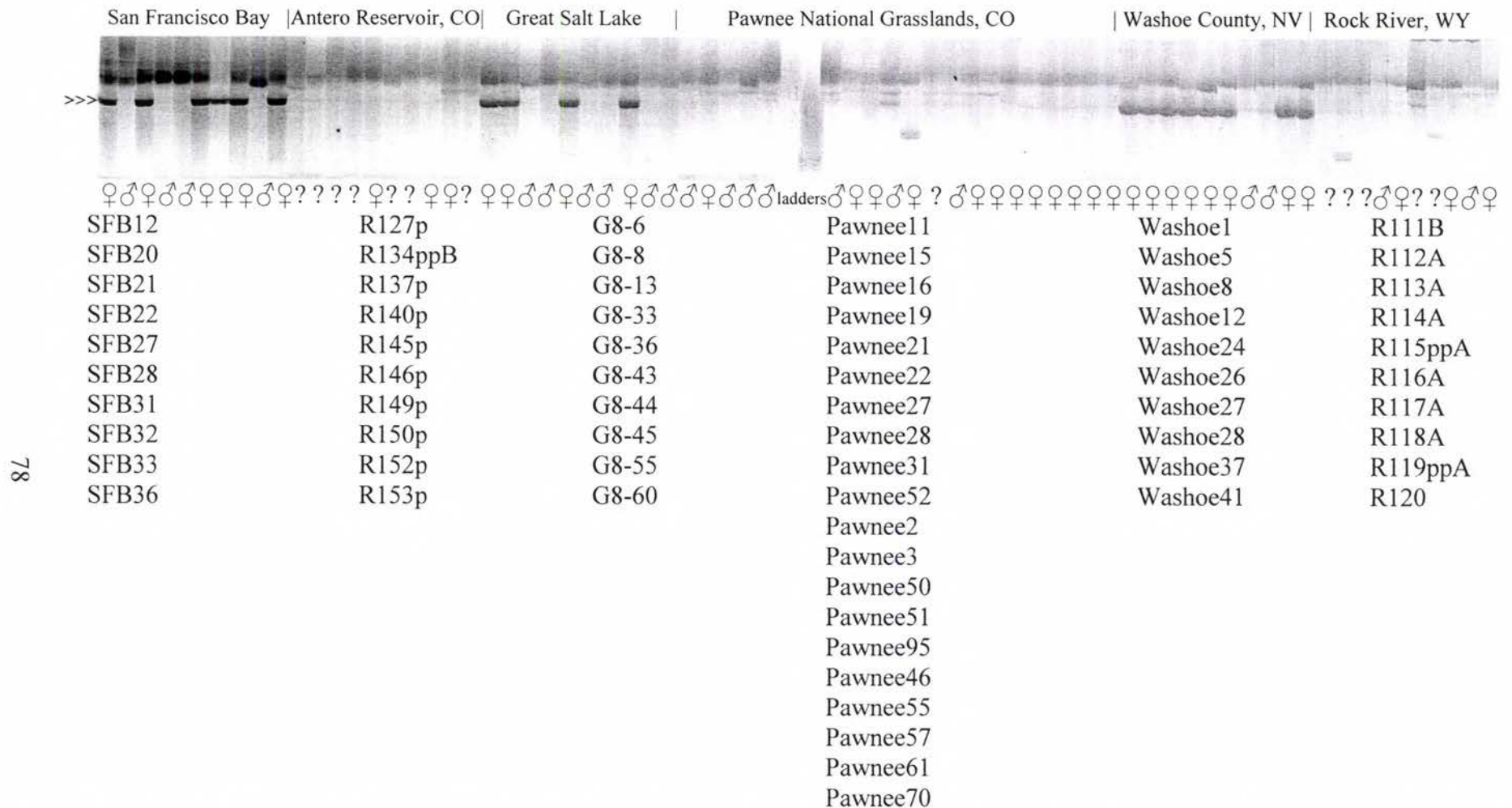


Fig. 2-7. RAPD bands amplified by primer OPF13 from samples of *Distichlis spicata* s.l. collected in the western United States. San Francisco Bay, Great Salt Lake, and Washoe County are 40-chromosome populations. Antero Reservoir, Pawnee, and Rock River are 38-chromosome populations. Sample names are listed below the image, population names above the gel lanes. Known sexes of plants are indicated. A question mark indicates the sex of the plant is not known. Arrowheads mark the location of the band present in females (Eppley, 1998).

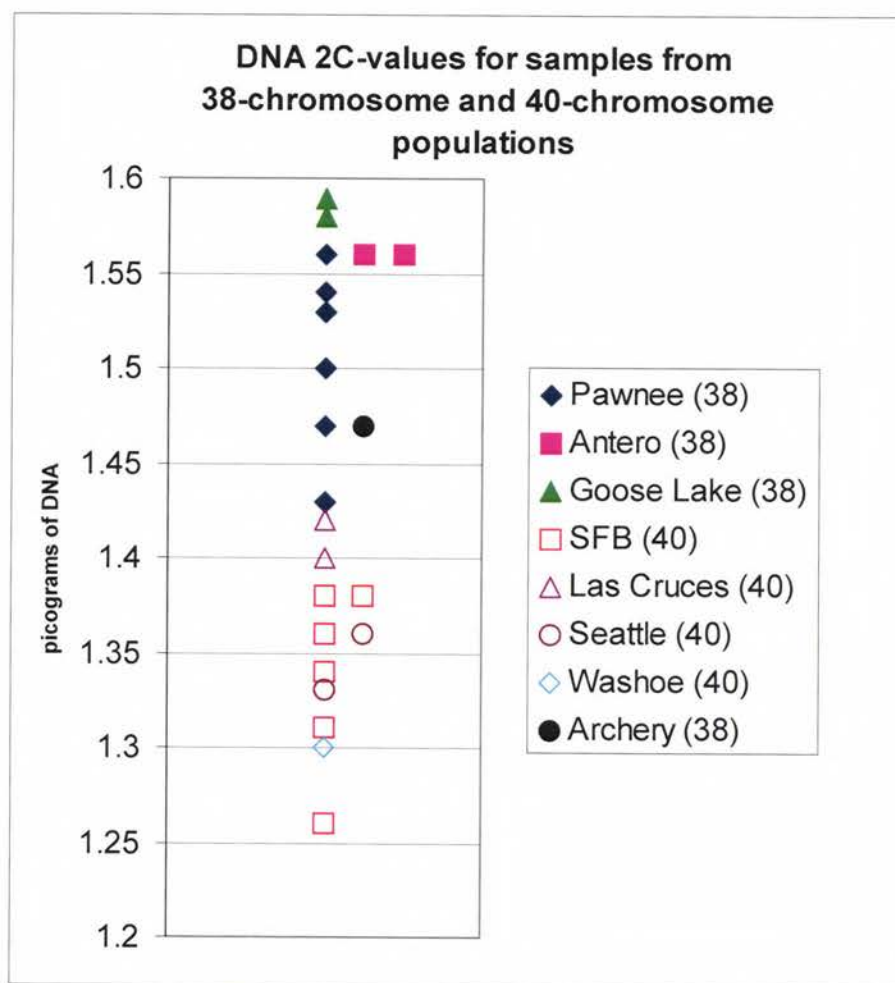


Fig. 2-8. DNA 2C-values for 38-chromosome (solid symbols) and 40-chromosome (open symbols) plants of *Distichlis spicata* s.l. collected in the western United States.

Flowering—Plants from the Pawnee location flowered about one month earlier than plants from the San Francisco Bay, Washoe, and Great Salt Lake locations (Fig. 2-9).

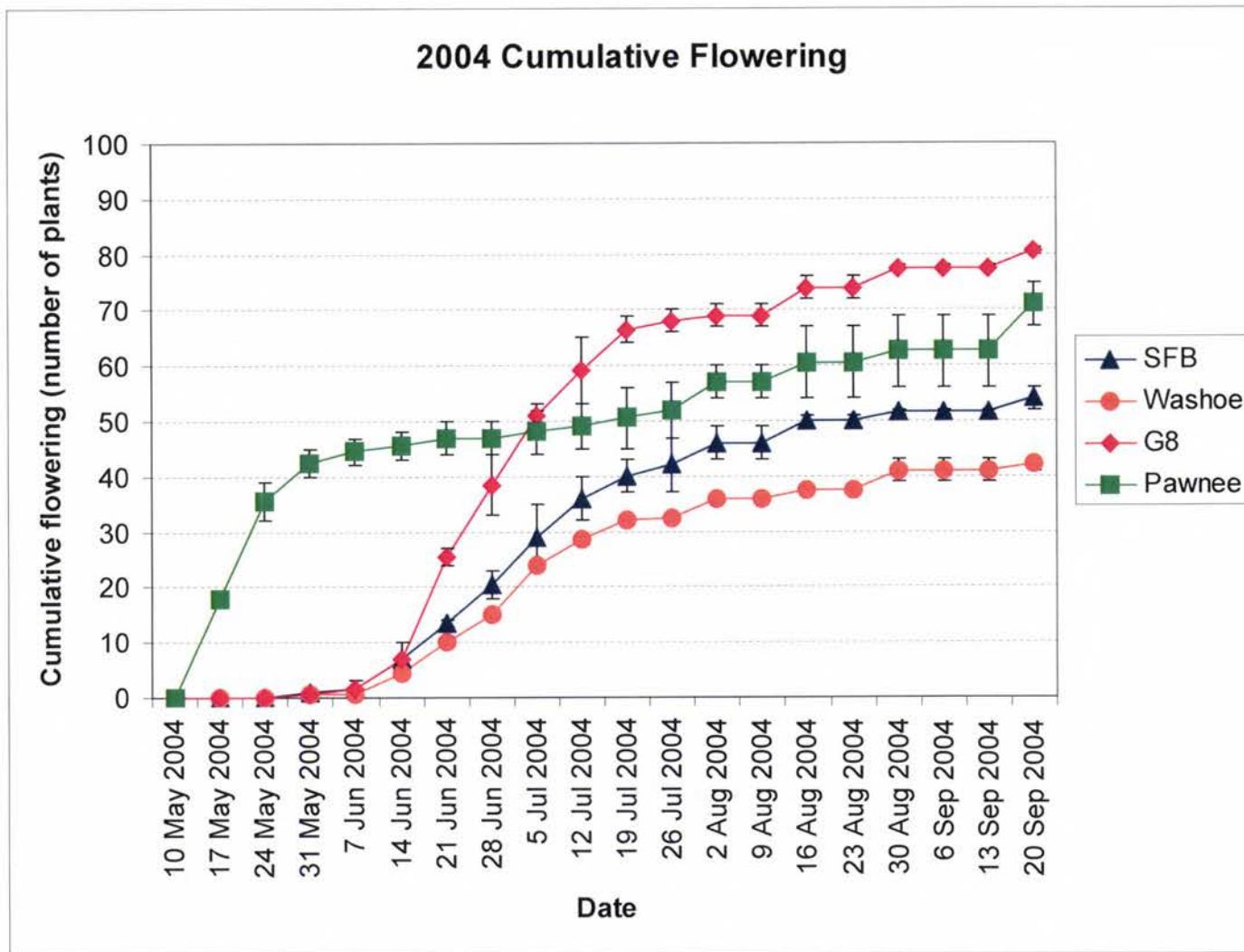


Fig. 2-9a. Cumulative flowering in 2004 for four populations of *Distichlis spicata* s.l. planted in a common garden at Fort Collins, Colorado, USA. Two vegetative replications of each plant were used. Standard error bars are shown.

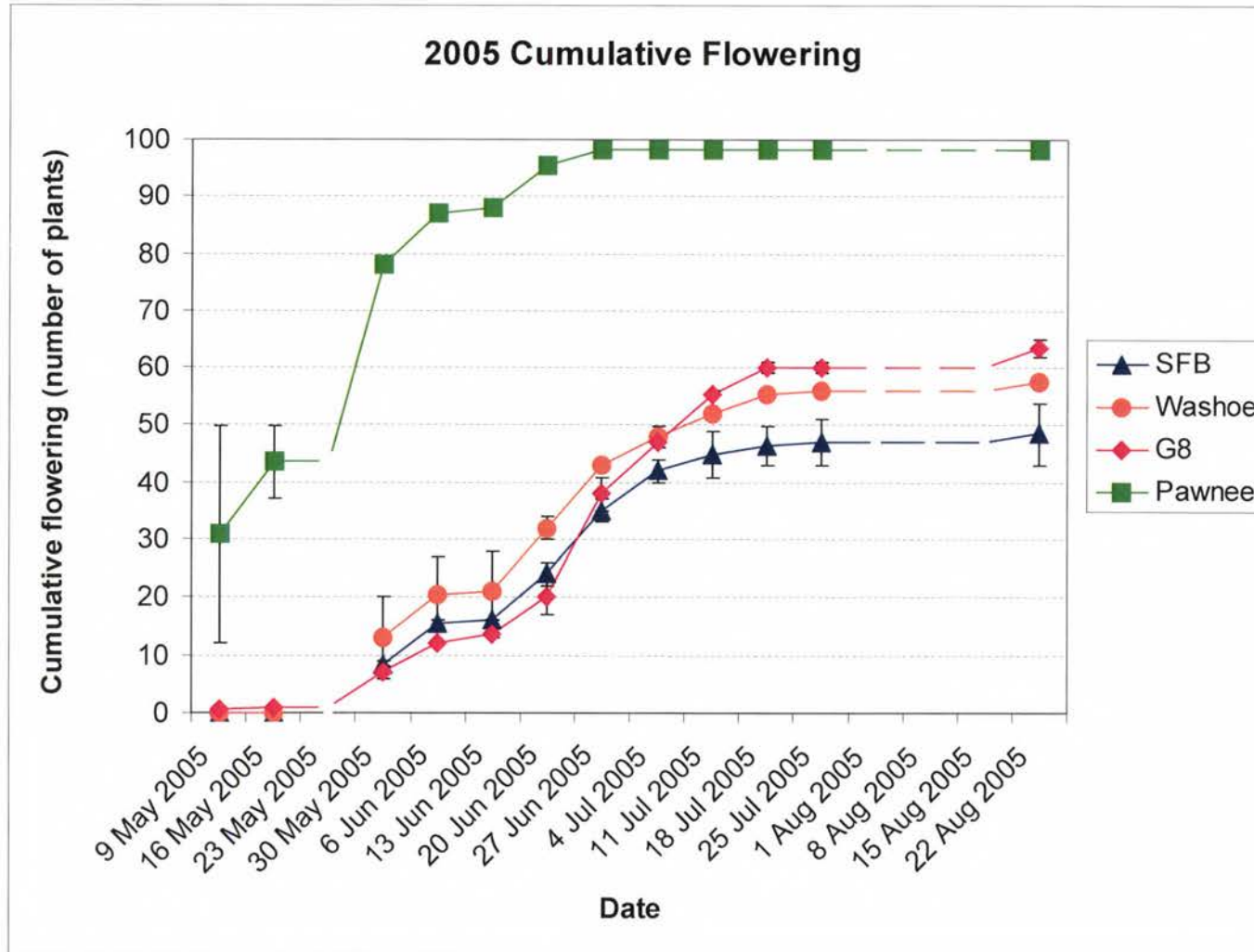


Fig. 2-9b. Cumulative flowering in 2005 for four populations of *Distichlis spicata* s.l. planted in a common garden at Fort Collins, Colorado, USA. Two vegetative replications of each plant were used. Standard error bars are shown. Missing symbols indicate no data was collected that week.

DISCUSSION AND CONCLUSIONS

Several congruent lines of evidence suggest that *D. spicata* s.l. in western North America consists of two species. These species are distinguished from each other by their chromosome numbers, chloroplast haplotypes, microsatellite profiles, RAPD profiles, and DNA content. One species encompasses plants from the West Coast and several inland locations in Nevada, Utah, and southern New Mexico. The other species consists of plants distributed only inland among the populations surveyed. The populations from Seattle, San Francisco Bay, Washoe, Great Salt Lake, Truth or Consequences, and Las Cruces had a predominant somatic chromosome number of 40. The plants from these six populations were closely related genetically, having almost identical chloroplast haplotypes and similar microsatellite profiles. Among these, the three populations used for RAPD profiling – the San Francisco Bay, Washoe, and Great Salt Lake populations -- had similar patterns. These genetic similarities are reflected in the positions of the populations in the trees based on chloroplast haplotype (Fig. 2-2), microsatellite profile (Fig. 2-3), and OPD16 RAPD profile (Fig. 2-6). The OPF13 RAPD primer that amplified a sex-diagnostic band in female plants from coastal California (Eppley, 1998) also amplified a sex-diagnostic band for female plants from San Francisco Bay, Washoe, and Great Salt Lake (Fig. 2-7). DNA 2C-values (Fig. 2-8) for a subset of 40-chromosome plants from Seattle, San Francisco Bay, Washoe, and Las Cruces averaged 1.349 pg, 12% lower than the DNA 2C-values for plants from 38-chromosome populations.

In contrast to the 40-chromosome populations, the populations from Pawnee, Wray, Rock River, Antero, Albuquerque, Honey Lake and Goose Lake had a

predominant chromosome number of 38 or a higher ploidy based on multiples of that number. Chloroplast haplotypes (Fig. 2-2) were more varied among the 38-chromosome plants than among the 40-chromosome plants, but all the variations are more similar to each other than to the almost-monomorphic chloroplast haplotype found in the 40-chromosome lineage. The profiles for microsatellite alleles (Fig. 2-3) were related among these populations, as were the profiles for OPD16 RAPD bands (Fig. 2-6). In addition to these similarities, the OPF13 RAPD primer that amplified a sex-diagnostic band in 40-chromosome female plants did not amplify a band in 38-chromosome female plants (Fig. 2-7). DNA 2C-values (Fig. 2-8) for a subset of plants from Pawnee, Antero, Goose Lake, and Archery averaged 1.526 pg, 13% higher than the DNA 2C-values for plants from 40-chromosome populations.

The regression lines showing the trend toward greater genetic distance with increasing geographic distance for the 38-chromosome and 40-chromosome lineages in Fig. 2-4 cross the X-axis at about 39 km, suggesting that each chromosome lineage is panmictic (fully interbreeding) within itself when populations are separated by this distance or less. The regression line for pairs involving one 38-chromosome and one 40-chromosome population does not cross the X-axis at a positive number, indicating that these two lineages are not panmictic even when they are sympatric.

DNA 2C-values—A gradient of DNA content corresponding to environment has been reported in some species (Price et al., 1981; Ceccarelli et al., 1991), but this study did not find a gradient corresponding to moisture, elevation, or latitude in *D. spicata* s.l. A significant divergence in 2C-DNA content corresponded to chromosome number.

Crossing experiment--Results from the crossing experiment are consistent with the indication from the genetic data of two separate lineages. The progeny showed unstable chromosome numbers, suggesting that mitosis does not proceed normally in hybrids between the two chromosome lineages. The expected somatic chromosome number in a hybrid is 39. A chromosome complement of 38, found in two progeny, suggests that one chromosome was eliminated. A chromosome number of 40 or more, found in one progeny and also earlier by Reid (2001), suggests that one or more chromosomes were duplicated beyond the normal duplication in advance of mitosis, or that nondisjunction or abnormal distribution of chromosomes to daughter nuclei had occurred. Meiosis in hybrids was not examined in this study, so it is not known if the hybrids have normal fertility.

Flowering times--Results from the common garden experiment are congruent with the genetic data indicating that the two lineages do not hybridize in natural settings. The 38-chromosome plants flowered about one month earlier than the 40-chromosome plants (Fig. 2-9), suggesting a possible mechanism for the continued separation of the two lineages despite their proximity in some areas. Different flowering times would reduce gene flow between the lineages when they occur in proximity, as the Washoe and Honey Lake populations do. The 40-chromosome plants originated from different environments – sea level and coastal in the case of San Francisco Bay, 1,200 m above sea level and a dry plain in the case of Washoe, 1,280 m above sea level and a lake in the case of Great Salt Lake – yet began flowering nearly the same week (early June in 2004, late May in 2005) and showed similar S-shaped curves of cumulative flowering over the summer. The 38-chromosome plants, represented by the Pawnee population, began

flowering about one month earlier than the 40-chromosome plants, although they originated at an elevation of 1,500 m above sea level, similar to the elevation at Great Salt Lake, and in a dry environment similar to that at Washoe. Latitudes of origin are similar for all four populations. Latitude varies from 38 North to 41 North for the 40-chromosome populations. The latitude of the 38-chromosome population is 40.8 North. This suggests that the difference in flowering is not a result of adaptation to different latitude, altitude, or availability of water.

Exceptions to the main patterns--Sample 07008 from Seattle had a longer than normal trnS-psbD chloroplast segment with a Hin6I restriction site in it, unlike all other 40-chromosome plants in this study. Fragment lengths for this chloroplast segment were considerably different from those in 38-chromosome plants, suggesting that the sample had an insertion that contained a novel Hin6I restriction site, rather than a sequence corresponding to the site in 38-chromosome plants. Fragment lengths for the other chloroplast segments in Seattle 07008 were characteristic for the “no Hin6I site” group and this sample clustered with the “no Hin6I site” plants in the chloroplast diagram (Fig. 2-2).

Although both the 38-chromosome lineage and the 40-chromosome lineage are believed to be tetraploid, most gel lanes showed a maximum of two alleles per microsatellite locus. Four alleles are potentially present in autotetraploid plants. The presence of only two alleles suggests that two of the four haploid chromosome complements lacked priming sites for these microsatellite loci. While most gel lanes showed only two alleles, nineteen plants had three alleles at one or more loci. Twelve of these 19 plants had somatic chromosome numbers of 56, 57, 74, or ca. 74, indicating that

they were hexaploid or octoploid. The third allele at a locus in these plants thus can be attributed to the presence of additional chromosome complements beyond the tetraploid complement. However the remaining seven plants that displayed a third allele had chromosome numbers of $2n = 38$ or $2n = 40$. One plant from Pawnee, two plants from Rock River, and four plants from Washoe had third alleles that could not be explained by the presence of additional sets of chromosomes. The third alleles in these seven plants had lengths identical to alleles commonly present in other plants. In the absence of sequencing, it is assumed from the length that these were not artifacts of the amplification process. It is possible that local tandem duplications have created additional microsatellite loci or that rare crossing-over events have transferred microsatellite loci to chromosomes that did not originally possess them.

Possible origin of the chromosome lineages--Grasses having three or more sets of the basic number (x) of chromosomes representative of their subfamily are considered to be polyploids (Gould and Shaw, 1983). The subfamily Chloridoideae, of which *Distichlis* is a member, has two basic chromosome numbers, $x = 9$ and $x = 10$ (Reeder, 1977; Gould and Shaw, 1983; Roodt and Spies, 2003a; Hilu, 2004). Chloridoid grasses with 38 or 40 somatic chromosomes are therefore considered to be tetraploids. Most of the polyploid species in the subfamily Chloridoideae are allopolyploids (Roodt and Spies, 2003b), that is, they are the result of hybridization between plants of two or more different species and they contain haploid genomes originating from more than one species. Evidence from meiosis (Reid, 2001) suggests that both of the lineages in this study are allotetraploids. The 40-chromosome plants form 20 bivalents, or sometimes 19 bivalents and two monovalents, at diakinesis. The 38-chromosome plants form 19

bivalents at diakinesis. The presence of bivalents rather than multivalents in a polyploid plant is consistent with a hybridization event combining two different genomes that do not synapse with each other during meiosis. Deficient octoploid individuals (those with ca. 74 chromosomes) from 38-chromosome regions do show multivalents at diakinesis (Reid, 2001), suggesting that more than two sets of homologs are synapsing during meiosis. These deficient octaploid plants may have been derived via autopoloidy from the 38-chromosome tetraploids. Autopoloidy involves the accumulation of extra same-species chromosome sets above the number usually carried by members of a species. This can occur through fertilization involving unreduced gametes or by spontaneous doubling of the chromosome complement.

The two basic chromosome numbers in the Chloridoid grass subfamily can be combined in various ways to produce both 38-chromosome and 40-chromosome plants. An allotetraploid derived from two species each having 20 chromosomes would have 40 chromosomes, the number found in the “coastal” *D. spicata* lineage and also in *D. littoralis*. An allotetraploid derived from one species with 20 chromosomes and one species with 18 chromosomes would have 38 chromosomes, the number found in the “inland” *D. spicata* lineage and also in *D. eludens* (Reeder, 1967). The distinctive chloroplast haplotypes found in the two *D. spicata* lineages, including the presence or absence of the *Hin6I* restriction site and other restriction sites characteristic of each lineage, suggest that different maternal parents were involved in the polyploidization events that may have given rise to these lineages. The presence of the *Hin6I* restriction site in chloroplasts of *D. littoralis* and *D. eludens* hints at a common maternal parent in the backgrounds of these two species and the 38-chromosome lineage of *D. spicata*,

distinct from the maternal parent of the 40-chromosome lineage of *D. spicata*. An alternative to the hypothesis of a common maternal origin is the hypothesis that introgression of the chloroplast occurred via hybridization among *D. littoralis*, *D. eludens*, and the 38-chromosome lineage of *D. spicata* in past eras. Modern-day introgression seems unlikely because *D. littoralis* is found in coastal regions where it would be more likely to hybridize with the 40-chromosome lineage of *D. spicata* than with either *D. eludens* or the 38-chromosome lineage of *D. spicata*, both of which are distributed inland.

The microsatellite and RAPD phylogenies are consistent with the chromosome numbers and chloroplast haplotypes that defined the two lineages in this study. This congruence of multiple molecular traits suggests that the two groups had different genetic sequences at their inception and there has been little homogenizing gene flow between them subsequently. The presence of two alleles per plant for most of the microsatellite loci in most of the populations in this study, coupled with the presence of three alleles for the microsatellites in some of the hexaploid and deficient octoploid plants in the Pawnee population, suggests that two copies of the targeted microsatellites exist in tetraploid plants while three copies exist in the hexaploid and deficient octoploid plants. This supports the idea that the tetraploid plants are allotetraploids, having two copies of one kind of genome and two copies of another kind of genome, commonly represented by such formulas as AABB for the known allotetraploid *Triticum durum* Desf. and AADD for the known allotetraploid *Gossypium barbadense* L.

Microsatellite results for *D. eludens* and *D. littoralis* offer limited support for the allopolyploid hypothesis. Microsatellite bands were amplified only for locus 4 in the *D.*

littoralis and *D. eludens* samples. At this locus, *D. eludens* had one allele not found in the *D. spicata* lineages and one allele found in both of the lineages. The two *D. littoralis* samples had four different alleles not found in the *D. spicata* populations or in the Florida or *D. eludens* samples. The amplification at only one locus could indicate the sharing of only one genome with the *D. spicata* lineages, the mutation over time of priming sites associated with the other three microsatellite loci, or a lack of close relationship among the taxa and a merely coincidental occurrence of alleles amplifiable by the primers. Like the two *D. spicata* lineages, *D. littoralis* and *D. eludens* specimens contain two alleles per individual at locus 4, suggesting that these species are allotetraploids.

Under one scenario, *D. eludens*, *D. littoralis*, and the two lineages of *D. spicata* would form a reticulated network of species derived by hybridization and polyploidization from diploid ancestors, similar to the patterns known from wheat (Hancock, 1992), rice (Oka, 1988; Ge et al., 1999; Brar and Ramos, 2008), and cotton (Hancock, 1992). In these latter cases, many of the diploid ancestors are extant. The A and D genomes of wheat exist as diploids as well as in tetraploid and hexaploid relatives. The B, C, E, G, and F genomes of rice exist as diploid species as well as being components of tetraploid species. The B wheat genome and the D, H, J, and K rice genomes exist only as components of modern-day tetraploid or hexaploid species (Hancock, 1992; Brar and Ramos, 2008). No diploid species or diploid progenitors of *Distichlis* are currently known. However, among the South American *Distichlis* species, *D. spicata* is the only one for which a chromosome number has been reported. Chromosome counts of the South American species may reveal whether diploids exist and whether tetraploid 38-chromosome and 40-chromosome species are present.

Molecular surveys of the South American *Distichlis* species would show whether these share the chloroplast haplotypes and microsatellite profiles found in this survey.

Other origins for the 38- and 40-chromosome lineages are possible.

Autopolyploidy of a 20-chromosome species followed by diploidization (i.e., modification to reduce the similarity of homologs so they will not form multivalents during meiosis) could have created the 40-chromosome lineage that seen in this study. Subsequent loss of a pair of chromosomes and gain of a *Hin6I* restriction site in the chloroplast could have given rise to the 38-chromosome lineage. However, the higher DNA content of the 38-chromosome lineage suggests that it did not originate via a simple loss of chromosomes from the lower-DNA-content 40-chromosome lineage. As an alternative, the variation in C-values might be due to an increase in repetitive DNA. As an additional alternative, autopolyploidy of an 18-chromosome species followed by diploidization could have created a 36-chromosome lineage, with the 38- and 40-chromosome lineages derived by subsequent gain of one or two pairs of chromosomes and loss or gain of a *Hin6I* restriction site in the chloroplast. The apparent gain of pairs of chromosomes could be mediated by incomplete diploidization. In this scenario, a few chromosome pairs that had not yet completed diploidization would form multivalents that might break as they were pulled apart at anaphase. The broken fragments would be drawn to one or the other daughter cell and would develop into additional pairs of chromosomes. The lower DNA content of the 40-chromosome lineage might result from loss of DNA associated with multiple breakage events involved in developing the higher number of chromosomes. Over time, the 38- and the 40-chromosome lineages could have become non-interfertile and would evolve along separate paths, acquiring different

microsatellite and RAPD profiles, to produce the genetically distinguishable populations now present in North America.

Two other molecular studies involving *D. spicata* (Ram et al., 2004; Bell and Columbus, 2008) reached divergent conclusions about genetic structure within the species. Using material from across North and South America, Bell and Columbus (2008) constructed two phylogenies of *Distichlis*. One phylogeny was based on a nuclear ribosomal ITS sequence and the other was based on *ndhF* and *trnL-F* chloroplast sequences. In both phylogenies *D. spicata* was polyphyletic. Chromosome counts were not done, so it is not known how their finding of polyphyly relates to the finding of distinct chromosome lineages in this study. Ram et al. (2004) used RAPDs to characterize genetic variation between coastal and inland types of 37 specimens of *D. spicata* from 32 locations in North and South America. Their Mantel test showed a very weak correlation ($R^2 = 0.036$) between genetic distance and geographic distance. An AMOVA found that less than 5% of the genetic variation was related to the distinction between inland and coastal origin. Ram et al. (2004) concluded that gene flow was widespread in North America and there was no genetic basis for distinguishing between inland and coastal types.

The conclusions of this study are exactly the opposite of those reached by Ram et al. (2004). It is possible that knowledge of the differing chromosome numbers allowed for the grouping of samples in a way that revealed the pattern of genetic differences. Because several 40-chromosome populations occur inland, the inland group assembled by Ram et al. (2004) may have contained both 38- and 40-chromosome plants. Their inland accessions from Great Salt Lake, southern Arizona, and southern California are

potentially of the 40-chromosome type, based on the examinations of populations and single accessions in this study. Mixing of chromosome types in the inland group would have reduced the apparent differences between the inland and coastal groups, leading to the conclusion that there was a great deal of gene flow and little geographic structure in the *D. spicata* populations of North America.

Current distribution of the two lineages--Chromosome numbers from single collections (Reid, 2001, and unpublished data) connect the populations examined in this study to plants distributed elsewhere in North America (Fig. 2-1). Plants apparently belonging to the 40-chromosome lineage include accessions from the Gulf Coast of Florida near Tampa Bay, from northern and central Arizona, southern California, eastern Colorado, and Utah. Plants apparently belonging to the 38-chromosome lineage include accessions from Idaho, South Dakota, Kansas, Nebraska, Nevada, Wyoming, northern California, the Texas Panhandle, and western Colorado.

The distribution of the 38-chromosome lineage seems similar to that of the original *D. stricta* (Torr.) Rydberg, i.e., from Saskatchewan, Canada, to Washington state and California in the west, and to Missouri and Texas in the east. This encompasses much of the western and Great Plains portions of the United States. Rydberg (1905) mentioned Fort Collins, Denver, and Pueblo as sites where *D. stricta* was found. Numerous accessions from these areas belong to the 38-chromosome lineage, suggesting that the *D. stricta* described by Rydberg was based on observations of the 38-chromosome lineage.

The distribution of the 40-chromosome lineage may encompass the entire Pacific Coast and Gulf Coast. The single accession from the Gulf Coast of Florida had 40 chromosomes and a chloroplast haplotype like that typically found in 40-chromosome

populations in the western states. The Florida sample had several microsatellite alleles not found in the western *D. spicata* populations, along with one allele that was specific to the 40-chromosome lineage and several that were found in both lineages. None of the alleles in the Florida sample was specific to the 38-chromosome lineage. Information on the Atlantic Coast populations is lacking. When a taxonomic distinction has been made in previous treatments, the Atlantic Coast populations have been considered “coastal”. None of the chromosome counts for coastal *D. spicata* have been made from Atlantic Coast populations.

The traditional approximation of distributions reflected in the common names “coastal saltgrass” for *D. spicata* and “inland saltgrass” for *D. spicata* subspecies/variety *stricta* seems to be inadequate in light of the 40-chromosome populations found in such non-coastal regions as northern Utah, western Nevada, and southern New Mexico.

Until a reliable morphological character is found, the simplest test to distinguish between the two lineages is the presence or absence of the *Hin6I* restriction site in the chloroplast. The results of this test can be visualized on an agarose gel, making it suitable for many basic laboratories.

The present data do not allow a conclusion regarding the origin of the two lineages. However the cohesion of five traits over such a broad geographic area, the genetic divergence between the lineages, and the cytogenetic and molecular links between the 38-chromosome lineage and *D. eludens* and *D. littoralis* suggest that the 38-chromosome lineage of *D. spicata* s.l. is a distinct species rather than a lineage-sorting product or a subspecies/variety of the 40-chromosome lineage.

Summary--The data are not consistent with a genetic gradient or a continuum of variation across the range of North American *Distichlis spicata* s.l. Nor are they consistent with a hypothesis of genetic isolation via current geographic barriers, since both chromosome lineages occur on both sides of the two most obvious geographic barriers, which are the Continental Divide and the deserts that separate the West Coast from the Great Plains. Nor are the data consistent with genetic isolation by distance. Direct, present-day gene flow among the more distantly-located populations in each chromosome group is unlikely, yet geographic distances within each chromosome group were not reflected in genetic relationships in this study. For example, the populations at Honey Lake and Washoe are at opposite ends of the genetic distance tree (Fig. 2-3) but are separated by only 50 km with no apparent geographic barrier, while the populations at Seattle and Las Cruces cluster close together genetically but are about 2,200 km apart. Populations are more related to other populations in their chromosome lineage, whether nearby or far away, than to neighboring populations of the other lineage. The genetic similarities shown within each chromosome group are probably due to some factor besides present-day gene flow. Multiple mutations and lineage sorting could have produced the patterns seen in this study, but the number of mutations is so large and the lineage sorting is congruent across so many traits that this hypothesis is considered unlikely. A more likely hypothesis is that differing origins in the past produced the two lineages. The genetic divergence between the two lineages, the different flowering times, and the chromosome irregularities in the progeny from the forced cross indicate that these lineages probably are no longer hybridizing in nature. The species name *D. stricta* (Torr.) Rydberg should be revived to identify the 38-chromosome lineage.

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Chapter 3: Importance of the Research, Perspectives, and Future Directions

The taxonomic status of *Distichlis stricta* has been in flux for more than a century. Correct placement of this grass would be helpful for conservation and restoration efforts, for plant breeding projects, and for accurate understanding of the evolution of the world's botanical resources.

The results from the current study confirm Reid's (2001) finding that two chromosome races of *Distichlis spicata* s.l. are found in North America. New locations of each chromosome race have been found, further developing our understanding of geographic distributions. The chromosome races of *Distichlis spicata* s.l. have been characterized using molecular methods. Chloroplast haplotype, microsatellite alleles, presence or absence of a sex-diagnostic RAPD band in females, DNA C-value, and flowering time appear to join chromosome number in a suite of traits distinguishing the two races. These results suggest that the races are separate phylogenetic lineages. Some isolation by distance is evident within each chromosome lineage when the lineages are considered separately, suggesting that there may be some within-lineage gene flow or a common origin in the past (Fig. 2-4). No relationship between genetic distance and geographic distance exists between the 38-chromosome lineage and the 40-chromosome lineage, suggesting that gene flow may not occur across the lineages or that they had somewhat different origins in the past (Fig. 2-4). Different flowering times may contribute to isolation of the lineages. Regression analysis from the populations studied

in this project indicates that the lineages may not hybridize in natural settings, although they can be crossed in the greenhouse. On one hand, the 38-chromosome lineage is separated from the 40-chromosome lineage by lineage-exclusive traits (chromosome number, characteristic chloroplast haplotypes, microsatellite alleles, RAPD bands, sex marker). On the other hand, the 38-chromosome lineage is genetically linked by the presence of a *Hin6I* restriction site in the *trnS-psbD* segment of the chloroplast to *D. eludens* and *D. littoralis*, two recognized species in the subtribe Monanthochlœe. Taken as a whole, this suggests that the 38-chromosome lineage is a genetic entity at the species level with *D. eludens* and *D. littoralis*, despite the difficulty of distinguishing between the two lineages of *D. spicata* s.l. via morphological examination. The lineages will be referred to as *D. spicata* (40 chromosomes) and *D. stricta* (38 chromosomes) in this chapter.

The importance of chromosome counts

Excellent chromosome preparations are necessary for correct identification of *Distichlis* specimens because chromosome number may have taxonomic significance. Fuzzy spreads and inadequate distance between chromosomes make accurate counting difficult. Nielson (1956) may have viewed 38-chromosome spreads in his investigation of variability in *D. stricta* in western North America, but the poor quality of his preparations caused him to defer to the published reports of $2n = 40$.

Examination of multiple samples from the sites in this study confirms the desirability of counting several individuals in a population to interpret chromosome number. B chromosomes are usually obvious because they are much smaller than the A

set, but aneuploidy in the A set of chromosomes can lead to incorrect interpretation. Specimens of *D. stricta*, the 38-chromosome species, with two supernumerary chromosomes (of the A set) may be mistaken for *D. spicata*, the 40-chromosome species. Specimens of 38-chromosome *D. stricta* plants may be mistaken for deficient aneuploids of *D. spicata*, the 40-chromosome species. Examination of multiple specimens collected at a location should reveal the more common euploid number.

Reeder's (1977) two aneuploid specimens of *D. spicata* from Albany County, Wyoming, with $2n = 38$ may have been legitimate aneuploids of the 40-chromosome species or may have been a second missed opportunity to discover the 38-chromosome species. The results of this study show that the 38-chromosome species is widespread in the plains and foothills just east of the Rocky Mountains and the samples from Albany County, Wyoming, belong to the 38-chromosome species. Additional sampling by Reeder in that region of Wyoming might have clarified the interpretation of the chromosome number in his specimens. However, Reeder (1977) probably is correct that the 42-chromosome plant from Salt Lake City was an aneuploid of the 40-chromosome species, which occurs around Great Salt Lake. The value of distribution maps for *D. spicata* s.l. is apparent from the complex pattern evident in Chapter 2-Fig. 1. Additional sampling, over a larger area and at a finer scale, is needed to clarify the very rough outlines of the distribution revealed by this study and by Reid (2001).

Hypotheses for species formation in Monanthochloe

From the standpoint of basic science, the results from this study provide a suggestion for the formation of species within the subtribe Monanthochloinae. Both

dysploidy, an evolutionarily significant change in chromosome number involving less than a complete haploid set, and alloploidy, hybridization between different species, are possible routes to the development of the chromosome numbers found in the Monanthochloinae. Members of the Monanthochloinae have been believed, on the basis of published counts (Stebbins and Löve, 1941; Bowden, 1960; Rahn, 1961; Reeder, 1967, 1971, 1977) for some species, to have $2n = 40$. But *D. eludens* (previously the monotypic *Reederochloa eludens*), a member of the Monanthochloinae with an exceedingly restricted distribution, was reported (Reeder, 1967) to have a somatic chromosome number of 38. Reid's (2001) discovery that *D. stricta*, a very widespread species in the Monanthochloinae, also has the somatic chromosome number 38 provides support for the notion that $2n = 38$ is not an exception to the general rule or an example of aneuploidy, as Reeder (1977) once termed it, but rather a normal result of natural processes operating in the Monanthochloinae.

The results of this project suggest that alloploidy is a more likely cause than dysploidy for the existence of two 38-chromosome species in the Monanthochloinae. The 38-chromosome *D. stricta* plants contain more nuclear DNA than the 40-chromosome *D. spicata* plants, despite the smaller number of chromosomes in *D. stricta*. One would expect more DNA with more chromosomes, if the two species shared the same chromosome makeup except for the missing or additional pair. Therefore, the dysploidy hypothesis seems inconsistent with the facts. The alloploidy hypothesis is consistent with different C-values. Sets of chromosomes originating from parents with different genomes could produce tetraploid hybrids with different C-values, for example, AABB for one species and AACC for the other species.

Future research should focus on the allopolyploid hypothesis. Identification of individual chromosomes has been done in wheat, but may prove difficult in *Distichlis* because the chromosomes are small. Cytological and molecular characterization of the South American species are essential, as these may share parents with the North American species or may *be* parents of the North American species if diploids are found. Approaches using fluorescent in situ hybridization or genomic in situ hybridization may be useful in identifying the extant genomes in the Monanthochloinae and possibly one or more ancestors if these survive.

Distinct species status

In light of the current uncertainty regarding the status and even the existence of *D. stricta*, it is perhaps surprising that botanists of an earlier century believed they were able to distinguish between *D. stricta* and *D. spicata* using only morphological means. The first recorded specimen of *D. stricta* was collected by Edwin James of Major Stephen Long's expedition as the explorers passed through northeastern New Mexico (James, 1820; Goodman and Lawson, 1995). The specimen was identified by Torrey (1824) as a different species from the already known *Uniola spicata*, later to be renamed *Distichlis spicata*. Torrey (1828) identified another grass specimen collected in Missouri during the Long Expedition as *U. spicata*, showing that he was aware of the morphology of that species distinct from *U. stricta*. Nearly 120 years later, Steyermark (1940) collected a specimen of *D. spicata* in Saline County, Missouri, in the probable vicinity of Torrey's *U. spicata* from the Long Expedition (Goodman and Lawson, 1995). Steyermark

published this as the farthest inland collection of *D. spicata*, differentiating it from *D. stricta*, which was at that time still recognized by some as a distinct species.

Doubts about the separate identity of *D. stricta* may have been raised by the presence of both species in California and Nevada in a complex distribution that belied the simplistic dichotomy of coast versus inland that is implied by the common names for these species. The distribution of *D. stricta*, the 38-chromosome species, can fairly be described as inland, corresponding to its common name, inland saltgrass. But *D. spicata*, the 40-chromosome species, is present at some inland locations as well as in the coastal regions from which it derives its common name. Early botanists, eager to discover new species in unexplored regions and having only a few widely separated specimens to examine, might have easily discerned the differences. Later botanists, reviewing samples that filled in the geographic gaps between the initial collections, might have interpreted the complex pattern as merely natural variation in a widely distributed single species.

Per Axel Rydberg, who transferred *U. stricta* to *Distichlis*, had a reputation as a “splitter” (Williams, 2003), a taxonomist with a propensity toward naming new species based on minor morphological differences in situations where more cautious taxonomists would see a morphologically variable species or would opt for naming subspecies/varieties. Ironically, Rydberg was not responsible for the original publication of *D. stricta* as a distinct species. That was done by John Torrey.

Because the results of this project support renewed recognition of a previously recognized species, a return to the old species name seems appropriate. Scribner (1894) first moved *stricta* to the genus *Distichlis*, but as a variety, not as a species. Rydberg

(1905) recognized both the correct genus and the correct taxonomic level in his classification of the plant as *Distichlis stricta* (Torr.) Rydb.

The need to identify D. spicata and D. stricta by plant morphology

Despite the increasing use of molecular methods in botanical research, taxonomists typically still rely on morphological discontinuities to decide where to draw lines separating plant species. Indeed, a very common question in response to the presentation of the data in this dissertation was, “Do you have a morphological character?” This is motivated by the practical consideration of being able to identify species in the field, without the delay and inconvenience of resorting to laboratory analysis to distinguish one species from another. Accordingly, the development of a morphological key that reliably differentiates *D. spicata* from *D. stricta* is important.

One practical use for a morphological key is in determining which species should be used to vegetate, revegetate, or restore lands in the western United States. Use of the proper species is advisable for many projects and is necessary for true restoration projects. Sellers of seeds and vegetative material need to be able to correctly describe what they are selling and buyers need to be able to determine which species is appropriate for their project without recourse to laboratory investigation. Currently, seeds advertised as “inland saltgrass” may actually be 40-chromosome “coastal” *Distichlis spicata* even though they were collected from an inland site. A reliable field key would facilitate correct labeling by sellers and would help buyers determine which species is present in their area of interest.

Plant breeders may find it useful to be able to distinguish between the two *Distichlis* species. Whether a breeder wishes to confine breeding efforts to only one species or wishes to experiment with variation produced by interspecific crosses, correct identification of the starting material is important.

A more speculative purpose that may or may not turn out to be important is research relating to Carson's wandering skipper, a federally listed endangered butterfly (U.S. Fish and Wildlife Service, 2007). Adult skippers feed on nectar from forbs and shrubs and lay their eggs exclusively on *Distichlis* plants. Suitable habitat must have nectar-producing forbs and shrubs growing close to *Distichlis*. Development projects have eliminated several skipper populations and only four populations remain (U.S. Fish and Wildlife Service, 2007), all of them along the border between California and Nevada, where both *D. spicata* and *D. stricta* occur. It is not known whether the skipper utilizes both *D. spicata* and *D. stricta*. Utilization of only one species of *Distichlis* would further restrict the already limited number of locations that are suitable for future recovery efforts targeting Carson's wandering skipper. The species of *Distichlis* should be determined at existing skipper sites to see whether both species are being utilized. The answer may influence the selection of possible colonization sites as wildlife managers attempt to return the skipper to non-endangered status. At present, *Distichlis* species identification requires laboratory methods, since no reliable field key exists. The plants at the Honey Lake skipper site are *D. stricta*. The species at other skipper sites have not been determined.

Detailed knowledge about the native flora is important as we progress toward better conservation and utilization of the natural resources of the United States. Correct

conclusions and correct interpretations depend on copious amounts of correct data. This study contributes to the fund of data that can be used to understand and identify *Distichlis spicata* and *Distichlis stricta* in the western United States.

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Appendix 1: Chromosome numbers

An empty cell indicates that no count was obtained for the sample.

accession name	source	2n
SFB1	San Francisco Bay, CA	40
SFB6	San Francisco Bay, CA	40
SFB12	San Francisco Bay, CA	42 + 7B
SFB20	San Francisco Bay, CA	40
SFB21	San Francisco Bay, CA	40
SFB22	San Francisco Bay, CA	40
SFB27	San Francisco Bay, CA	40
SFB28	San Francisco Bay, CA	40
SFB31	San Francisco Bay, CA	40
SFB32	San Francisco Bay, CA	40 + 3B
SFB33	San Francisco Bay, CA	
SFB36	San Francisco Bay, CA	40
SFB38	San Francisco Bay, CA	40
SFB39	San Francisco Bay, CA	40 + 1B
SFB41	San Francisco Bay, CA	40
SFB51	San Francisco Bay, CA	40
SFB61	San Francisco Bay, CA	40
SFB67	San Francisco Bay, CA	40
SFB89	San Francisco Bay, CA	40
SFB96	San Francisco Bay, CA	40
Washoe1	Smoke Creek Desert, NV	40
Washoe2	Smoke Creek Desert, NV	40
Washoe4	Smoke Creek Desert, NV	40
Washoe5	Smoke Creek Desert, NV	40
Washoe8	Smoke Creek Desert, NV	
Washoe9	Smoke Creek Desert, NV	40
Washoe12	Smoke Creek Desert, NV	40
Washoe16	Smoke Creek Desert, NV	40
Washoe24	Smoke Creek Desert, NV	40

accession name	source	2n
Washoe26	Smoke Creek Desert, NV	
Washoe27	Smoke Creek Desert, NV	40
Washoe28	Smoke Creek Desert, NV	41
Washoe37	Smoke Creek Desert, NV	40
Washoe41	Smoke Creek Desert, NV	40
Washoe44	Smoke Creek Desert, NV	
Washoe54	Smoke Creek Desert, NV	40
Washoe81	Smoke Creek Desert, NV	40
Washoe84	Smoke Creek Desert, NV	
Washoe95	Smoke Creek Desert, NV	40
Washoe96	Smoke Creek Desert, NV	40
G8-6	Great Salt Lake, UT	40
G8-7	Great Salt Lake, UT	41
G8-10	Great Salt Lake, UT	
G8-13	Great Salt Lake, UT	42
G8-19	Great Salt Lake, UT	40
G8-20	Great Salt Lake, UT	40
G8-29	Great Salt Lake, UT	40
G8-33	Great Salt Lake, UT	41
G8-39	Great Salt Lake, UT	40
G8-37	Great Salt Lake, UT	40
G8-43	Great Salt Lake, UT	40
G8-44	Great Salt Lake, UT	40
G8-45	Great Salt Lake, UT	40
G8-48	Great Salt Lake, UT	40
G8-55	Great Salt Lake, UT	40
G8-58	Great Salt Lake, UT	40
G8-60	Great Salt Lake, UT	40
G8-64	Great Salt Lake, UT	40

accession name	source	2n
G8-70	Great Salt Lake, UT	40
G8-77	Great Salt Lake, UT	40
07007p	Grays Harbor near Seattle, WA	40
07008p	Grays Harbor near Seattle, WA	
07011p1	Grays Harbor near Seattle, WA	40
07014p	Grays Harbor near Seattle, WA	
07019p	Grays Harbor near Seattle, WA	
07020p	Grays Harbor near Seattle, WA	
07021p	Grays Harbor near Seattle, WA	
07022p	Grays Harbor near Seattle, WA	40
R111Bp	near Rock River, WY	38
R112App	near Rock River, WY	38
R113A	near Rock River, WY	38
R114A	near Rock River, WY	38 + 4B
R115ppA	near Rock River, WY	38
R116A	near Rock River, WY	38
R117Ap	near Rock River, WY	38
R118A	near Rock River, WY	38
R119ppA	near Rock River, WY	38
R120	near Rock River, WY	38
R125p	Antero Reservoir, CO	38
R127p	Antero Reservoir, CO	
R130p	Antero Reservoir, CO	38
R133p	Antero Reservoir, CO	38
R134ppB	Antero Reservoir, CO	38
R135p	Antero Reservoir, CO	
R137p	Antero Reservoir, CO	
R140p	Antero Reservoir, CO	38
R141p	Antero Reservoir, CO	38

accession name	source	2n
R143p	Antero Reservoir, CO	38
R144p	Antero Reservoir, CO	38
R145p	Antero Reservoir, CO	38
R146p	Antero Reservoir, CO	38
R148p	Antero Reservoir, CO	38
R149p	Antero Reservoir, CO	38
R150p	Antero Reservoir, CO	38
R151p	Antero Reservoir, CO	38
R152p	Antero Reservoir, CO	38
R153p	Antero Reservoir, CO	38
R155p	Antero Reservoir, CO	38
R156	Wray, CO	38
R157a	Wray, CO	
R159a	Wray, CO	38
R160	Wray, CO	38
R161	Wray, CO	38
R162p	Wray, CO	57
R165	Wray, CO	
R166	Wray, CO	38
R169p	Wray, CO	38
R170a	Wray, CO	38
R171	Wray, CO	38
R174	Wray, CO	
R179	Wray, CO	38
R180	Wray, CO	
R181	Wray, CO	38
R182	Wray, CO	38
R183	Wray, CO	38
R184	Wray, CO	38

accession name	source	2n
R186	Wray, CO	38
R187	Wray, CO	38
Paw2	Pawnee Grasslands, CO	57
Paw3	Pawnee Grasslands, CO	57
Paw10	Pawnee Grasslands, CO	38
Paw11	Pawnee Grasslands, CO	38
Paw14	Pawnee Grasslands, CO	38
Paw15	Pawnee Grasslands, CO	38
Paw16	Pawnee Grasslands, CO	38 + 1B
Paw18	Pawnee Grasslands, CO	38
Paw19	Pawnee Grasslands, CO	38
Paw21	Pawnee Grasslands, CO	38
Paw22	Pawnee Grasslands, CO	38
Paw24	Pawnee Grasslands, CO	57
Paw25	Pawnee Grasslands, CO	38
Paw27	Pawnee Grasslands, CO	38
Paw28	Pawnee Grasslands, CO	38
Paw31	Pawnee Grasslands, CO	38 + 1B
Paw46	Pawnee Grasslands, CO	74
Paw50	Pawnee Grasslands, CO	56
Paw51	Pawnee Grasslands, CO	56
Paw52	Pawnee Grasslands, CO	38
Paw55	Pawnee Grasslands, CO	74
Paw57	Pawnee Grasslands, CO	ca. 74
Paw61	Pawnee Grasslands, CO	ca. 74
Paw70	Pawnee Grasslands, CO	74
Paw78	Pawnee Grasslands, CO	38
Paw86	Pawnee Grasslands, CO	38
Paw87	Pawnee Grasslands, CO	38

accession name	source	2n
Paw95	Pawnee Grasslands, CO	57
Paw97	Pawnee Grasslands, CO	38
Paw98	Pawnee Grasslands, CO	38
05133p	Honey Lake, CA	38
05140p	Honey Lake, CA	38
05142p	Honey Lake, CA	38
05150p	Honey Lake, CA	38
05152p	Honey Lake, CA	38
05156p	Honey Lake, CA	38
05165p1	Honey Lake, CA	38
05166p	Honey Lake, CA	39
05167p	Honey Lake, CA	38
05168p	Honey Lake, CA	38
05173p	Honey Lake, CA	38
05174p	Honey Lake, CA	41 + 2B
05176p2	Honey Lake, CA	38
05179p	Honey Lake, CA	38
05180p	Honey Lake, CA	38
05181p	Goose Lake, CA	38
05201p3	Goose Lake, CA	38
05202p3	Goose Lake, CA	38
05203p2	Goose Lake, CA	38
05204p2	Goose Lake, CA	38
05209#C	Goose Lake, CA	41
05210p3	Goose Lake, CA	41
05220p1	Goose Lake, CA	38
05223p1	Goose Lake, CA	38
05225p1#A	Goose Lake, CA	38
05226p1	Goose Lake, CA	38

accession name	source	2n
05227p2	Goose Lake, CA	38
05235	Goose Lake, CA	57
05236p1	Goose Lake, CA	38
05237p2	Goose Lake, CA	38
Tom Lp1	near Albuquerque, NM	38
Tom Mp1	near Albuquerque, NM	38
Tom Np1	near Albuquerque, NM	38
Tom Op1	near Albuquerque, NM	38
Tom Pp1	near Albuquerque, NM	38
Tom Ap	near Truth or Consequences, NM	40
Tom Bp	near Truth or Consequences, NM	42
Tom Dp1	near Truth or Consequences, NM	40 - 42
Tom Epp	near Truth or Consequences, NM	
Tom Fpp	near Truth or Consequences, NM	40
Tom Gp3	near Truth or Consequences, NM	40
Tom Hp1	near Truth or Consequences, NM	42
Tom Ip1	near Truth or Consequences, NM	42
Tom Jp	near Las Cruces, NM	40
Tom Rp1	near Las Cruces, NM	40
Tom Sp1	near Las Cruces, NM	41
Tom Tp1	near Las Cruces, NM	42
Tom Up	near Las Cruces, NM	40
Tom Vp2	near Las Cruces, NM	42
Tom Xp1	near Las Cruces, NM	40
Tom Yp2	near Las Cruces, NM	40
Tom Zp	near Las Cruces, NM	40
Tom AAp	near Las Cruces, NM	40
Tom BBp1	near Las Cruces, NM	40
Tom CCp1	near Las Cruces, NM	40

accession name	source	2n
Tom DDp2	near Las Cruces, NM	40
Tom EEp1	near Las Cruces, NM	42
Florida	Gulf Coast of Florida	40
Monan CA	coast of California	40
Monan TX	coast of Texas	40
Archery5	Fort Collins, CO	38
Arc5XW16-1	hand cross	38
Arc5XW16-2	hand cross	39
Arc5XW16-3	hand cross	39
Arc5XW16-4	hand cross	40
Arc5XW16-5	hand cross	39
Arc5XW16-6	hand cross	39
Arc5XW16-7	hand cross	38
Arc5XW16-8	hand cross	
Arc5XW16-9	hand cross	
Arc5XW16-10	hand cross	39

Appendix 2: Chloroplast restriction site data

Column headings indicate the name of the digestion enzyme and the length of the band in base pairs. A plus sign indicates the presence of a restriction site. A minus sign indicates the absence of a restriction site.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1		Hin480	Hin525	Taq492	Taq280	Taq270	Taq260	Taq250	Taq246	Taq245	Taq230	Taq228	Taq226	Taq220	Taq206
2	SFBY012	-	-	-	+	-	-	-	+	-	-	+	-	+	-
3	SFBY020	-	-	-	+	-	-	-	+	-	-	+	-	+	-
4	SFBY021	-	-	-	+	-	-	-	+	-	-	+	-	+	-
5	SFBY022	-	-	-	+	-	-	-	+	-	-	+	-	+	-
6	SFBY027	-	-	-	+	-	-	-	+	-	-	+	-	+	-
7	SFBY028	-	-	-	+	-	-	-	+	-	-	+	-	+	-
8	SFBY031	-	-	-	+	-	-	-	+	-	-	+	-	+	-
9	SFBY032	-	-	-	+	-	-	-	+	-	-	+	-	+	-
10	SFBY033	-	-	-	+	-	-	-	+	-	-	+	-	+	-
11	SFBY036	-	-	-	+	-	-	-	+	-	-	+	-	+	-
12	ANTR127	+	-	-	+	-	-	-	+	-	-	+	-	+	-
13	ANTR134	+	-	-	+	-	-	-	+	-	-	+	-	+	-
14	ANTR137	+	-	-	+	-	-	-	+	-	-	+	-	+	-
15	ANTR140	+	-	-	+	-	-	-	+	-	-	+	-	+	-
16	ANTR145	+	-	-	+	-	-	-	+	-	-	+	-	+	-
17	ANTR146	+	-	-	+	-	-	-	+	-	-	+	-	+	-
18	ANTR149	+	-	-	+	-	-	-	+	-	-	+	-	+	-
19	ANTR150	+	-	-	+	-	-	-	+	-	-	+	-	+	-
20	ANTR152	+	-	-	+	-	-	-	+	-	-	+	-	+	-
21	ANTR153	+	-	-	+	-	-	-	+	-	-	+	-	+	-
22	GATE006	-	-	-	+	-	-	-	+	-	-	+	-	+	-
23	GATE007	-	-	-	+	-	-	-	+	-	-	+	-	+	-
24	GATE013	-	-	-	+	-	-	-	+	-	-	+	-	+	-
25	GATE033	-	-	-	+	-	-	-	+	-	-	+	-	+	-
26	GATE036	-	-	-	+	-	-	-	+	-	-	+	-	+	-
27	GATE043	-	-	-	+	-	-	-	+	-	-	+	-	+	-
28	GATE044	-	-	-	+	-	-	-	+	-	-	+	-	+	-
29	GATE045	-	-	-	+	-	-	-	+	-	-	+	-	+	-
30	GATE055	-	-	-	+	-	-	-	+	-	-	+	-	+	-
31	GATE060	-	-	-	+	-	-	-	+	-	-	+	-	+	-
32	PAWN011	+	-	-	+	-	-	-	+	-	-	+	-	+	-
33	PAWN015	+	-	-	+	+	-	+	-	-	-	-	-	+	-

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
34	PAWN016	+	-	-	+	-	-	-	+	-	-	+	-	+	-
35	PAWN019	+	-	-	+	-	-	-	+	-	-	+	-	+	-
36	PAWN021	+	-	-	+	-	-	-	+	-	-	+	-	+	-
37	PAWN022	+	-	-	+	-	-	-	+	-	-	+	-	+	-
38	PAWN027	+	-	-	+	-	-	-	+	-	-	+	-	+	-
39	PAWN028	+	-	-	+	-	-	-	+	-	-	+	-	+	-
40	PAWN031	+	-	-	+	-	-	-	+	-	-	+	-	+	-
41	PAWN052	+	-	-	+	-	-	-	+	-	-	+	-	+	-
42	PAWN002	+	-	-	+	-	-	-	+	-	-	+	-	+	-
43	PAWN003	+	-	-	+	-	-	-	+	-	-	+	-	+	-
44	PAWN050	+	-	-	+	-	-	-	+	-	-	+	-	+	-
45	PAWN051	+	-	-	+	-	-	-	+	-	-	+	-	+	-
46	PAWN095	+	-	-	+	-	-	-	+	-	-	+	-	+	-
47	PAWN046	+	-	-	+	-	-	-	+	-	-	+	-	+	-
48	PAWN055	+	-	-	+	-	-	-	+	-	-	+	-	+	-
49	PAWN057	+	-	-	+	-	-	-	+	-	-	+	-	+	-
50	PAWN061	+	-	-	+	-	-	-	+	-	-	+	-	+	-
51	PAWN070	+	-	-	+	-	-	-	+	-	-	+	-	+	-
52	WASH001	-	-	-	+	-	-	-	+	-	-	+	-	+	-
53	WASH005	-	-	-	+	-	-	-	+	-	-	+	-	+	-
54	WASH008	-	-	-	+	-	-	-	+	-	-	+	-	+	-
55	WASH012	-	-	-	+	-	-	-	+	-	-	+	-	+	-
56	WASH024	-	-	-	+	-	-	-	+	-	-	+	-	+	-
57	WASH026	-	-	-	+	-	-	-	+	-	-	+	-	+	-
58	WASH027	-	-	-	+	-	-	-	+	-	-	+	-	+	-
59	WASH028	-	-	-	+	-	-	-	+	-	-	+	-	+	-
60	WASH037	-	-	-	+	-	-	-	+	-	-	+	-	+	-
61	WASH041	-	-	-	+	-	-	-	+	-	-	+	-	+	-
62	RRIV111	+	-	-	+	-	-	-	+	-	-	+	-	+	-
63	RRIV112	+	-	-	+	-	-	-	+	-	-	+	-	+	-
64	RRIV113	+	-	-	+	-	-	-	+	-	-	+	-	+	-
65	RRIV114	+	-	-	+	+	-	+	-	-	-	+	-	+	-
66	RRIV115	+	-	-	+	-	-	-	+	-	-	+	-	+	-

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
67	RRIV116	+	-	-	+	+	-	+	-	-	-	-	-	+	-
68	RRIV117	+	-	-	+	-	-	-	+	-	-	+	-	+	-
69	RRIV118	+	-	-	+	-	-	-	+	-	-	+	-	+	-
70	RRIV119	+	-	-	+	+	-	+	-	-	-	-	-	+	-
71	RRIV120	+	-	-	+	-	-	-	+	-	-	+	-	+	-
72	SFBY001	-	-	-	+	-	-	-	+	-	-	+	-	+	-
73	SFBY006	-	-	-	+	-	-	-	+	-	-	+	-	+	-
74	SFBY038	-	-	-	+	-	-	-	+	-	-	+	-	+	-
75	SFBY039	-	-	-	+	-	-	-	+	-	-	+	-	+	-
76	SFBY041	-	-	-	+	-	-	-	+	-	-	+	-	+	-
77	SFBY051	-	-	-	+	-	-	-	+	-	-	+	-	+	-
78	SFBY061	-	-	-	+	-	-	-	+	-	-	+	-	+	-
79	SFBY067	-	-	-	+	-	-	-	+	-	-	+	-	+	-
80	SFBY089	-	-	-	+	-	-	-	+	-	-	+	-	+	-
81	SFBY096	-	-	-	+	-	-	-	+	-	-	+	-	+	-
82	ANTR125	+	-	-	+	-	-	-	+	-	-	+	-	+	-
83	ANTR130	+	-	-	+	-	-	-	+	-	-	+	-	+	-
84	ANTR133	+	-	-	+	-	-	-	+	-	-	+	-	+	-
85	ANTR135	+	-	-	+	-	-	-	+	-	-	+	-	+	-
86	ANTR141	+	-	-	+	-	-	-	+	-	-	+	-	+	-
87	ANTR143	+	-	-	+	-	-	-	+	-	-	+	-	+	-
88	ANTR144	+	-	-	+	-	-	-	+	-	-	+	-	+	-
89	ANTR148	+	-	-	+	-	-	-	+	-	-	+	-	+	-
90	ANTR151	+	-	-	+	-	-	-	+	-	-	+	-	+	-
91	ANTR155	+	-	-	+	-	-	-	+	-	-	+	-	+	-
92	GATE010	-	-	-	+	-	-	-	+	-	-	+	-	+	-
93	GATE019	-	-	-	+	-	-	-	+	-	-	+	-	+	-
94	GATE020	-	-	-	+	-	-	-	+	-	-	+	-	+	-
95	GATE029	-	-	-	+	-	-	-	+	-	-	+	-	+	-
96	GATE037	-	-	-	+	-	-	-	+	-	-	+	-	+	-
97	GATE048	-	-	-	+	-	-	-	+	-	-	+	-	+	-
98	GATE058	-	-	-	+	-	-	-	+	-	-	+	-	+	-
99	GATE064	-	-	-	+	-	-	-	+	-	-	+	-	+	-

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
100	GATE070	-	-	-	+	-	-	-	+	-	-	+	-	+	-
101	GATE077	-	-	-	+	-	-	-	+	-	-	+	-	+	-
102	PAWN010	+	-	-	+	-	-	-	+	-	-	+	-	+	-
103	PAWN014	+	-	-	+	+	-	+	-	+	-	-	-	+	-
104	PAWN018	+	-	-	+	+	-	+	-	+	-	-	-	+	-
105	PAWN024	+	-	-	+	-	-	-	+	-	-	+	-	+	-
106	PAWN025	+	-	-	+	-	-	-	+	-	-	+	-	+	-
107	PAWN078	+	-	-	+	+	-	+	-	+	-	-	-	+	-
108	PAWN086	+	-	-	+	-	-	-	+	-	-	+	-	+	-
109	PAWN087	+	-	-	+	+	-	+	+	-	-	-	-	+	-
110	PAWN097	+	-	-	+	-	-	-	+	-	-	+	-	+	-
111	PAWN098	+	-	-	+	-	-	-	+	-	-	+	-	+	-
112	WASH002	-	-	-	+	-	-	-	+	-	-	+	-	+	-
113	WASH004	-	-	-	+	-	-	-	+	-	-	+	-	+	-
114	WASH009	-	-	-	+	-	-	-	+	-	-	+	-	+	-
115	WASH016	-	-	-	+	-	-	-	+	-	-	+	-	+	-
116	WASH044	-	-	-	+	-	-	-	+	-	-	+	-	+	-
117	WASH054	-	-	-	+	-	-	-	+	-	-	+	-	+	-
118	WASH081	-	-	-	+	-	-	-	+	-	-	+	-	+	-
119	WASH084	-	-	-	+	-	-	-	+	-	-	+	-	+	-
120	WASH095	-	-	-	+	-	-	-	+	-	-	+	-	+	-
121	WASH096	-	-	-	+	-	-	-	+	-	-	+	-	+	-
122	WRAY156	+	-	-	+	+	-	+	-	-	-	-	-	+	-
123	WRAY157	+	-	-	+	-	-	-	+	-	-	+	-	+	-
124	WRAY159	+	-	-	+	+	-	+	-	-	-	-	-	+	-
125	WRAY160	+	-	-	+	-	-	-	+	-	-	+	-	+	-
126	WRAY161	+	-	-	+	-	-	-	+	-	-	+	-	+	-
127	WRAY162	+	-	-	+	-	-	-	+	-	-	+	-	+	-
128	WRAY165	+	-	-	+	+	-	+	-	-	-	-	-	+	-
129	WRAY166	+	-	-	+	-	-	-	+	-	-	+	-	+	-
130	WRAY169	+	-	-	+	-	-	-	+	-	-	+	-	+	-
131	WRAY170	+	-	-	+	-	-	-	+	-	-	+	-	+	-
132	WRAY171	+	-	-	+	-	-	-	+	-	-	+	-	+	-

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
133	WRAY174	+	-	-	+	-	-	-	+	-	-	+	-	+	-
134	WRAY179	+	-	-	+	+	-	+	-	-	-	-	-	+	-
135	WRAY180	+	-	-	+	-	-	-	+	-	-	+	-	+	-
136	WRAY181	+	-	-	+	-	-	-	+	-	-	+	-	+	-
137	WRAY182	+	-	-	+	-	-	-	+	-	-	+	-	+	-
138	WRAY183	+	-	-	+	+	-	+	-	-	-	-	-	+	-
139	WRAY184	+	-	-	+	+	-	+	-	-	-	-	-	+	-
140	WRAY186	+	-	-	+	-	-	-	+	-	-	+	-	+	-
141	WRAY187	+	-	-	+	-	-	-	+	-	-	+	-	+	-
142	HONY133	+	-	-	+	-	-	-	+	-	-	+	-	+	-
143	HONY140	+	-	-	+	-	-	-	+	-	-	+	-	+	-
144	HONY142	+	-	-	+	-	-	-	+	-	-	+	-	+	-
145	HONY150	+	-	-	+	-	-	-	+	-	-	+	-	+	-
146	HONY152	+	-	-	+	-	-	-	+	-	-	+	-	+	-
147	HONY156	+	-	-	+	-	-	-	+	-	-	+	-	+	-
148	HONY165	+	-	-	+	-	-	-	+	-	-	+	-	+	-
149	HONY166	+	-	-	+	-	-	-	+	-	-	+	-	+	-
150	HONY167	+	-	-	+	-	-	-	+	-	-	+	-	+	-
151	HONY168	+	-	-	+	-	-	-	+	-	-	+	-	+	-
152	HONY173	+	-	-	+	-	-	-	+	-	-	+	-	+	-
153	HONY174	+	-	-	+	-	-	-	+	-	-	+	-	+	-
154	HONY176	+	-	-	+	-	-	-	+	-	-	+	-	+	-
155	HONY179	+	-	-	+	-	-	-	+	-	-	+	-	+	-
156	HONY180	+	-	-	+	-	-	-	+	-	-	+	-	+	-
157	GOOS181	+	-	-	+	-	-	-	+	-	-	+	-	+	-
158	GOOS201	+	-	-	+	-	-	-	+	-	-	+	-	+	-
159	GOOS202	+	-	-	+	-	-	-	-	+	-	-	+	-	+
160	GOOS203	+	-	-	+	-	-	-	-	+	-	-	+	-	+
161	GOOS204	+	-	-	+	-	-	-	+	-	-	+	-	+	-
162	GOOS209	+	-	-	+	-	-	-	+	-	-	+	-	+	-
163	GOOS210	+	-	-	+	-	-	-	-	+	-	-	+	-	+
164	GOOS220	+	-	-	+	-	-	-	+	-	-	+	-	+	-
165	GOOS223	+	-	-	+	-	-	-	+	-	-	+	-	+	-

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
166	GOOS225	+	-	-	+	-	-	-	+	-	-	+	-	+	-
167	GOOS226	+	-	-	+	-	-	-	+	-	-	+	-	+	-
168	GOOS227	+	-	-	+	-	-	-	+	-	-	+	-	+	-
169	GOOS235	+	-	-	+	-	-	-	+	-	-	+	-	+	-
170	GOOS236	+	-	-	+	-	-	-	+	-	-	+	-	+	-
171	GOOS237	+	-	-	+	-	-	-	-	+	-	-	+	-	+
172	TOMALBL	+	-	-	+	-	-	-	+	-	-	+	-	+	-
173	TOMALBM	+	-	-	+	-	-	-	+	-	-	+	-	+	-
174	TOMALBN	+	-	-	+	-	-	-	+	-	-	+	-	+	-
175	TOMALBO	+	-	-	+	-	-	-	+	-	-	+	-	+	-
176	TOMALBP	+	-	-	+	-	-	-	+	-	-	+	-	+	-
177	TOMTOCA	-	-	-	+	-	-	-	+	-	-	+	-	+	-
178	TOMTOCB	-	-	-	+	-	-	-	+	-	-	+	-	+	-
179	TOMTOCD	-	-	-	+	-	-	-	+	-	-	+	-	+	-
180	TOMTOCE	-	-	-	+	-	-	-	+	-	-	+	-	+	-
181	TOMTOCF	-	-	-	+	-	-	-	+	-	-	+	-	+	-
182	TOMTOCG	-	-	-	+	-	-	-	+	-	-	+	-	+	-
183	TOMTOCH	-	-	-	+	-	-	-	+	-	-	+	-	+	-
184	TOMTOCI	-	-	-	+	-	-	-	+	-	-	+	-	+	-
185	TOMCRUJ	-	-	-	+	-	-	-	+	-	-	+	-	+	-
186	TOMCRUR	-	-	-	+	-	-	-	+	-	-	+	-	+	-
187	TOMCRUS	-	-	-	+	-	-	-	+	-	-	+	-	+	-
188	TOMCRUT	-	-	-	+	-	-	-	+	-	-	+	-	+	-
189	TOMCRUU	-	-	-	+	-	-	-	+	-	-	+	-	+	-
190	TOMCRUV	-	-	-	+	-	-	-	+	-	-	+	-	+	-
191	TOMCRUX	-	-	-	+	-	-	-	+	-	-	+	-	+	-
192	TOMCRUY	-	-	-	+	-	-	-	+	-	-	+	-	+	-
193	TOMCRUZ	-	-	-	+	-	-	-	+	-	-	+	-	+	-
194	TOMCRAA	-	-	-	+	-	-	-	+	-	-	+	-	+	-
195	TOMCRBB	-	-	-	+	-	-	-	+	-	-	+	-	+	-
196	TOMCRCC	-	-	-	+	-	-	-	+	-	-	+	-	+	-
197	TOMCRDD	-	-	-	+	-	-	-	+	-	-	+	-	+	-
198	TOMCREE	-	-	-	+	-	-	-	+	-	-	+	-	+	-

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
199	SEAT007	-	-	-	+	-	-	-	+	-	-	+	-	+	-
200	SEAT008	-	+	-	+	-	-	-	+	-	-	+	-	+	-
201	SEAT011	-	-	-	+	-	-	-	+	-	-	-	-	+	-
202	SEAT014	-	-	-	+	-	-	-	+	-	-	+	-	+	-
203	SEAT019	-	-	-	+	-	-	-	+	-	-	+	-	+	-
204	SEAT020	-	-	-	+	-	-	-	+	-	-	-	-	+	-
205	SEAT021	-	-	-	+	-	-	-	+	-	-	+	-	+	-
206	SEAT022	-	-	-	+	-	-	-	+	-	-	+	-	+	-
207	REEDERO	+	-	+	-	-	-	-	+	-	-	-	-	-	-
208	MONANCA	+	-	-	+	-	+	-	+	-	+	-	-	+	-
209	MONANTX	+	-	-	+	-	+	-	+	-	+	-	-	+	-
210	FLORIDA	-	-	-	+	-	-	-	+	-	+	-	-	+	-

	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE
1	Taq202	Taq201	Taq173	Taq170	Taq167	Taq165	Taq155	Taq154	Taq149	Taq147	Taq146	Tru247	Tru244	Tru229	Tru228	Tru222
2	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
3	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
4	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
5	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
6	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
7	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
8	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
9	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
10	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
11	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	+
12	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
13	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
14	+	-	-	+	-	+	-	-	-	-	-	-	+	-	-	+
15	+	-	-	+	-	+	-	-	-	-	-	-	+	-	-	+
16	+	-	-	+	-	+	-	-	-	-	-	-	+	-	-	+
17	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
18	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
19	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
20	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
21	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
22	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
23	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
24	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
25	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
26	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
27	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
28	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
29	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
30	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
31	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
32	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
33	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+

	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE
34	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
35	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
36	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
37	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
38	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
39	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
40	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
41	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
42	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
43	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
44	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
45	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
46	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
47	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
48	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
49	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
50	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
51	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
52	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
53	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
54	+	-	+	-	+	-	-	-	-	-	-	+	-	-	-	+
55	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
56	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
57	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
58	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
59	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
60	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
61	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
62	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
63	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
64	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
65	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
66	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+

	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE
67	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
68	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
69	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
70	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
71	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
72	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	+
73	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	+
74	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	+
75	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	+
76	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	+
77	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	+
78	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	+
79	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	+
80	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	+
81	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	+
82	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
83	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
84	+	-	-	-	-	+	-	-	+	-	-	-	-	+	-	+
85	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+
86	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+
87	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+
88	+	-	-	+	-	-	+	-	-	-	-	-	+	+	-	+
89	+	-	-	-	-	-	+	+	+	-	-	-	-	+	-	+
90	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+
91	+	-	-	-	+	-	-	-	-	-	-	+	+	-	-	+
92	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	+
93	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	+
94	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	+
95	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	+
96	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	+
97	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	+
98	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	+
99	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	+

	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE
100	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
101	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
102	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
103	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
104	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
105	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
106	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
107	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
108	+	-	-	+	-	+	-	-	-	-	-	-	+	-	-	+
109	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
110	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
111	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
112	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
113	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
114	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
115	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
116	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
117	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
118	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
119	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
120	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
121	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
122	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
123	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
124	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
125	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
126	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
127	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
128	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
129	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
130	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
131	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
132	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+

	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE
133	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
134	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
135	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
136	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
137	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
138	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
139	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
140	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
141	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
142	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
143	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
144	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
145	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
146	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
147	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
148	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
149	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
150	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
151	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
152	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
153	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
154	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
155	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
156	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
157	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
158	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
159	-	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
160	-	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
161	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
162	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
163	-	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
164	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
165	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+

	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE
166	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
167	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
168	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
169	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
170	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
171	-	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
172	+	-	-	+	-	+	-	-	-	-	-	-	+	-	-	+
173	+	-	-	+	-	+	-	-	-	-	-	-	+	-	-	+
174	+	-	-	+	-	+	-	-	-	-	-	-	+	-	-	+
175	+	-	-	+	-	+	-	-	-	-	-	-	+	-	-	+
176	+	-	-	+	-	+	-	-	-	-	-	-	+	-	-	+
177	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
178	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
179	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
180	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
181	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
182	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
183	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
184	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
185	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
186	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
187	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
188	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
189	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
190	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
191	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
192	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
193	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
194	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
195	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
196	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
197	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+
198	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	+

	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE
199	+	-	+	-	-	-	-	-	-	-	-	+	-	-	-	+
200	+	-	+	-	+	-	-	-	-	-	-	+	-	-	-	+
201	+	-	+	-	-	-	-	-	-	-	-	+	-	-	-	+
202	+	-	+	-	+	-	-	-	-	-	-	+	-	-	-	+
203	+	-	+	-	-	-	-	-	-	-	-	+	-	-	-	+
204	+	-	+	-	-	-	-	-	-	-	-	+	-	-	-	+
205	+	-	-	-	+	-	-	-	-	-	-	+	-	-	-	+
206	+	-	+	-	-	-	-	-	-	-	-	+	-	-	-	+
207	-	-	-	-	+	-	-	+	-	-	+	-	-	-	+	+
208	+	-	-	-	-	-	+	-	-	+	-	-	-	+	-	+
209	+	-	-	-	-	-	+	-	-	+	-	-	-	+	-	+
210	+	-	+	-	+	-	-	-	-	-	-	+	-	-	-	+

	AF	AG	AH	AI	AJ	AK	AL	AM	AN	AO	AP	AQ	AR
1	Tru216	Tru214	Tru212	Tru211	Tru210	Tru207	Tru197	Tru191	Tru190	Tru171	Bsu360	Bsu335	Bsu300
2	-	-	-	-	-	+	-	+	-	-	+	-	+
3	-	-	-	-	-	+	-	+	-	-	+	-	+
4	-	-	-	-	-	+	-	+	-	-	+	-	+
5	-	-	-	-	-	+	-	+	-	-	+	-	+
6	-	-	-	-	-	+	-	+	-	-	+	-	+
7	-	-	-	-	-	+	-	+	-	-	+	-	+
8	-	-	-	-	-	+	-	+	-	-	+	-	+
9	-	-	-	-	-	+	-	+	-	-	+	-	+
10	-	-	-	-	-	+	-	+	-	-	+	-	+
11	-	-	-	-	-	+	-	+	-	-	+	-	+
12	-	+	-	-	-	+	-	+	-	-	-	+	+
13	-	+	-	-	-	+	-	+	-	-	-	+	+
14	-	-	-	-	-	+	-	+	-	-	-	+	+
15	-	-	-	-	-	+	-	+	-	-	-	+	+
16	-	-	-	-	-	+	-	+	-	-	-	+	+
17	-	+	-	-	-	+	-	+	-	-	-	+	+
18	-	+	-	-	-	+	-	+	-	-	-	+	+
19	-	+	-	-	-	+	-	+	-	-	-	+	+
20	-	+	-	-	-	+	-	+	-	-	-	+	+
21	-	+	-	-	-	+	-	+	-	-	-	+	+
22	-	-	-	-	-	+	-	+	-	-	+	-	+
23	-	-	-	-	-	+	-	+	-	-	+	-	+
24	-	-	-	-	-	+	-	+	-	-	+	-	+
25	-	-	-	-	-	+	-	+	-	-	+	-	+
26	-	-	-	-	-	+	-	+	-	-	+	-	+
27	-	-	-	-	-	+	-	+	-	-	+	-	+
28	-	-	-	-	-	+	-	+	-	-	+	-	+
29	-	-	-	-	-	+	-	+	-	-	+	-	+
30	-	-	-	-	-	+	-	+	-	-	+	-	+
31	-	-	-	-	-	+	-	+	-	-	+	-	+
32	-	+	-	-	-	+	-	+	-	-	-	+	+
33	-	+	-	-	-	+	+	-	-	-	-	+	+

	AF	AG	AH	AI	AJ	AK	AL	AM	AN	AO	AP	AQ	AR
34	-	+	-	-	-	+	-	+	-	-	-	+	+
35	-	+	-	-	-	+	-	+	-	-	-	+	+
36	-	+	-	-	-	+	-	+	-	-	-	+	+
37	-	+	-	-	-	+	-	+	-	-	-	+	+
38	-	+	-	-	-	+	-	+	-	-	-	+	+
39	-	+	-	-	-	+	-	+	-	-	-	+	+
40	-	+	-	-	-	+	-	+	-	-	-	+	+
41	-	+	-	-	-	+	-	+	-	-	-	+	+
42	-	+	-	-	-	+	-	+	-	-	-	+	+
43	-	+	-	-	-	+	-	+	-	-	-	+	+
44	-	+	-	-	-	+	-	+	-	-	-	+	+
45	-	+	-	-	-	+	-	+	-	-	-	+	+
46	-	+	-	-	-	+	-	+	-	-	-	+	+
47	-	+	-	-	-	+	-	+	-	-	-	+	+
48	-	+	-	-	-	+	-	+	-	-	-	+	+
49	-	+	-	-	-	+	-	+	-	-	-	+	+
50	-	+	-	-	-	+	-	+	-	-	-	+	+
51	-	+	-	-	-	+	-	+	-	-	-	+	+
52	-	-	-	-	-	+	-	+	-	-	+	-	+
53	-	-	-	-	-	+	-	+	-	-	+	-	+
54	-	-	-	-	-	+	-	+	-	-	+	-	+
55	-	-	-	-	-	+	-	+	-	-	+	-	+
56	-	-	-	-	-	+	-	+	-	-	+	-	+
57	-	-	-	-	-	+	-	+	-	-	+	-	+
58	-	-	-	-	-	+	-	+	-	-	+	-	+
59	-	-	-	-	-	+	-	+	-	-	+	-	+
60	-	-	-	-	-	+	-	+	-	-	+	-	+
61	-	-	-	-	-	+	-	+	-	-	+	-	+
62	-	+	-	-	-	+	-	+	-	-	-	+	+
63	-	+	-	-	-	+	-	+	-	-	-	+	+
64	-	+	-	-	-	+	-	+	-	-	-	+	+
65	-	+	-	-	-	+	-	+	-	-	-	+	+
66	-	+	-	-	-	+	-	+	-	-	-	+	+

	AF	AG	AH	AI	AJ	AK	AL	AM	AN	AO	AP	AQ	AR
67	-	+	-	-	-	+	+	-	-	-	-	+	+
68	-	+	-	-	-	+	-	+	-	-	-	+	+
69	-	+	-	-	-	+	-	+	-	-	-	+	+
70	-	+	-	-	-	+	+	-	-	-	-	+	+
71	-	+	-	-	-	+	+	+	-	+	+	+	+
72	-	-	-	-	-	+	+	+	-	+	+	-	+
73	-	-	-	-	-	+	+	+	-	+	+	-	+
74	-	-	-	-	-	+	+	+	-	+	+	-	+
75	-	-	-	-	-	+	+	+	-	+	+	-	+
76	-	-	-	-	-	+	+	+	-	+	+	-	+
77	-	-	-	-	-	+	+	+	-	+	+	-	+
78	-	-	-	-	-	+	+	+	-	+	+	-	+
79	-	-	-	-	-	+	+	+	-	+	+	-	+
80	-	-	-	-	-	+	+	+	-	+	+	-	+
81	-	-	-	-	-	+	+	+	-	+	+	-	+
82	-	-	-	-	-	+	+	+	-	-	-	+	+
83	-	-	-	-	-	+	+	+	-	-	-	+	+
84	-	-	-	-	-	+	+	+	-	-	-	+	+
85	-	-	-	-	-	+	+	+	-	-	-	+	+
86	-	-	-	-	-	+	+	+	-	-	-	+	+
87	-	-	-	-	-	+	+	+	-	-	-	+	+
88	-	-	-	-	-	+	+	+	-	-	-	+	+
89	-	-	-	-	-	+	+	+	-	-	-	+	+
90	-	-	-	-	-	+	+	+	-	-	-	+	+
91	-	-	-	-	-	+	+	+	-	-	-	+	+
92	-	-	-	-	-	+	+	+	-	+	+	-	+
93	-	-	-	-	-	+	+	+	-	+	+	-	+
94	-	-	-	-	-	+	+	+	-	+	+	-	+
95	-	-	-	-	-	+	+	+	-	+	+	-	+
96	-	-	-	-	-	+	+	+	-	+	+	-	+
97	-	-	-	-	-	+	+	+	-	+	+	-	+
98	-	-	-	-	-	+	+	+	-	+	+	-	+
99	-	-	-	-	-	+	+	+	-	+	+	-	+

	AF	AG	AH	AI	AJ	AK	AL	AM	AN	AO	AP	AQ	AR
100	-	-	-	-	-	+	-	+	-	-	+	-	+
101	-	-	-	-	-	+	-	+	-	-	+	-	+
102	-	-	-	-	-	+	-	+	-	-	-	+	+
103	-	+	-	-	-	+	-	-	-	-	-	+	+
104	-	+	-	-	-	+	-	-	-	-	-	+	+
105	-	-	-	-	-	+	-	+	-	-	-	+	+
106	-	-	-	-	-	+	-	+	-	-	-	+	+
107	-	+	-	-	-	+	-	-	-	-	-	+	+
108	-	-	-	-	-	+	-	+	-	-	-	+	+
109	-	+	-	-	-	+	-	-	-	-	-	+	+
110	-	-	-	-	-	+	-	+	-	-	-	+	+
111	-	-	-	-	-	+	-	+	-	-	-	+	+
112	-	-	-	-	-	+	-	+	-	-	+	-	+
113	-	-	-	-	-	+	-	+	-	-	+	-	+
114	-	-	-	-	-	+	-	+	-	-	+	-	+
115	-	-	-	-	-	+	-	+	-	-	+	-	+
116	-	-	-	-	-	+	-	+	-	-	+	-	+
117	-	-	-	-	-	+	-	+	-	-	+	-	+
118	-	-	-	-	-	+	-	+	-	-	+	-	+
119	-	-	-	-	-	+	-	+	-	-	+	-	+
120	-	-	-	-	-	+	-	+	-	-	+	-	+
121	-	-	-	-	-	+	-	+	-	-	+	-	+
122	-	+	-	-	-	+	-	-	-	-	-	+	+
123	-	-	-	-	-	+	-	+	-	-	-	+	+
124	-	+	-	-	-	+	-	-	-	-	-	+	+
125	-	-	-	-	-	+	-	+	-	-	-	+	+
126	-	-	-	-	-	+	-	+	-	-	-	+	+
127	-	-	-	-	-	+	-	+	-	-	-	+	+
128	-	+	-	-	-	+	-	-	-	-	-	+	+
129	-	-	-	-	-	+	-	+	-	-	-	+	+
130	-	-	-	-	-	+	-	+	-	-	-	+	+
131	-	-	-	-	-	+	-	+	-	-	-	+	+
132	-	-	-	-	-	+	-	+	-	-	-	+	+

	AF	AG	AH	AI	AJ	AK	AL	AM	AN	AO	AP	AQ	AR
133	-	-	-	-	-	+	-	+	-	-	-	+	+
134	-	+	-	-	-	+	-	-	-	-	-	+	+
135	-	-	-	-	-	+	-	+	-	-	-	+	+
136	-	-	-	-	-	+	-	+	-	-	-	+	+
137	-	-	-	-	-	+	-	+	-	-	-	+	+
138	-	+	-	-	-	+	-	-	-	-	-	+	+
139	-	+	-	-	-	+	-	-	-	-	-	+	+
140	-	-	-	-	-	+	-	+	-	-	-	+	+
141	-	-	-	-	-	+	-	+	-	-	-	+	+
142	-	-	-	-	-	+	-	+	-	-	-	+	+
143	-	-	-	-	-	+	-	+	-	-	-	+	+
144	-	-	-	-	-	+	-	+	-	-	-	+	+
145	-	-	-	-	-	+	-	+	-	-	-	+	+
146	-	-	-	-	-	+	-	+	-	-	-	+	+
147	-	-	-	-	-	+	-	+	-	-	-	+	+
148	-	-	-	-	-	+	-	+	-	-	-	+	+
149	-	-	-	-	-	+	-	+	-	-	-	+	+
150	-	-	-	-	-	+	-	+	-	-	-	+	+
151	-	-	-	-	-	+	-	+	-	-	-	+	+
152	-	-	-	-	-	+	-	+	-	-	-	+	+
153	-	-	-	-	-	+	-	+	-	-	-	+	+
154	-	-	-	-	-	+	-	+	-	-	-	+	+
155	-	+	-	-	-	+	-	+	-	-	-	+	+
156	-	-	-	-	-	+	-	+	-	-	-	+	+
157	-	-	-	-	-	+	-	+	-	-	-	+	+
158	-	+	-	-	-	+	-	+	-	-	-	+	+
159	-	+	-	-	-	+	-	+	-	-	-	+	+
160	-	+	-	-	+	-	-	-	+	-	-	+	+
161	-	+	-	-	+	-	-	-	+	-	-	+	+
162	-	-	-	-	-	+	-	+	-	-	-	+	+
163	-	+	-	-	+	-	+	-	+	-	-	+	+
164	-	+	-	-	-	+	-	+	-	-	-	+	+
165	-	+	-	-	-	+	-	+	-	-	-	+	+

	AF	AG	AH	AI	AJ	AK	AL	AM	AN	AO	AP	AQ	AR
166	-	+	-	-	-	+	-	+	-	-	-	+	+
167	-	+	-	-	-	+	-	+	-	-	-	+	+
168	-	+	-	-	-	+	-	+	-	-	-	+	+
169	-	-	-	-	-	+	-	+	-	-	-	+	+
170	-	+	-	-	-	+	-	+	-	-	-	+	+
171	-	+	-	-	+	-	-	-	+	-	-	+	+
172	-	-	-	-	-	+	-	+	-	-	-	+	+
173	-	-	-	-	-	+	-	+	-	-	-	+	+
174	-	-	-	-	-	+	-	+	-	-	-	+	+
175	-	-	-	-	-	+	-	+	-	-	-	+	+
176	-	-	-	-	-	+	-	+	-	-	-	+	+
177	-	-	-	-	-	+	-	+	-	-	+	-	+
178	-	-	-	-	-	+	-	+	-	-	+	-	+
179	-	-	-	-	-	+	-	+	-	-	+	-	+
180	-	-	-	-	-	+	-	+	-	-	+	-	+
181	-	-	-	-	-	+	-	+	-	-	+	-	+
182	-	-	-	-	-	+	-	+	-	-	+	-	+
183	-	-	-	-	-	+	-	+	-	-	+	-	+
184	-	-	-	-	-	+	-	+	-	-	+	-	+
185	-	-	-	-	-	+	-	+	-	-	+	-	+
186	-	-	-	-	-	+	-	+	-	-	+	-	+
187	-	-	-	-	-	+	-	+	-	-	+	-	+
188	-	-	-	-	-	+	-	+	-	-	+	-	+
189	-	-	-	-	-	+	-	+	-	-	+	-	+
190	-	-	-	-	-	+	-	+	-	-	+	-	+
191	-	-	-	-	-	+	-	+	-	-	+	-	+
192	-	-	-	-	-	+	-	+	-	-	+	-	+
193	-	-	-	-	-	+	-	+	-	-	+	-	+
194	-	-	-	-	-	+	-	+	-	-	+	-	+
195	-	-	-	-	-	+	-	+	-	-	+	-	+
196	-	-	-	-	-	+	-	+	-	-	+	-	+
197	-	-	-	-	-	+	-	+	-	-	+	-	+
198	-	-	-	-	-	+	-	+	-	-	+	-	+

	AF	AG	AH	AI	AJ	AK	AL	AM	AN	AO	AP	AQ	AR
199	-	-	-	-	-	+	-	+	-	-	+	-	+
200	-	-	-	-	-	+	-	+	-	-	+	-	+
201	-	-	-	-	-	+	-	+	-	-	+	-	+
202	-	-	-	-	-	+	-	+	-	-	+	-	+
203	-	-	-	-	-	+	-	+	-	-	+	-	+
204	-	-	-	-	-	+	-	+	-	-	+	-	+
205	-	-	-	-	-	+	-	+	-	-	+	-	+
206	-	-	-	-	-	+	-	+	-	-	+	-	+
207	+	-	-	+	-	-	-	+	-	+	-	+	+
208	+	-	+	-	-	+	-	+	-	+	-	+	+
209	+	-	+	-	-	+	-	+	-	+	-	+	+
210	-	-	-	-	-	+	-	+	-	-	+	-	+

Appendix 3: Microsatellite data

The number “1” indicates the absence of a band for a particular sample in cases where the primers amplify alleles in other samples from the population.

The word “null” indicates the absence of a band for a particular sample in cases where too few plants were examined to know whether or not the primers amplify alleles in the population.

If more than two alleles were present for a sample, the third allele is indicated in the column headed “3rd band” and the ploidy level of the sample, if known, is indicated as “6X” or “8X” for hexaploid or octaploid. The third allele was not used in analyses. Bands allocated to the category of “third allele” were never unique, rare, or private.

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	Sample	Pop	Locus1-Dist1		Locus2-Dist3		3rd band	Locus3-Dist10		3rd band	Locus4-Dist22		3rd band
2	SFBY012	SFBY	236	224	200	200		1	1		166	154	
3	SFBY020	SFBY	242	226	200	200		180	180		166	154	
4	SFBY021	SFBY	230	230	200	200		1	1		166	154	
5	SFBY022	SFBY	222	222	192	192		194	194		166	154	
6	SFBY027	SFBY	236	236	212	200		180	180		166	154	
7	SFBY028	SFBY	238	226	212	200		180	180		166	154	
8	SFBY031	SFBY	238	238	200	200		1	1		166	154	
9	SFBY032	SFBY	238	230	212	200		180	180		166	154	
10	SFBY033	SFBY	238	228	212	200		183	183		166	154	
11	SFBY036	SFBY	238	228	208	208		180	180		166	154	
12	SFBY001	SFBY	240	224	208	204		182	182		166	154	
13	SFBY006	SFBY	242	226	200	200		180	180		166	154	
14	SFBY038	SFBY	236	222	200	200		182	182		166	154	
15	SFBY039	SFBY	240	236	200	200		1	1		166	158	
16	SFBY041	SFBY	234	220	204	204		1	1		166	154	
17	SFBY051	SFBY	227	221	214	208		180	180		166	154	
18	SFBY061	SFBY	232	218	211	211		182	182		166	154	
19	SFBY067	SFBY	226	218	208	204		180	180		166	154	
20	SFBY089	SFBY	238	226	208	200		1	1		166	154	
21	SFBY096	SFBY	238	225	200	200		183	183		166	154	
22	GATE006	GATE	224	224	212	212		1	1		166	154	
23	GATE007	GATE	242	242	212	212		180	180		166	154	
24	GATE013	GATE	242	228	200	200		1	1		166	154	
25	GATE033	GATE	220	220	200	200		1	1		166	154	
26	GATE036	GATE	240	240	208	204		1	1		166	154	
27	GATE043	GATE	240	240	212	212		182	182		166	154	
28	GATE044	GATE	240	228	200	200		184	184		166	154	
29	GATE045	GATE	240	234	204	200		180	180		166	154	
30	GATE055	GATE	240	230	204	200		184	184		166	154	
31	GATE060	GATE	254	242	208	208		1	1		166	154	
32	GATE010	GATE	240	226	211	211		182	182		166	154	
33	GATE019	GATE	230	221	204	204		1	1		166	154	

	A	B	C	D	E	F	G	H	I	J	K	L	M
34	GATE020	GATE	240	230	204	200		196	182		166	154	
35	GATE029	GATE	240	226	212	212		1	1		166	166	
36	GATE037	GATE	236	228	208	200		183	180		166	154	
37	GATE048	GATE	240	226	211	211		1	1		166	154	
38	GATE058	GATE	254	238	211	211		1	1		166	154	
39	GATE064	GATE	238	227	208	208		1	1		170	166	
40	GATE070	GATE	240	226	211	211		182	182		166	154	
41	GATE077	GATE	230	216	208	204		194	194		166	154	
42	WASH001	WASH	260	230	212	200		1	1		166	154	
43	WASH005	WASH	256	236	204	200		206	206		184	154	166
44	WASH008	WASH	256	230	224	224		206	206		166	154	
45	WASH012	WASH	258	258	212	200		1	1		184	154	166
46	WASH024	WASH	260	260	204	200		1	1		166	154	
47	WASH026	WASH	256	230	204	200		1	1		166	154	
48	WASH027	WASH	260	234	204	200		1	1		184	154	166
49	WASH028	WASH	254	254	224	200		206	206		165	154	
50	WASH037	WASH	230	230	240	212		206	206		184	154	166
51	WASH041	WASH	254	254	200	200		1	1		166	154	
52	WASH002	WASH	256	256	240	240		206	206		166	154	
53	WASH004	WASH	256	232	240	240		206	197		166	154	
54	WASH009	WASH	232	232	212	212		206	197		166	154	
55	WASH016	WASH	232	232	224	206		206	206		166	166	
56	WASH044	WASH	232	232	224	213		206	206		166	153	
57	WASH054	WASH	260	232	204	200		206	206		166	158	
58	WASH081	WASH	230	216	212	212		206	206		166	153	
59	WASH084	WASH	256	230	204	200		206	206		166	166	
60	WASH095	WASH	260	232	240	240		197	197		166	158	
61	WASH096	WASH	232	232	204	200		206	206		166	153	
62	TOMTOCA	TORC	238	225	208	208		182	180		166	154	
63	TOMTOCB	TORC	242	226	212	200		180	180		166	154	
64	TOMTOCD	TORC	252	248	208	196		194	194		166	154	
65	TOMTOCE	TORC	246	230	212	212		182	182		166	154	
66	TOMTOCF	TORC	236	218	212	212		192	192		166	154	

	A	B	C	D	E	F	G	H	I	J	K	L	M
67	TOMTOCG	TORC	244	225	214	204		194	194		166	154	
68	TOMTOCH	TORC	244	244	214	204		1	1		166	154	
69	TOMTOCI	TORC	244	226	212	200		180	180		166	154	
70	TOMCRUJ	CRUC	220	206	212	200		180	180		166	154	
71	TOMCRUR	CRUC	240	224	212	212		182	182		166	154	
72	TOMCRUS	CRUC	238	230	212	204		182	180		165	153	
73	TOMCRUT	CRUC	240	240	212	204		180	180		166	154	
74	TOMCRUU	CRUC	230	230	212	200		1	1		166	154	
75	TOMCRUV	CRUC	240	226	212	200		1	1		166	154	
76	TOMCRUX	CRUC	244	221	212	200		180	180		166	154	
77	TOMCRUY	CRUC	256	232	212	204		1	1		166	154	
78	TOMCRUZ	CRUC	224	224	208	204		182	182		166	154	
79	TOMCRAA	CRUC	224	224	208	204		182	182		166	154	
80	TOMCRBB	CRUC	238	222	212	200		194	180		166	154	
81	TOMCRCC	CRUC	246	232	212	204		183	183		166	154	
82	TOMCRDD	CRUC	224	224	208	204		182	182		166	154	
83	TOMCREE	CRUC	240	226	212	200		1	1		166	154	
84	SEAT007	SEAT	238	228	200	200		1	1		166	154	
85	SEAT008	SEAT	270	228	228	228		1	1		166	154	
86	SEAT011	SEAT	228	228	1	1		1	1		166	154	
87	SEAT014	SEAT	228	228	224	200		1	1		166	154	
88	SEAT019	SEAT	270	228	212	200		1	1		166	154	
89	SEAT020	SEAT	234	228	228	200		1	1		166	154	
90	SEAT021	SEAT	234	234	200	200		1	1		166	154	
91	SEAT022	SEAT	230	228	228	200		1	1		166	154	
92	ANTR127	ANTR	228	228	196	192		1	1		1	1	
93	ANTR134	ANTR	228	220	1	1		190	182		166	154	
94	ANTR137	ANTR	230	226	192	192		190	182		166	154	
95	ANTR140	ANTR	228	214	196	192		184	182		166	154	
96	ANTR145	ANTR	228	228	196	192		184	182		166	154	
97	ANTR146	ANTR	228	214	192	192		190	184		166	154	
98	ANTR149	ANTR	228	228	192	192		1	1		166	154	
99	ANTR150	ANTR	1	1	192	192		1	1		166	154	

	A	B	C	D	E	F	G	H	I	J	K	L	M
100	ANTR152	ANTR	228	214	196	192		182	182		166	154	
101	ANTR153	ANTR	228	214	196	192		182	182		166	154	
102	ANTR125	ANTR	228	214	196	192		182	182		166	166	
103	ANTR130	ANTR	1	1	196	196		194	194		166	166	
104	ANTR133	ANTR	228	214	192	192		1	1		166	166	
105	ANTR135	ANTR	230	227	192	192		190	182		166	166	
106	ANTR141	ANTR	1	1	196	196		196	182		166	150	
107	ANTR143	ANTR	224	224	196	196		196	192		166	150	
108	ANTR144	ANTR	1	1	196	196		196	182		166	150	
109	ANTR148	ANTR	228	228	192	192		1	1		166	154	
110	ANTR151	ANTR	228	228	192	192		1	1		166	154	
111	ANTR155	ANTR	233	228	200	200		190	190		166	166	
112	PAWN011	PAWN	1	1	216	192		182	182		166	154	
113	PAWN015	PAWN	220	206	228	192		198	190		178	170	
114	PAWN016	PAWN	206	206	204	200		198	198		178	166	
115	PAWN019	PAWN	1	1	200	192		194	190		166	166	
116	PAWN021	PAWN	1	1	200	192		182	182		170	158	
117	PAWN022	PAWN	1	1	212	200		1	1		166	154	
118	PAWN027	PAWN	230	230	240	196		188	182		170	158	
119	PAWN028	PAWN	1	1	240	192		190	182		170	158	
120	PAWN031	PAWN	1	1	212	200		182	182		166	154	
121	PAWN052	PAWN	1	1	192	192		198	186		166	154	
122	PAWN002	PAWN	230	230	216	192	6X 196	190	188		170	158	
123	PAWN003	PAWN	230	230	216	192	6X 196	190	188		170	170	
124	PAWN050	PAWN	1	1	196	192	6X 212	190	184		178	150	
125	PAWN051	PAWN	1	1	196	192	6X 212	190	184		178	150	
126	PAWN095	PAWN	1	1	192	192		188	184	6X 190	178	166	
127	PAWN046	PAWN	1	1	196	192	8X 208	188	184		178	150	
128	PAWN055	PAWN	226	226	196	192	8X 208	188	184		178	150	8X 166
129	PAWN057	PAWN	1	1	196	192	8X 208	188	184		178	150	
130	PAWN061	PAWN	226	226	196	192	8X 208	188	184		178	150	
131	PAWN070	PAWN	1	1	196	192	8X 208	188	184		178	150	
132	PAWN010	PAWN	1	1	192	192		182	182		170	150	

	A	B	C	D	E	F	G	H	I	J	K	L	M
133	PAWN014	PAWN	220	206	228	192		198	190		178	170	
134	PAWN018	PAWN	220	206	228	196		198	190		178	150	
135	PAWN024	PAWN	1	1	196	192	6X 208	188	184		178	150	
136	PAWN025	PAWN	232	216	240	196		188	182		170	170	
137	PAWN078	PAWN	227	227	228	211	213	182	182		170	158	
138	PAWN086	PAWN	230	220	196	192		190	190		178	150	
139	PAWN087	PAWN	228	226	228	204		190	182		178	170	
140	PAWN097	PAWN	228	210	213	211		190	182		154	154	
141	PAWN098	PAWN	248	224	196	192		198	184		166	154	
142	RRIV111	RRIV	222	222	228	196		190	188		184	150	166
143	RRIV112	RRIV	230	216	204	204		188	188		184	178	174
144	RRIV113	RRIV	226	226	204	204		188	188		166	154	
145	RRIV114	RRIV	230	216	196	192		190	190		166	150	
146	RRIV115	RRIV	1	1	204	196		188	188		182	174	
147	RRIV116	RRIV	226	226	192	192		190	188		184	150	
148	RRIV117	RRIV	230	230	196	192		188	188		170	158	
149	RRIV118	RRIV	226	226	196	192		182	182		178	150	
150	RRIV119	RRIV	230	230	192	192		188	182		150	150	
151	RRIV120	RRIV	230	230	196	192		182	182		184	174	
152	WRAY156	WRAY	1	1	213	196		182	182		166	153	
153	WRAY157	WRAY	228	228	213	200		198	198		1	1	
154	WRAY159	WRAY	1	1	204	192		182	182		1	1	
155	WRAY160	WRAY	1	1	213	213		186	182		166	166	
156	WRAY161	WRAY	224	224	204	200		186	182		166	166	
157	WRAY162	WRAY	1	1	204	196		196	190		178	170	6X 166
158	WRAY165	WRAY	1	1	216	204		186	182		166	153	
159	WRAY166	WRAY	228	228	204	192		184	182		165	150	
160	WRAY169	WRAY	1	1	204	192		198	182		170	170	
161	WRAY170	WRAY	242	242	204	192		186	184		170	170	
162	WRAY171	WRAY	1	1	208	208		186	186		166	166	
163	WRAY174	WRAY	240	226	204	200		182	182		166	150	
164	WRAY179	WRAY	226	226	204	192		198	190		170	170	
165	WRAY180	WRAY	220	206	200	200		182	182		166	150	

	A	B	C	D	E	F	G	H	I	J	K	L	M
166	WRAY181	WRAY	1	1	212	192		198	190		178	178	
167	WRAY182	WRAY	1	1	204	192		198	184		166	166	
168	WRAY183	WRAY	1	1	204	196		168	168		170	170	
169	WRAY184	WRAY	233	233	204	192		197	190		178	178	
170	WRAY186	WRAY	233	233	204	200		197	197		166	166	
171	WRAY187	WRAY	233	233	213	204		198	182		166	154	
172	HONY133	HONY	230	230	1	1		188	188		198	198	
173	HONY140	HONY	232	232	192	192		188	188		194	194	
174	HONY142	HONY	1	1	1	1		196	186		178	152	
175	HONY150	HONY	1	1	1	1		190	190		182	182	
176	HONY152	HONY	1	1	200	192		196	196		182	182	
177	HONY156	HONY	1	1	192	192		190	190		194	182	
178	HONY165	HONY	230	230	1	1		196	186		194	190	
179	HONY166	HONY	1	1	1	1		196	196		186	186	
180	HONY167	HONY	230	230	192	192		196	188		178	152	
181	HONY168	HONY	232	232	192	192		196	186		152	152	
182	HONY173	HONY	232	232	1	1		188	188		182	152	
183	HONY174	HONY	1	1	1	1		196	196		194	166	
184	HONY176	HONY	232	232	192	192		196	196		178	178	
185	HONY179	HONY	1	1	1	1		196	196		198	152	
186	HONY180	HONY	230	230	1	1		188	188		178	178	
187	GOOS181	GOOS	1	1	1	1		196	196		202	202	
188	GOOS201	GOOS	1	1	1	1		190	190		198	178	
189	GOOS202	GOOS	230	230	1	1		188	188		206	206	
190	GOOS203	GOOS	236	236	1	1		188	188		186	178	
191	GOOS204	GOOS	233	233	192	192		186	186		1	1	
192	GOOS209	GOOS	234	234	200	200		194	194		178	178	
193	GOOS210	GOOS	233	233	200	200		202	202		178	178	
194	GOOS220	GOOS	233	233	200	200		188	188		1	1	
195	GOOS223	GOOS	232	232	192	192		1	1		206	178	
196	GOOS225	GOOS	232	232	200	200		188	188		178	178	
197	GOOS226	GOOS	232	232	192	192		1	1		178	178	
198	GOOS227	GOOS	232	232	1	1		190	188		178	178	

	A	B	C	D	E	F	G	H	I	J	K	L	M
199	GOOS235	GOOS	232	232	1	1		188	188		154	154	
200	GOOS236	GOOS	233	233	1	1		206	206		190	178	
201	GOOS237	GOOS	233	233	204	192		202	202		170	170	
202	TOMALBL	ALBU	1	1	200	196		190	190		166	166	
203	TOMALBM	ALBU	1	1	200	196		190	190		166	166	
204	TOMALBN	ALBU	1	1	200	192		190	190		206	206	
205	TOMALBO	ALBU	1	1	200	200		190	190		178	178	
206	TOMALBP	ALBU	1	1	196	192		192	190		206	170	
207	REEDER		null		null			null			160	158	
208	MONANCA		null		null			null			221	110	
209	MONANTX		null		null			null			220	109	
210	FLORIDA		230	200	224	220		184	165		170	176	