AN ABSTRACT OF THE THESIS OF

Lemma W. Mengistu for the degree of Doctor of Philosophy in Crop Science presented on July 2, 1998. Title: Genetic Diversity and Herbicide Resistance in Annual Bluegrass (*Poa annua* L.).

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George W. Mueller-Warrant

We surveyed the genetic diversity of *Poa annua* using randomly amplified polymorphic DNA (RAPD) from 1357 individual plants. One mechanism of resistance to metribuzin and diuron was inferred from DNA sequence analysis of the *psb*A gene.

Gene diversity and differentiation parameters and analysis of molecular variance (AMOVA) revealed the presence of significant variability in *P. annua* among sites, collection dates within sites and within collection dates. The mean Nei gene diversity statistic (h) for all populations was 0.241, and the total diversity (H_T) was 0.245. A greater proportion of the diversity, however, was within populations (H_S = 0.209) rather than among populations (G_{ST} = 0.146). Diversity among herbicide susceptible populations was greater (G_{ST} = 0.125) than among resistant populations (G_{ST} as low as 0.01). Genetic diversity was lower in most populations from high herbicide selection pressure areas than from low, indicating that selection pressure played a vital role in shaping the genetic structure of *P. annua*. Low G_{ST} and high H_S imply high gene flow among populations.

The herbicide-binding region of a *psb*A gene DNA fragment from metribuzin and diuron resistant and susceptible accessions of *P. annua* was selectively amplified using PCR. Sequence analysis of this fragment revealed that six accessions of *P. annua* (one resistant to both herbicides as well as five diuron resistant whose reaction to metribuzin was not tested) exhibited a substitution of valine to isoleucine at position 219 of the D1 protein of the *psb*A gene. This is the same mutation as reported for *Chlamydomonas* and *Synechococcus* through site-selected or site-directed mutagenesis and in cell cultures of *Chenopodium rubrum.* This mutation has been reported to cause resistance to metribuzin, diuron and ioxynil. Our results show that this newly discovered mutation in higher plants could occur under conditions in western Oregon grass seed fields. The absence of *psb*A mutation in certain other accessions resistant to diuron and metribuzin implies the existence of other mechanisms of resistance. To our knowledge no higher plant has ever been reported to exhibit resistance in the field to photosystem II inhibitors due to any *psb*A mutations other than amino acid position 264.

Genetic Diversity and Herbicide Resistance in Annual Bluegrass (*Poa annua* L.)

by

Lemma W. Mengistu

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APPROVED:

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Major professor, representing Crop Science

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Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Lemma W. Mengistu, Author

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

Page

LIST OF FIGURES

LIST OF TABLES

GENETIC DIVERSITY AND HERBICIDE RESISTANCE IN ANNUAL BLUEGRASS (*Poa annua* L.)

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

OBJECTIVES AND GOALS OF THESIS

The objective of my thesis is to help modify old and develop new seed production and weed control practices that will prolong our ability to control *Poa annua* by delaying the advent and minimizing the extent of resistance to herbicides and cultural weed control practices. This thesis was therefore initiated in order to understand the genetic diversity and the genetics of herbicide resistance in *P. annua*.

My specific goals were: 1) To determine and quantify the extent of genetic diversity within and among populations of *P. annua* of western Oregon grass seed crops. 2) To detect whether time of germination of *P. annua* or site and herbicide use histories of specific fields have association with the diversity of the species. 3) To determine whether the herbicide selection pressure has a role in shaping the population structure of *P. annua*. 4) To determine the mechanism of resistance to diuron and metribuzin and possibly identify and sequence the site mutation resulting in the resistance as well as characterize the resistance mechanisms in terms of number of genes and inheritance of resistance.

ORGANIZATION OF THESIS

This thesis is organized in to 4 chapters followed by a reference list. Chapter 1 provides a review of literature relevant to the thesis covering the biology of *P. annua*. Chapter 2 entitled "Genetic diversity of annual bluegrass (*Poa annua* L.) in western Oregon grass seed crops" deals with measurements of the total genetic diversity, within population diversity, and among population diversity, in various structured populations of *P. annua*. Chapter 3 entitled " A *psb*A mutation (219 valine to isoleucine) causes resistance to metribuzin and diuron in annual bluegrass (*Poa annua* L.)" deals with identification of the mechanism of resistance and characterization of identified mutants by sequence analysis. Chapter 4 provides comprehensive conclusions drawn from the thesis research and is followed by a bibliography containing all references cited within the thesis.

THE PLANT ANNUAL BLUEGRASS (*POA ANNUA* L.)

Annual bluegrass (*Poa annua* L.) is also known by several other common names such as goose grass, winter grass, and annual meadowgrass. The species belongs to the genus *Poa*. Anton and Connor (1995) made an extensive review of the floral biology and reproduction in the genus *Poa*. In summary, the genus is estimated to consist of 400-500 species, has many varied floral forms because most elements from hermaphroditism to diocism are expressed, and, in addition to these sexual forms, apomixis is common. The center for the origin of the genus *Poa* is commonly postulated as Eurasia, with migration to North America and later to South America. *Poa* is a genus that shows interesting

phenomena because it has a widely diversified floral biology and varied reproductive biology derived from initial self-incompatible hermaphroditism. A higher concentration of diversity is found in the New World than the Old World, and, within the New World, South America possesses more dimorphic and monomorphic sex forms than North America. Although pathways to all the systems of reproduction are well known, selection pressures favoring the evolution of any of the sex forms are unknown (Anton and Connor, 1995).

History

Warwick's (1979) review of the history of *P. annua* indicated that the species had spread from its postulated center of origin in the Mediterranean throughout the temperate regions of the world. It was noted that the seeds of *P. annua* have been recovered from adobe bricks in California dating to about 1797. *P. annua* is a very successful colonizer, found in both disturbed and undisturbed sites, and tolerates varied environmental conditions. Its importance in agriculture is due more to its persistence presence in numerous crops than the substantial yield loss (Holm et al., 1997). *P. annua* is widely distributed across the world where it has been reported as a weed (Figure 1).

Evolution of the species *Poa annua*

Poa annua may have evolved through crossing of *P. infirma* Kunth and *P. supina* Schrader (Tutin, 1952). Tutin (1957) also postulated that *P. annua* (tetraploid) arose on

the northern side of the Mediterranean from a cross between *P. infirma* (diploid) and *P. supina* (diploid) in the late glacial or during an interglacial period. However, after examining chromosomes of all three species, Koshy (1968) suggested that the cross might have been between an unknown species and either *P. infirma* or *P. supina*, or

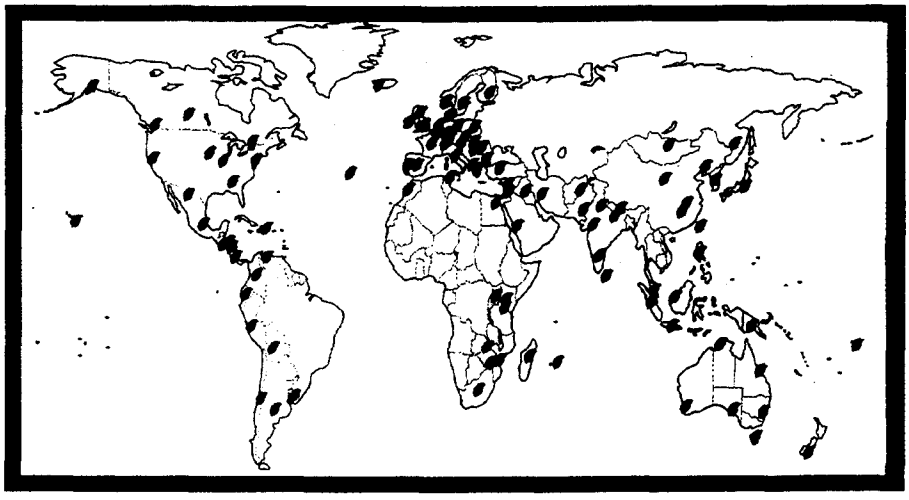


Figure 1. The distribution of *Poa annua* L. across the world where it has been reported as a weed. (Adapted from Holm et al., 1997)

alternatively, that karyotype modification had occurred. Recently Darmency and Gasquez (1997) investigated the possibility of spontaneous hybridization between *P. infirma* and *P. supina* as a first step in the origin of *P. annua*. They obtained spontaneous hybrids between the two diploids *P. infirma* and *P. supina* at the rate of 0.2%. No reciprocal maternal effect was observed. Almost all hybrids survived, grew normally, but were sterile. Hybrids were diploid, and resembled in morphology the tetraploid *P.*

annua. Isozyme patterns showed additivity of the parental profiles, and were very similar to those found in *P. annua* populations. The ease with which hybrids were obtained and the similarity of their morphological and isozyme pattern to *P. annua* suggests that the two diploid species are closely related to allotetraploid species and are perhaps its parent species (Darmency and Gasquez, 1997). The authors emphasized that the 0.2% rate of natural hybrids in the progeny of a single plant grown alongside an individual of the other species suggests that a very few plants and a very short period contact between the two diploid species are sufficient to produce a large number of hybrids. Among those hybrids a few could produce unreduced gametes and create the allotetraploid species. However, cytological evidence suggests the involvement of another as yet unidentified ancestor species in the origins of *P. annua* cannot yet be ruled out (Darmency and Gasquez, 1997).

Poa annua has a 2*n*-chromosome count of 28 and plants are normally self-pollinated, with 0 to 15 % outcrossing in natural populations depending on environmental and population conditions. This type of breeding system is suggested to be adaptively advantageous to a colonizing species (Ellis, 1973). The most frequent number of chromosomes found from Canadian collections of *P. annua* was also reported to be $2n = 28$. However, the number $2n = 14$, has also been reported, and this from four separate locations: Australia, California, France and Tunisia (Warwick, 1979). Ellis (1973) further postulated that the breeding system, chromosome pairing, in polyploid and phenotypic plasticity may interrelate to form a single integrated system for the regulation of variability in *P. annua*. Hovin (1957) reported that temperatures of 29 C or more kill

pollen or anthers of *P. annua* and argued this to be the cause for its absence in tropical climates. It lacks tolerance to extremes of temperature, particularly soil temperature, and moisture (Warwick, 1979).

Poa annua is sometimes confused with two other *Poa* species: *Poa pratensis* L. (Kentucky bluegrass) and *Poa compressa* L. (Canada bluegrass). *P. annua* may be distinguished from these species primarily by its growth habit. It behaves either as an annual or a short-lived perennial that roots at nodes, whereas both *P. pratensis* and *P. compressa* are rhizomatous perennials. Other features include 1) for *P. compressa*, a strongly flattened stem; 2) for *P. pratensis*, lemmas which are copiously webbed at the base; and 3) for *P. annua*, the presence of wrinkles on the leaves (although these may not always be visible) (Warwick, 1979).

Two life-history forms in *Poa annua*

One important and unique feature of *P. annua* is the presence of annual and perennial types. Annual types are usually erect and flower within 45 days after germination, while perennial types are slower to flower (Tutin, 1957). Annuals dominate cultivated areas and perennials thrive in closely mowed, well-managed areas such as golf courses, pastures, and bowling greens (Holm et al., 1997). Annual types are often referred to as variety *annua* and the perennial as variety *reptans*. Warwick and Briggs (1978a) reported that the proportion of annuals to perennials in less managed habitats to be 2:1, with winter collections possessing higher proportion of annuals than spring collections.

Warwick (1979) reviewed the taxonomic and synecological studies indicating two main variants of *P. annua*, between which exist a genetically determined difference in habitat and reproductive behavior. These include: 1) quick flowering, short-lived plants with erect growth habit, and 2) prostrate or semi-prostrate perennial plants which are slower to flower and fruit. Although many people characterized these as two sub-species, ssp. *annua* and ssp. *reptans*, the author argued that they do not represent good sub-species since they are not geographically isolated, but rather tend to exhibit differential proportions in any given population.

Evidence has been provided for population differentiation of *P. annua* in a mosaic environment of 1) golf course greens and adjacent roughs, 2) pasture and opportunistic sites, and 3) bowling greens and their adjacent flowerbeds. The prostrate perennial variant dominated in golf course greens, and an erect type in a pasture and bowling green sites (Warwick, 1979). Gibeault and Goetze's (1973) survey of golf courses in Oregon and Washington showed that turf that was irrigated infrequently tended to have annual types of *P. annua*, whereas perennial types were common under moist conditions.

Ellis et al. (1970) analyzed morphological variation within and between populations of *P. annua* by using univariate, multivariate, and distance statistics on seven populations. The results revealed significant differences for both within and between populations. Law et al. (1977) also made comparative experiments among two life-history forms. In summary, density-independent (r selection), referred to as opportunist, and density-dependent (k selection), referred to as pasture populations, have shown to

have genetic differences. The patterns of evolutionary development of r and k selection is represented by dN/dt = rN(K-N/K) where dN/dt is change in population size with time, constant r innate capacity of population increase (i.e. birth rate minus death rate), N is population size, and K is the highest population that can be maintained in a constant environment or often considered as carrying capacity of a habitat (Radosevich and Holt, 1984).

Flowering, photoperiod and vernalization in *Poa annua*

Poa annua is generally regarded as day-neutral, and flowering begins in early spring in the Northern Hemisphere (Holm et al., 1997). Annual types flower at all day lengths, have a moderate nodal rooting capacity and mature in 3 to 4 months. However, perennial types flower more abundantly in short days with cool temperatures, have a high rooting ability and can live up 1.5 to 4 years (Wells, 1974; Netland, 1984). *P. annua* is a C_3 plant and has a higher rate of photosynthesis in short (8-hr) than long (16-hr) days (Burian and Winter, 1976).

Koshy (1969) conducted excellent breeding system studies and reviewed *P. annua* literature. In summary, pollen normality of all nine populations was high with no significant differences among populations. One important feature of the biology of *P. annua* was noted to be the occurrence of female florets in otherwise hermaphrodite spiklets. At least 72.6 % of apical florets are females, the remainder being doubtful hermaphrodite spiklets. According to Koshy (1969) the most notable feature of the

breeding system of *P. annua* is probably its ability to mature viable seeds in panicles which are removed from the plant soon after pollination. The author has shown the rapidity of seed maturation and seed formation following pollination, even though the panicles were removed from the plant the same day pollination occurred. Seeds were viable in only 1 to 2 days after pollination (Beard, 1973). This high degree of versatility of seed formation by *P. annua* undoubtedly contributes to its conspicuous success as a weed. This also explains why, in frequently mowed turf and lawns, *P. annua* retains an efficient sexual reproductive system even under most intensive mowing systems.

Hovin (1958) found that high night temperatures arrested the normal development of *P. annua* anthers without noticeably damaging the pistillate organs when plants with emerging panicles were transferable to a glasshouse with controlled night temperature. Pistillate panicles emerged 11 to 19 days after initiation of a treatment characterized by 29 C maximum day temperature and 20 C minimum night temperature. Following controlled pollination, approximately 96% of seeds resulted in F1 hybrids in this naturally self-pollinated species.

Recently, Johnson and White (1997a) studied the vernalization requirements of selected genotypes of *P. annua*. They found that vernalization requirements differed between annuals and perennials, as well as among perennials. Annuals did not respond to vernalization. Vernalization was the primary floral induction mechanism in perennial genotypes. Vernalization requirements, or lack of them, are critical to the differences in flowering patterns among *P. annua* populations. The requirements influence

reproductive timing, and therefore, life history. Variation in vernalization along with photoperiod requirements may explain the diversity within the species *P. annua*, ranging from annual to perennial (Johnson and White, 1997a). In the perennial genotypes of *P. annua* flowering either required a cold treatment (vernalization), or flowering was enhanced by it. This responsiveness of the perennial genotypes to vernalization caused the largest number of flowers to be produced in spring (Johnson and White, 1997a). One locus and two alleles controlled the pattern of flowering in *P. annua* genotypes where continual flowering was completely dominant to seasonal flowering (Johnson and White, 1998).

Johnson and White (1997a) also reported significant variation in response to photoperiod among genotypes of *P. annua* without cold treatment, during cold treatments, and after cold treatment requirements were satisfied. Although the literature reports *P. annua* only as day-neutral, many genotypes may show either long-day or short-day sensitivity or day-neutrality depending on vernalization status. Only the annual type fits the typical day-neutral description. These photoperiodic responses may also explain some of the variation in flowering habits observed in the field (Johnson and White, 1997b).

A study conducted by Johnson and White (1997b) on photoperiodism revealed that the annual type was not responsive to treatments and was day-neutral while the perennials responded differently. For example, in the absence of cold treatment, one perennial genotype was induced in long days (16 h), two perennials were induced to

flower in short days (8 h), and one perennial was not induced in either, indicating that photoperiodism is associated with perenniality in *P. annua.*

The soil seed bank dynamics and seed dormancy in *Poa annua*

Although there can exist great variation, *P. annua* seeds generally have a short after-ripening period before they will germinate (Holm et al., 1997). Naylor and Abdalla (1982) found that freshly collected seeds resulting in a 0 to 100% germination at 20 C. It was indicated that all populations of their collections gave 95% germination at 15/25 or 20/30 C night day temperatures. However, if the difference between high and low temperatures exceeded 24 C, germination dropped markedly (Holm et al., 1997). According to Sgambatti- Araujo, (1978), as presented by (Holm et al., 1997), seeds from perennial plants are not dormant, but four stages of dormancy exist in annual plants. These include: 1) 0 to 1 month with no germination unless stratified at 4 C, 2) from 1.5 to 2.5 months, germination is controlled by the lemma and palea, 3) between 2.5 and 8 months, light favors germination, and 4) seeds older than 8 months germinate readily.

Wu et al. (1987) studied the genetic differentiation in temperature-enforced seed dormancy among golf course populations of *P. annua* in Davis, California. The *P. annua* populations' seeds were collected randomly from three sites: golf green, fairway and rough, each subjected to three categories of management, resulting in nine seed collections. After imposing seed storage treatments and temperature treatments during germination, Wu et al. (1987) reported that there was rapid genetic differentiation in

temperature-enforced seed dormancy among populations of *P. annua* at the micro-ecological level. The study revealed that the seed germination indices of the less managed populations from the golf roughs were uniformly low (less than 30% with mean index of 5%). Conversely, germination of the golf green populations were high, with the index for most seeds higher than 60%. A wide range of seed germination indices was reported in seed families of the fairway populations, ranging from 10 to 90% with mean index of 49% having a bimodal distribution. Wu et al. (1987) indicated that at 25 C, seed germination of the rough populations was almost completely inhibited. The golf green populations had the highest percentage germination at 25 C, with no difference from their percentage of germination at 12 C. The authors further speculated that disruptive selection for temperature-enforced seed dormancy might have been operating among the golf course *P. annua* populations. Based on their results from seed storage and germination experiments, they implied that increased seed germination through alternative temperature storage might be of ecological significance.

Seed storage moisture in addition to seed storage temperature plays a role in annual blue grass seed dormancy and viability. Standifer and Wilson (1988) reported that nearly all *P. annua* seeds stored at temperatures in the range of 5 to 35 C in air-dry soil remained dormant for 6 months regardless of storage temperature. The authors argue that this report differs from other reports of low temperatures breaking seed dormancy in *P. annua*. However, in moist soil storage between 5 and 35 C, Standifer and Wilson (1988) reported that seeds were dormant for at least 1 month at all test temperatures. Seeds

stored for two months at 30 and 35 C showed conditional dormancy. Seeds start to lose viability after two months at 35 C and were dead after 7 months.

The soil seed bank of *P. annua* is very rich in terms of the number of seeds per unit area. Large number of *P. annua* grass seeds can be produced per plant. The number of seeds per plant in *P. annua* has been estimated from 1050 to 2250 seeds per plant, with weights of 0.1 to 0.2 g per 1000 seeds. Studies of the soil seed bank showed the number of *P. annua* seeds to be incredibly high. As many as 118,600/m^2 in surveyed fields have been documented. Moreover, *P. annua* often constitutes a major portion of the seed reservoir of arable fields (Holm et al., 1997). Another estimate made by Lush (1988a) indicated that *P. annua* and *Agrostis* species made up nearly all of the ground cover, and were always present in the green, with a total number of seedlings per unit area reaching 270,000 m^2. Lush (1988b) also studied the seed bank of the greens by making 17 rounds of seed collections throughout the year for two years. In summary, *P. annua* was by far the most abundant species in the seed bank with a maximum of 210,000/ m^2 propagules recorded and an average at the peak season of 168,000/ m^2. After a pulse of germination, seed numbers declined to a base of about 30,000 seeds/m^2. Lush (1988b) explained that the behavior of the *P. annua* seed bank fluctuation in that the seeds that germinated rapidly (the transient seed bank) and the seeds that were carried over (the persistent seed bank) were genetically different.

The dynamics of the soil seed bank is related to the tillage practices used in arable fields. Deep plowing reduced seed populations more than disking (900 versus 3300/m^2 to

40cm depths). Large increases in seed populations occur in years of high rainfall because cultivation is much less effective. *P. annua* seeds became 62% of the total seed bank after 9 yr. of vegetable cropping. When *P. annua* did not produce seed and no crops were grown, the rate of seed loses over 5 yr. was 22 to 26% per yr. in non-tilled areas and 30 to 36% per yr. in tilled areas. Rate of seed loses reached 56% per yr. when soils were disturbed seven times annually (Holm et al., 1997).

Perennation

The annual variant of *P. annua* maintains itself solely by seeds that over-winter on or under the surface of the soil. The perennial variant over-winters successfully at any stage of plant development, with uncertain longevity estimates from 13 months to 2-3 years (Warwick, 1979). The seed dispersal estimated by Law (1975) was less than 0.5 m from the parent plant. Lightness of the grain would aid in wind dispersal, but dispersal by birds is substantial and the main carrier of seeds is probably man, and one of the main means of its transport is his mower (Warwick, 1979).

Vegetative reproduction

Warwick (1979) has reviewed the vegetative reproduction capability of *P. annua*. In summary, the perennial variant of *P. annua,* unlike the annual variant, forms a mass of tillers that develop adventitious roots at the nodes. The non-flowering tillers appear to have the potential of becoming established as separate individuals, with vegetative

reproduction indeed important in determining the reproductive characteristics of populations.

Poa annua as a weed

Poa annua is considered a weed in turf because it tends to leave ornamental and functional swards with a spotty appearance. These include its light green color, constant seed head production, shallow root system, disease, smog, drought and high temperature susceptibility, and annual or short-lived perennial growth habit (Warwick, 1979). Tutin (1957) and Warwick (1979) have also characterized a number of morphological and other characters of *P. annua* that help the species to its success as a weed. These include: 1) great phenotypic and genotypic variability, 2) rapid germination and generally short life cycle, 3) small size (enabling to escape notice), 4) lack of ease in being uprooted and great powers of survival when uprooted, 5) development of shallow root system in response to soil compaction, 6) small, light seeds which are highly palatable to birds and stick readily to feet, shoes, implements, etc. *P. annua* is one of the worst weeds confronting grass seed growers in the Willamette Valley. Its presence in harvested seeds complicates seed cleaning and reduces crop value if seed can not be cleaned to *Poa*-free status (Mueller-Warrant, 1996).

Poa annua, however, may not be considered as a weed under certain conditions. For example, in pastures, *P. annua* is not so clearly classifiable as a weed, for although it may account for as much as 50% of perennial ryegrass swards, herbage production and

quality is not affected (Warwick, 1979). *P. annua* is not regarded as an invader that displaces other plants, but rather a highly opportunistic colonizer (Wells and Haggar, 1984).

Competition

Wells (1974) reviewed the biology of *P. annua* and its significance in grasslands, including competition and ingress by *P. annua*. In summary, low plant density and a high degree of rectangularity in the sown species led to poor competitive ability of the sward and consequent increase of *P. annua*. Distribution of *P. annua* resulted in four to six fold higher seedling numbers of *Capsella* and *Senecio* when *Poa* was patchy rather than randomly distributed. Inhibition by mulch was greater for *Senecio* than for *Poa*, and *Senecio* became relatively less competitive as dead *Poa* abundance increased, leading to eventual dominance by *Poa* in sites newly colonized by both species (Bergelson, 1990). In New Zealand (Cullen and Meeklah, 1959, as presented by Wells, 1974) less *P. annua* invaded newly sown ryegrass pasture when the sowing rate was increased from 11 to 33 kg/ha. In Belgium, meadow fescue (*Festuca pratensis*) swards grown for seed production were invaded by *P. annua* when rows were sown 40 cm apart but not when 20 cm apart (Vyncke, 1968, as reported by Wells, 1974). Newly sown ryegrass in Britain allowed 45 percent more *P. annua* to invade when the ryegrass seed was sown in rows than when it was broadcast (Wells, 1974).

McNeilly (1981) made a competition study using six populations of *P. annua* and the cultivar S-23 of *Lolium perenne* in a glass house. The seed-derived populations of *P. annua* studied were from three open habitats and three closed habitats. Pure stands of *P. annua* and *Lolium perrene*, and 50:50 mixtures of two species were compared after 13 weeks growth when uncut, and when cut at 5, 7, 9, 11 and 13 weeks to a height of 1.5 cm. The results indicated that populations from closed habitats performed better in the less favorable conditions imposed by cutting or competition or both these conditions than those from open habitats, whereas this situation was reversed in their absence. Under the cutting and competition treatment, the *P. annua* populations from closed habitats significantly out-yielded their *Lolium* associate.

With competition, uncut *P. annua* populations from open habitats significantly outyielded *Lolium* whilst two of the populations from closed habitats, one adjacent to one of the open populations, were significantly out-yielded by *Lolium*. Based on these results, McNeilly (1981) suggested that population differentiation in *P. annua* has occurred over short distances in response to competition or grazing or mowing, and that competitive ability is not a fixed characteristic of a species. A similar study was also conducted later by McNeilly (1984) using 8 populations where 4 populations were from open and the other 4 from closed habitats, but in this case to determine inter-population variation in response to competition and cutting. McNeilly (1984) reported that significant differences were found between genotypes within both populations when cut in pure stands and mixture with *Lolium*. With uncut and pure stands, inter-genotypic differences were found only in the open population. The closed population was

significantly more variable when uncut and in mixed stands than in any other treatment combination, whilst the open population did not vary significantly with treatment. McNeilly (1984) considered this as confirmation of the occurrence of genetically based inter-population differences in competitive ability in *P. annua.*

Mueller-Warrant (1996) reported the existence of an inverse relationship between *P. annua* ground cover and perennial ryegrass seed yield. Increasing *P. annua* density in the range between 13 and 42% ground cover reduced crop seed yield by 198 lb/a, indicating the capability of *P. annua* competing with perennial ryegrass. However, yield reduction due to herbicide damage to the crop in attempts to reduce *Poa* was not ruled out (Mueller-Warrant, 1996). Bergelson's (1996) report of competition study between groundsel and *P. annua* revealed that the interaction between the two species includes time lag with earlier generations affecting later ones. She also found that bluegrass should dominate whenever litter accumulates and that groundsel dominates if the litter decomposes quickly.

Poa annua as an alternate host to diseases of crops

Since *P. annua* shows susceptibility to many pathogens it can act as an alternate host for certain crop diseases. Masterman et al. (1994) studied the role *P. annua* in the epidemology of Barley Yellow Dwarf Virus (BYDV) in autumn-sown cereals in Scotland. In summary, it was shown that *P. annua* was often abundant in winter barley fields during the summer, and in the subsequent stubble fields in late summer and

autumn. It has also been found that *P. annua* was frequently BYDV infected and aphid infested. As cereals mature in the summer, aphids move from the crop onto the *P. annua*, transferring BYDV inoculum. The authors suggest that *P. annua* plays a major role in the green bridge.

Lee et al. (1997) have detected and characterized a phytoplasma associated with annual bluegrass (*P. annua*) white leaf disease in southern Italy. In their studies, they used five samples of *P. annua* showing white leaf symptoms and eleven showing no apparent symptoms collected in the fall of 1995 from fields near Caserta in southern Italy. The phytoplasma associated with *P. annua* white leaf disease was identified as a new member of phytoplasma 16S rRNA group X 1 (16Sr XI) (type strain, rice yellow dwarf phytoplasma). It was indicated that the *P. annua* white leaf phytoplasma is most closely related to Bermuda grass white leaf phytoplasma found in Asia. The authors indicated that this to be the first report that a plant pathogenic phytoplasma belonging to group 16Sr XI is present on the European continent. The white leaf disease is not known to occur in the United States. An RFLP analysis of nested –PCR products with restriction enzymes *Mse*I and *Rsa*I indicated that all the *P. annua* plants contained a very similar or identical phytoplasma (Lee et al., 1997).

Poa annua response to human manipulation

Use of acid-forming fertilizers that result in low soil pH is not favorable for *P. annua* (Warwick, 1979; Lush 1988a). Herbage yield of *P. annua* increases linearly with

increased use of nitrogen, phosphorus and potassium. *P. annua* withstands grazing, poor aeration, and the increased bulk density of compacted soils. Mowing is important factor in influencing the distribution patterns of the two variants. The perennial/prostrate type better withstand clipping damage as compared to the annual/erect type. In less frequently watered turf mainly annual types were present, whereas perennial types can be anticipated in more frequently irrigated areas (Warwick, 1979).

The influence of tillage on germination of *P. annua* has been well documented. Maximum emergence (92%) of imbibed seeds occurs at 1 cm, with 45% emergence at 0 cm and 25% at 3 cm (Williams, 1983). When seeds were mixed with soil to 2.5, 7.5, and 15-cm depths and tilled, 71, 68, and 63%, respectively, had emerged after 5 years (Roberts and Feast, 1972). Emergence was 25% less when the soil was left untilled (Holm et al., 1997). Germinating seeds tolerate 46 C for 6 hr but are killed at 49 C (Wells, 1974). In arable culture, cultivation practices designed to desiccate the plants, unless they prevent development before anthesis, clearly have only a partial effect in crippling the further establishment of progeny from seed (Koshy, 1969).

Ascard (1995) studied the effects of flame weeding on several weed species at different developmental stages in order to determine the dose and timing of flaming needed to control different weed species in Sweden. Of the many species tested, only *P. annua* was not completely killed with a single flame treatment, regardless of developmental stage or propane dose (Ascard, 1995). With later flame treatment, the

emergence and growth of *P. annua* even increased at high doses indicating the species to be very tolerant to flaming (Ascard, 1995).

Biological control of Poa annua

The insect families Aphididae, Coleoptera and Lepidoptera contain many species that attack *P. annua*. *P. annua* has been reported to possess severe susceptibility to dozens of fungal pathogens (see, Warwick, 1979, for review). Zhou and Neal (1995) reported that two strains of the bacterium *Xanthomonas campestris* pv. *poannua* from California and Michigan have resulted in control of the annual and perennial types of *P. annua* at levels of 92 and 82%, respectively in growth chamber tests, but only 11 and 7% in field tests. Control of established *P. annua* in the field was improved to a maximum of about 40% by increasing inoculation frequency to three times per week for 4 weeks, but recovery occurred 2 to 5 weeks after inoculations were discontinued. In greenhouse and growth chamber studies, *P. annua* of both annual and perennial types were controlled by one or more applications of *X. campestris* pv. *poannua* (Haygood, 1992; Roberts et al., 1985; and Webber et al., 1992). In field tests up to 85% of control of annuals was achieved when *X. campestris* pv. *poannua* was applied at 10^9 bacterial colony forming units (cfu)/ml three to four times at two week intervals (Haygood, 1992; Johnson, 1994).

Herbicides to control Poa annua

PRE herbicides effectively control annual types of *P. annua* in both warm and cool-season turfgrasses. POST herbicides control the weed in turfgrasses except golf course putting greens. Ethofumesate can be applied to cool-season turfgrasses and dormant bermudagrass, but herbicides such as atrazine, pronamide, and simazine are limited for use only on dormant warm-season grasses (see, Johnson and Murphy, 1995, for review).

Poa annua has been confirmed resistant to many herbicides currently used in grass seed production. Among the herbicides that *P. annua* is resistant to are: Karmex (diuron), Norton (ethofumesate), Lexone or Sencor (metribuzin), and Sinbar (terbacil) (Gamroth et al., 1996, Mueller-Warrant, 1996). Gamroth et al. (1996) reported that *P. annua* control was very good to excellent with glufosinate, glyphosate, and paraquat. However, all three of these herbicides are relatively non-selective.

Mueller-Warrant (1996) studied the response of herbicide-resistant *P. annua* in an established perennial ryegrass using registered and experimental herbicides under various residue management practices having 28 treatment combinations. In summary, none of the 28 treatments (25 herbicides and 3 checks) achieved better than 75% control of *P. annua*. It was reported that a majority of the treatments increased *P. annua* ground cover compared to the untreated full straw check. The presence of volunteer perennial ryegrass was suppressive to *P. annua*, and *P. annua* ground cover doubled when volunteer crop

seedlings were removed by treatments such as mechanical disturbance without herbicides. *P. annua* density increased even more when herbicide treatment injured the crop and opened up the canopy, reaching 49% ground cover for 0.25 lb/a oxyfluorfen, plus full-rate diuron (1.6 lb/a) applied through the flail (Mueller-Warrant, 1996).

Previous studies of the genetic diversity in *Poa annua*

Sweeney and Danneberger (1995) made RAPD characterization of *P. annua* populations in golf courses greens and fairways to assess genetic differences in adjacent populations. In summary, there were significant differences between the green and fairway populations for four of the RAPD markers and among holes within fairways for three markers. Based on their result they have suggested limited gene flow between adjacent populations of *P. annua*.

A study made by Darmency and Gasquez (1981) on population dynamics using morphological characters and isoenzymes in atrazine-resistant and susceptible populations of *P. annua* revealed that the resistant populations contained as much polymorphism as the susceptible ones. Based on esterase patterns they found that the resistant population (11 phenotypes) showed about the same phenotypic diversity as the susceptible population (13 phenotypes), which would not be expected in the case of founder effect and with low levels of outcrossing. One phenotype on an isozyme basis was reported to be much more frequent in each habitat (i.e. resistant and susceptible). Out of the total of 23 esterase phenotypes recorded only one was common to both

populations. Band A was fairly specific for the resistant populations with a frequency of 93% compared to 0% in the susceptible populations. Band B occurred in only 22% of the resistant individuals, but was found in all the susceptible plants. These patterns reveal strong differences in the genetic structure of both populations. An experiment to measure the degree of outcrossing using homozygous dominant esterase band E resulted in a 13% hybrid content, suggesting 10-16% natural outcrossing (Darmency and Gasquez, 1981). The genetic diversity within both susceptible and resistant populations is very high, as revealed by the esterase zymograms, which yielded 12 differential bands, one of which corresponds to one locus (with null allele). The resistant population is not monomorphic and the high degree of polymorphism could be related to the degree of allogamy (10 to 16% in experiments, although this is probably an over estimate of the outcrossing rate in natural habitats (Darmency and Gasquez, 1981).

Based on morphology and chromosome counts, Darmency and Gasquez (1981) reported that there was no difference in time or requirements for germination of seeds from resistant or susceptible populations. Young seedling chromosome counts from root-tip mitosis revealed both populations to be tetraploid ($2n = 28$). Observation under greenhouse conditions revealed that susceptible individuals had an erect growth habit whereas resistant individuals differ from the typical erect variants, and were short lived perennials showing greater rooting ability at the nodules than susceptible plants. Populations also differed in leaf morphology, where susceptible plants have wider leaves than resistant plants under the growth conditions used. Resistant plants flowered later than susceptible plants, and this difference tended to increase with short day conditions.

Both populations exhibit different pre-reproductive periods that may vary according to different day length and temperature conditions (from 2 to 29 days in their experiments). Although there was a morphological difference between the resistant and susceptible populations, the difference they found between that resistant and susceptible was not as striking as that found between prostrate and erect growth forms. They considered the resistant population to consist of a semi-prostrate growth form.

Darmency and Gasquez (1983) studied esterase polymorphism and growth form differentiation in *P. annua* using eight populations from two habitat types (four opportunist and four semi-permanent populations). In summary, it was demonstrated that growth forms were controlled by nine leaf esterase loci. Locus Est 1 proved highly correlated with growth form: homozygous AA were prostrate, whereas homozygous BB were erect. All populations were polymorphic both for esterase isozymes and growth form. The outcrossing rate was strongly dependent upon plant densities, where 30, 50, 60 and 100 plants/m^2 resulted in 2.8, 4.9, 6.0 and 12.7% outcrossing rates respectively. This however is in contrast to other species characteristics where increased density reduces long distance outcrossing rate specially if wind is the pollination agent (Ellstrand and Elam, 1993). It was demonstrated that both types of habitats contained both growth forms with mean frequencies of prostrate and erect plants of 12 and 88% respectively in opportunist populations and 32 and 68% in semi-permanent populations. They concluded that prostrate and erect plants exchange genes. It was also shown that out of 17 loci, nine, with a mean number of 1.7 alleles each, generated the esterase polymorphism. Disomic inheritance for all loci was in full accordance with the hypothesis of the allotetraploid

origin of *P. annua* ($2n = 4x = 28$). Darmency and Gasquez (1983) concluded that 1) both growth forms within each population have similar gene frequencies, and 2) prostrate and erect plants show similar frequencies of heterozygotes. In *P. annua* plant density appears to be an important factor influencing the proportion of heterozygotes.

Darmency and Gasquez (1983) found correlation in *P. annua* populations between esterase isozymes and developmental traits in two different growth variants. While the species *P. annua* is predominantly selfed, allozyme analysis showed that outcrossing ranged between 1 to 22% in populations with high density regardless of growth variant (Darmency et al., 1992). Darmency and Gasquez (1983) modified their previous statements that resistant population contained as much polymorphism as that found in susceptibles because they more recently had found less polymorphism in resistant populations than susceptible, with a polymorphism index of $R = 0.03$ while that of $S = 0.056$.

Warwick and Briggs (1978b) studied the impact of disruptive selection on population differentiation of *P. annua*. In summary, they found that in each site studied, the seed population was more variable than the adult, a finding compatible with the notion of disruptive selection. Differential response to clipping was observed; prostrate individuals from different populations were in selective advantage over erect individuals. Clipping was determined to have two effects: 1) greater percentage decrease in total dry matter production in the erect compared with prostrate, and 2) a greater percentage of the dry weight produced was removed by the cut in the erect forms compared with prostrate

forms. The coefficients of selection acting against erect plants under a regime of clipping ranged from 0.53 to 0.68. A selection coefficient of 0.77 was reported for prostrate plants in the absence of clipping (Warwick and Briggs, 1978b).

POPULATION GENETICS AND MOLECULAR MARKERS AS TOOLS FOR GENETIC DIVERSITY STUDIES

Evolutionary forces

Populations are important functional units of evolution and adaptation: mutation and migration provide new alleles within populations, mating systems shuffle alleles among genotypes, and selection and drift act on novel genotypes producing patterns of both stasis and evolution (Dobzhansky, 1937).

Mutation

A mutation is defined broadly as any change in a single nucleotide to a modification of a karyotype. Changes, insertions and deletions of individual nucleotides, occur at a very low rate which appears to be roughly the same in different parts of the genome and in different eukaryote species, approximately $4x10^{-9}$ per base pair per generation (Kimura, 1983). Mutation provides new alleles, whose frequencies are then governed by other forces.

Mutation rates vary between different regions of genomes and between different genomes within a cell (i.e., cytoplasmic versus nuclear). Comparing synonymous (silent, non amino acid changing) substitutions among nuclear and cytoplasmic encoded genes of plants, Wolfe et al. (1987) found substantially different rates of mutation. The mutation rate of nuclear (nDNA) substitutions are approximately two-fold higher than chloroplast (cpDNA) substitutions, which were roughly three-fold higher than mitochondrial (mtDNA) substitutions measured in nucleotide substitutions per site per year. These translate into $0.2\text{-}1.0\text{x}10^{-9}$ for mtDNA, $1.0\text{-}3.0\text{X}10^{-9}$ for cpDNA, and $5.0\text{-}30.0\text{x}10^{-9}$ for nDNA (Wolfe et al., 1987). Wolfe et al. (1987) reported that animals show an inverse pattern of mutation rate with synonymous substitutions in mtDNA being roughly five-fold higher than in nDNA, nDNA of plants and animals show roughly similar mutation rates, though rates may be somewhat higher among plants. Mutation rates at microsatellite loci are as high as 1/100 per generation (Weber and Wong, 1993). The higher rates of mutation within repetition loci may lead to levels of polymorphism that are not concordant when compared with non-repetition loci (e.g. Scribner et al., 1994).

Genetic drift

Genetic drift is a process of change in allele frequencies caused by impredictable consequences of Mendelian inheritance. Allele frequencies can change from generation to generation because only a sample of gametes will be represented in each generation. It is well known that population size is the most important determinant of genetic drift, with small populations being much more strongly affected than large ones.

Genetic drift depends on a variety of factors whose net effect can be summarized by a single quantity, the "effective population size" (N_e) (Wright, 1938). Genetic drift reduces genetic diversity in a population at a rate determined by effective population size. Hill (1972) indicated that the effective population size was always smaller than the census size depending on birth and death rates of each age class, and will be smaller if overlapping generations exist in populations.

In general, genetic drift reduces genetic diversity in a population at a rate determined by the effective population size. If H is the proportion of heterozygous loci in randomly mating population, then H is reduced by a factor of [1-1/(2Ne)] each generation by genetic drift (Crow and Kimura, 1970).

Natural and artificial selection

The importance of natural selection relative to other evolutionary forces has been vigorously debated since early in the history of population genetics (see Nei, 1987; and Ohta, 1992, for reviews). Selection within population occurs whenever inherited characteristics of individuals affect an individual's ability to survive and reproduce. We can distinguish natural selection, which is caused by naturally occurring environmental conditions affecting survival and reproduction, from artificial selection, which is imposed by humans for altering and improving domesticated species. Although the same principles play to both kinds of selection, there are a number of differences

between them. First, natural selection is typically relatively weak while artificial selection is almost always quite strong. Second, natural selection acts for entire lifetimes of species, possibly million of years, while artificial selection acts over a few and at most tens of generations. Finally, the way in which artificial selection is performed can be adjusted. In contrast, there is no goal in natural selection (see, Barbault and Sastrapradja, 1995, for review).

Selection is particularly effective at fixing alleles in populations when effective population sizes are small, and may have little effect when gene flow into populations is large (Ellstrand and Elam, 1993). Selection is also strongly affected by mating systems, and may therefore be extremely complex in natural populations (Lande et al., 1994). The survey of Linhart and Grant (1996) revealed the existence of population differentiation in plant populations resulting from selection. Among the listings of agents of selection in space that lead to local differentiation at microsite levels include fertilizers, herbicides, lawns or grazed areas, competition etc. Based on this survey, *P. annua* have been shown to differentiate within 5 to 10 meters distance for its dry weight when mowing was used as selection factor with a selection coefficient range of 0.53 to 0.77. When survival is considered as a character of differentiation, the herbicide use of simazine for six years time was indicated to show differentiation in *Senecio vulgaris*. Herbicide resistance was among the characters listed considered as the most affected localized differentiation plant characters.

Recombination/Mating system

Recombination in eukaryotic organisms rearranges alleles among chromosomes. Recombination does not itself create genetic diversity at each locus but it can create new combination of alleles at different loci by mixing alleles from different individuals. The associations of alleles within and between loci are largely a function of a population's mating system (relative amount of selfing vs. outcrossing). Monoecious plants may show highly structured mating systems with rates of selfing ranging from near zero to 100% (Hamrick and Godt, 1989).

Genetic Diversity

Genetic diversity is the heritable genetic variation within and among populations. The variability that we observe among individuals (phenotype) results partly from the interaction of genetic differences (genotype) with their surrounding environments. The genetic diversity occurs in the form of nucleotide variation within the genome. When it causes a change in a given protein the variants are termed alleles. The allelic variation occurs at various genetic loci, or gene positions in the genome. The genetically variable loci are termed polymorphic or are said to show polymorphism. The recognition that natural populations have high levels of genetic variation has recently followed from the application of allozyme electrophoresis (Hubby and Lewontin, 1966). It is also this

genetic diversity within species that allows a species the opportunity to evolve under changing environments and selection pressures.

Measures of genetic diversity and population differentiation

Studies of genetic variation in plants have typically used Nei's genetic diversity statistics or Wright's F statistics as tools for describing the extent of differentiation among populations (Nei, 1973; Wright, 1969; Hamrick and Godt, 1989).

Nei gene diversity and genetic distance

Nei (1973) presented a method by which the gene diversity (heterozygosity) of a subdivided population can be analyzed into its components, namely the gene diversity within and between populations. Nei (1973) proposed a shift in emphasis from heterozygosity to the more general concept of gene diversity (h), which is free from assumption of mutation, selection, migration, and mating system. The method is based on the identities of two randomly chosen genes within and between populations, and is independent of the number of alleles. The genetic diversity is also applicable to populations regardless of the mode of reproduction (i.e. sexual or asexual), whether gamete or diploid phases are considered, and whether nuclear or organelle genes are studied. Many genetic diversity studies use Nei's gene diversity statistic and different plant taxa do show different levels of diversity. For example, a Schoen and Brown (1991) survey using published isozyme data revealed the existence of correlation between

intraspecific variation in populations genetic diversity and effective population size with mating systems in plants. Their analyses reveal that inbreeding species have greater variation in effective number of populations (N_e) and greater average values of Nei's gene diversity statistic. For example, the mean, minimum, maximum, and range values of Nei's gene diversity statistic for eight self-fertilizing species with their standard errors were 0.125(0.024), 0.008(0.008), 0.294(0.026) and 0.286(0.021), respectively. In contrast, for nine outcrossing species the mean, minimum, maximum and range of Nei's gene diversity statistics and (standard errors) were 0.257(0.028), 0.174(0.038), 0.328(0.024) and 0.154(0.035), respectively.

Average sub-population diversity is referred to as H_S, average between population diversity is D_{ST}, and the sums comprise the total gene diversity, H_T. The analog of Wright's (1965) F_{ST} is then defined by Nei as a coefficient of gene differentiation (G_{ST}), and is calculated as the ratio of gene diversity between populations to total gene diversity. This is the measure of population subdivision most commonly used to quantify the distribution genetic variation in species (Hamarick and Godt, 1989). Both Nei's (1973) and Wright's (1965) methods are similar, and when calculated for a single locus both F_{ST} and G_{ST} are mathematically equivalent (Nei, 1977). Since G_{ST} is (almost) independent of mutation rate, total population size, number of alleles, and since it rapidly approaches equilibrium, it is quite natural to use G_{ST} for molecular data rather than F_{ST} (Crow, 1985).

Nei (1972) also formulated a genetic distance (D) based on the identity of genes between populations. It is defined as D = -log I, where I is the normalized identity of

genes between the two populations. The measure is applicable to any kind of organism without regard to ploidy or mating system. Since he relates it to the accumulated number of gene differences per locus, these measures of genetics distance have the following advantages. 1) It gives coefficient of kinship in simple way. 2) It measures the accumulated number of gene substitutions per locus. 3) If the rate of gene substitutions per year is constant, it is linearly related to evolutionary time. 4) In some migration models it is linearly related to geographical distance or area (Nei, 1972).

Wright's F statistics

When more than one population of a species is examined, measures of variation among populations are needed. In considering the overall level of differentiation among populations, Wright's F_{ST} is a commonly used measure and is a natural extension of his coefficient of inbreeding within populations (Wright, 1978). The S indicates that the subpopulation is being compared with the total population, denoted by T. In a group of populations, the overall inbreeding coefficient F_{IT} (the individual I compared to the total) can be attributed either to differences in allele frequency among populations (F_{ST}) or to deviations from Hardy-Weinburg genotype frequencies within each population (F_{IS}). Both F_{IS} and F_{IT} can be calculated either from pedigree (Wright 1965) or from the variance in allele frequency (Weir and Cockerham, 1984) at different levels of population subdivision. In addition, a correlation between alleles from different subpopulations (F_{ST}) can be calculated from F_{IS} and F_{IT}.

Analysis of molecular variance (AMOVA)

Excoffier et al. (1992) extended the analysis of variance to calculate F statistic analog (Φ statistics) from distance metrices based on molecular markers, and called this analysis of molecular variance (AMOVA). Excoffier's AMOVA has been most commonly used to analyze data from studies employing dominant markers. The method focuses on the hierarchical distribution of variance (diversity) calculated from pair-wise squared distances among individuals of hierarchical population subdivisions. The sum of squared differences (distances) between all observations (individuals) at a given level of hierarchy provides an estimate of the variance at that level. The hierarchical model employs within population, among population/within groups and among groups components of diversity. The ratio of variance component due to differences among populations relative to the total variance is an F_{ST} analog, Φ_{ST} (Excoffier et al 1992; Michalakis and Excoffier, 1996).

Excoffier et al. (1992) originally applied AMOVA to the analysis of mitochondrial hyplotype diversity by partitioning the total diversity into regions, populations and individuals. Huff et al. (1993) used this method to study the distribution of variation in randomly amplified polymorphic DNA (RAPD) phenotypes. RAPD markers are dominant and may cause biased estimates of population genetic parameters (e.g., Lynch and Milligan, 1994). The use of AMOVA by Huff et al. (1993) on RAPD analysis has attracted many researchers to use AMOVA in population genetic studies using RAPD markers (e.g. Nesbitt et al., 1995; Vicario et al., 1995; Yeh et al., 1995).

The reason for this is that Mendelian interpretations of molecular phenotypes can be complicated by dominance and other factors, applying gene diversity and F statistics approaches may be inappropriate in some circumstances. Dominant markers require that allele frequency estimates from diploid tissues rely on mating system assumptions. In addition, both population sample sizes and null homozygote frequencies strongly influence calculations of allele frequencies (Lynch and Milligan, 1994).

Any type of distance measure may be used in the AMOVA analysis, and distance measures do not require that mating system assumptions or calculations of allele frequencies be made. The approach is general enough to deal with any organism and to study any type of structure (hierarchical or otherwise) that one might wish to consider (Excoffier et al., 1992).

Genetic diversity in plants

Genetic diversity in plants is generally regarded to be higher than in animals. A larger number of mating systems produces richer variety of population genetic structures in plants than animals (Hamrick and Godt, 1989). For example, wind-pollinated species have higher levels of heterozygosity (H_S = 0.15 to 0.20) within populations, on average, than do animal-pollinated plants (H_S = 0.09 to 0.12), and both of these groups have higher levels than self-pollinated plants (H_S = 0.07). Because of the restricted gene flow between populations, self-pollinated plants show a greater degree of genetic differences amongst populations (F_{ST} = 0.51) than the species with mixed breeding systems (F_{ST} =

0.10 to 0.22) or outcrossing species (F_{ST} = 0.10 to 0.20). The geographic range of a plant species is also an important parameter controlling the amount of genetic diversity within and between populations. Plant species with small geographic ranges harbor less genetic variability within populations on average (H_S = 0.06) than those with "narrow" (H_S = 0.11), regional (H_S = 0.12) or widespread (H_S = 0.16) distributions (see, Hawksworth and Kalin-Aroyo, 1995, for a review). More data related to these parameters can be obtained from the survey report of Loveless and Hamrick (1984). The authors indicated the magnitude of H_T to be influenced by the proportion of polymorphic loci within a species, the number of alleles per locus, and the evenness of mean allele frequencies summed for a species. Based on the same survey, the H_T was reported to be higher for short-lived perennials and plants with both sexual and vegetative reproduction, while H_T was low for long-lived and sexually reproduced plants. For example, the H_T, H_S and G_{ST} for autogamous is 0.291, 0.128, and 0.523, respectively, while for mixed mating they were 0.242, 0.174 and 0.243, respectively. The authors indicated also high G_{ST} values to be characteristics of selfing species, annuals, and early successional species, where the G_{ST} for outcrossing annuals, mixed mating annuals and selfing annuals was 0.161, 0.269 and 0.560, respectively.

Molecular markers in genetic diversity studies

Genetic diversity studies have utilized various molecular markers to study genetic variability and genetic substructuring at the population level. Bisby (1995) reviewed the molecular tools used at the population level, and listed karyology (B-chromosomes,

bands), allozyme (heterozygosity), mtDNA sequence, DNA finger printing and micro-satellites to be the most appropriate tools to study genetic variability and genetic substructuring in populations. This review mostly focused on PCR based random amplified polymorphic DNA (RAPD) technique.

Random amplified polymorphic DNA (RAPD)

The development of the polymerase chain reaction (PCR) has provided methods for detecting DNA polymorphisms by amplifying specific DNA fragments and separating the fragments by gel electrophoresis to visualize polymorphism. Amplification of DNA using arbitrary oligonucleotides, usually 10 base pairs as primers, has been described as a way to detect random amplified polymorphic DNAs (RAPDs) (Williams et al., 1990; Welsh and McClelland, 1990). Short arbitrary primers (usually 8-10 bases) can be used to amplify random fragments of DNA as a result of tolerance of PCR mismatches between primers and the DNA template, mainly at lower temperatures (30-37 C). Only minute quantities (picograms) of template DNA are required, markers are easily scored with nonradioactive staining techniques, and an unlimited number of markers are available due to the random nature of primers. Many bands are obtained per primer and the technique is simple, inexpensive and rapid. However, RAPD markers are dominant (heterozygotes and homozygotes cannot be distinguished) and problems of reproducibility may exist since RAPDs do suffer from a sensitivity to changes in PCR conditions.

Basic assumptions in RAPD analysis

When comparing genotypes for the presence (1) or absence (0) of marker amplification, the informativeness of the results may vary depending on the comparison. The presence of a RAPD marker in both genotypes indicates high level of sequence homology at this site (Williams et al., 1992). In other words, the presence or absence of this specific PCR product is assumed to represent mutations in the primer binding sites of the genomic DNA. However, the RAPD polymorphism (band presence/absence) that is assumed to be resulted from nucleotide sequence mutations that prevent the amplification of a particular marker in some individuals, may be invalidated if many well-documented factors that can affect the reproducibility of RAPD fragments (see, Wolfe and Liston, 1998, for review). Wolfe and Liston (1998) have made an extensive review including the PCR technology and its role in plant systematics. The basic limitations of RAPD have been categorized as 1) deviations from the expectations of strict Mendelian inheritance (these may be caused by artificial (nongenetic) variation, organellar bands, and epigenetic interactions; and 2) problems of homology assessment (Wolfe and Liston, 1998).

Application of RAPD

Since its development in 1990, RAPD has been used as an important molecular genetic marker in various fields of plant sciences, such as introgression, genetic diversity, differentiation, ecology, and mapping. Wolfe and Liston's (1998) review revealed the range of taxonomic levels at which RAPD markers have been applied in plants that deal

with phenetic or cladistic estimation of relationships. RAPDs have been used to study at the level of intergeneric relationships on some taxa. Of all the studied genera the mean intergeneric parent polymorphism found by RAPD was 100%. At the interspecific level of studies, RAPD has shown a mean percent polymorphism of 87.7% while intraspecific mean polymorphism were reported to be 62%. For a single cultivar or among clones and inbred lines the mean percent polymorphism by RAPD was only 18.6%. Only few studies have incorporated homology testing (Wolfe and Liston, 1998). RAPD markers have been used for genetic analysis both in simple and complex (polyploid) genomes. For example, Thompson et al. (1997), have shown that the construction of genetic linkage map is possible by using RAPD for sweet potato, a hexaploid with 2*n* chromosome number of 90. The authors have found 60% 1:1 segregation ratio from 124 RAPD markers in a test cross that were useful for linkage determination. Hallden et al. (1996) studied the impact of template competition as a source of errors in RAPD analysis and reported that the error rates are consistent for such diverse genomes as *Brassica napus* (allotetraploid) and *Bacillus cerus* (haploid procaryote). In conclusion, they stated that the actual DNA sequences rather than the sequence copy number seems to be the important determinant for successful amplification of RAPD markers.

RAPD markers provide a powerful tool for the investigation of genetic variation in natural and domestic populations. Since the method is fast, simple and inexpensive, has no requirement for DNA sequence information and avoids radioactive probes, its use in population studies is advantageous if proper analysis is followed while interpreting RAPD markers.

Analysis of RAPD and considerations taken

It is obvious that techniques described in literature relevant to the measurement of the degree of genetic relationship using isozyme and RFLPs can be applied with slight modification to RAPD marker data. Gower (1985) and Jackson et al. (1989) have summarized options regarding the choice of genetic distance estimator for binary data. Most RAPD studies in plants that estimate phylogenetic relationships have used pair wise distance data analysis with UPGMA or neighbor-joining (NJ) clustering. Most of the similarities coefficients used to transform the RAPD binary data to similarity (distance) are Jackard or Dice. Both measures exclude negative matches (Wolfe and Liston, 1998). Some studies used simple matching coefficient that includes negative matches. Skorch et al. (1992) for example, suggested use of simple matching with a four contingency tables that view shared absence (0,0) comparisons to be evidence of homology providing nearly equivalent information to the shared presence (1,1) comparisons. The assumption for using the simple matching is that there are large numbers of RAPD marker loci distributed over the genomes, and therefore the proportion of differences found from looking at random sample of those loci will be a relatively good measure of the sequence differences between the genomes. However, Wolfe and Liston (1998) recommended not using simple matching for RAPD data because shared RAPD band absence may have many causes.

Since all pair wise comparisons of RAPD fragments are made with the assumption that co-migrating bands represent homologous loci, some studies fail to be

consistent as a result of using agarose gels. It has been reported that using polyacrylamide gel electrophoresis and silver staining can reduce such errors in homology assumptions due to better resolution. However, only few RAPD studies in plants have used these alternatives to agarose gel electrophoresis and EtBr staining (see, the review of Wolfe and Liston, 1998, for details of explanations and recommendations in using RAPD markers).

CHAPTER 2
GENETIC DIVERSITY OF ANNUAL BLUEGRASS (*POA ANNUA* L.) IN WESTERN OREGON GRASS SEED CROPS

ABSTRACT

A survey of the genetic diversity of annual bluegrass (*P. annua*) populations of the Willamette Valley was conducted using RAPD markers. A total of 1357 individual plants and 47 structured populations gave reproducible RAPD markers and were used to determine the genetic diversity of *P. annua* populations of the western Oregon grass seed fields. These 47 populations came from 16 sites collected in the fall, winter, and spring (no new seedlings were found at one site in the spring) in order to estimate between and among population diversity and the impact of site or time of germination on diversity.

All gene diversity statistics, simple analysis of frequency of occurrence, and AMOVA revealed the presence of significant variability in *P. annua* among sites, collection dates within sites and within collection dates. The Nei gene diversity statistics and population differentiation parameters indicated that *P. annua* populations were highly diverse. The mean Nei gene diversity for *P. annua* populations was (h) = 0.241 and the total diversity (H_T) = 0.245. A greater proportion of this diversity, however, was within populations rather than between populations. The within population diversity when the 47 populations were grouped as one was (H_S) = 0.209 while as three seasons it was (H_S) = 0.241, and when the populations were grouped to 16 sites, the within populations diversity (H_S) was = 0.224. The genetic diversity among 47 populations when considered as one group for the western Oregon grass seed crops fields resulted in

mean (G_{ST}) = 0.146. These same estimates were (G_{ST}) = 0.121, 0.142, and 0.133 for fall, winter, and spring populations, respectively. The mean among population diversity for the three seasons was (G_{CS} = 0.132) while among season diversity for all populations was only (G_{ST}) = 0.017. The fields where samples were collected from high herbicide selection pressures showed low differentiation among population with (G_{ST}) = 0.01, and low herbicide selection pressures showed greater differentiation among population with (G_{ST}) = 0.125. In high selection pressure areas populations were lower in gene diversity [as low as (h) = 0.155] while in areas of low selection pressure there was higher gene diversity (h) = 0.286. The site to site variability was greater in high selection pressure areas up to 69% or (G_{ST}) = 0.107 while the time of germination variability was greater in areas of low herbicide selection pressure (70%) or (G_{ST}) = 0.067.

The founding populations of *P. annua* of western Oregon grass seed crops were rich in diversity and a strong herbicide selection pressure played a role to shape the existing population diversity. The seed bank supply of *P. annua* in frequently herbicide treated fields along with considerable rate of outcrossing and gene flow must have been some of the factors involved in keeping the diversity at the current level despite high selection pressure of herbicides.

INTRODUCTION

Annual bluegrass (*Poa annua* L.) is a common weed wherever cool-season turfgrasses are grown. Its presence as a contaminant in grass seed is one of the most serious weed problems facing grass seed growers. Recent reductions in field burning in

the Pacific Northwest USA and cancellations of registrations for older herbicides have created increased reliance on the several remaining registered herbicides for *P. annua* control in grass seed crops. Not unexpectedly, biotypes of *P. annua* resistant to two widely used herbicides, diuron and ethofumesate, have now become serious threats to the continued production of *Poa*-free grass seed. Cool-season grass seed production and reports of herbicide resistant *P. annua* are concentrated in the Willamette Valley of Oregon. We therefore conducted a survey of the genetic diversity of *P. annua* in this region to learn more about evolutionary aspects of this weed. Our long range goal is to modify old and develop new seed production and weed control practices that will prolong our ability to control this species by delaying the advent and minimizing the extent of resistance to herbicides and cultural weed control practices.

Two life history forms of *P. annua* are now well known, annual subtypes that quickly flower and perennials that are slow to reach flowering (Tutin, 1957; Holm et al., 1997). Evidence of the existence of both of these types in combination is documented in various surveys (Warwick and Briggs, 1978a; Warwick, 1979). However, differentiation of the populations in response to the environment has also been revealed by several surveys (Gibeault and Goetze, 1973; Warwick, 1979). Genetic variation within and between the populations of *P. annua* has generally been significant (Ellis et al., 1970). One very significant biological property of this species, documented by Koshy, (1969) is the formation of viable seeds when panicles were removed from the plant soon after pollination. *P. annua* shows great diversity in vernalization requirements, from none for annuals to more exacting requirements for perennials (Johnson and White, 1997b).

Within perennials, vernalization needs varied from population to population. Seed dormancy studies showed the existence of rapid differentiation in temperature-enforced dormancy among populations (Wu et al., 1987). *Poa annua* possesses the greatest number of seeds per unit area in soil seed banks of any weed species (Holm et al., 1997). The seed dispersal distance estimated by Law (1975) was less than 0.5 m from parent plants. However, human activities and birds play substantial roles in further dispersion.

Sweeney and Danneberger (1995) used random amplified polymorphic DNA (RAPD) to characterize *P. annua* populations in adjacent golf course greens and fairways giving a limited amount of gene flow and the existence of genetic differences within and between populations. Other studies using isozymes indicated that outcrossing in *P. annua* reached 10-16% (Darmency and Gasquez, 1981), and even up to 22% if density of plants was high (Darmency et al., 1992).

In our study, genetic diversity was assayed using RAPD markers on individual seedlings collected in a structured design. Methods based on arbitrary primed-PCR (Welsh and McClelland, 1990) and RAPDs (Williams et al., 1990) have recently become widely used tools for studying genetic variation in natural populations. RAPD technique and Analysis of Molecular Variance – AMOVA (Excoffier et al., 1992) have been used in outcrossing buffalograss [*Buchloë dactyloides* (Nutt.) Engelm.] populations and demonstrated to show partitioning of variation within population, among populations and among regions (Huff et al., 1993). RAPDs are predominantly dominant markers so that it is difficult to distinguish homozygote from heterozygote RAPD phenotypes in diploid

tissue. The program POPGENE Version 1.21 (Yeh et al., 1997) was recently released to analyze RAPD and other data sets in structured populations. This software allows the user to specify whether the populations are in Hardy-Weinberg Equilibrium (HWE) or in disequilibrium (HWD), whether the marker is dominant or co-dominant, and allows entering of the inbreeding coefficient from known co-dominant markers study (F_{IS}) in the analysis where there is departure from HWE. POPGENE computes both comprehensive genetic statistics, e.g., RAPD frequencies using algorithm given by Chong et al. (1994), gene diversity, genetic distance, G-statistics, F-statistics, and complex genetic statistics, e.g. gene flow, neutrality tests, linkage disequilibria and multi-locus structure (Yeh et al., 1997). Studies of genetic variation in plants have typically used Nei's genetic diversity statistics or Wright's F-statistics as tools for describing the extent of differentiation among populations (Nei, 1973; Wright, 1969; Hamrick and Godt, 1989). It is well understood that mutation, selection, drift, migration and recombination can all shape the structure of plant populations. Understanding which factor is playing the vital role in shaping the populations of *P. annua* of western Oregon would therefore help us understand the dynamics of population status and might suggest possible management options.

MATERIALS AND METHODS

Plant collection

A total of 1578 2-leaf stage *P. annua* seedlings were collected from 10 grass seed production fields in the Willamette Valley of Oregon from early fall 1994 through early spring 1995 (Table 1). Two of the 10 fields were further subdivided on the basis of

Table 1. Annual bluegrass (*Poa annua*) accessions collected from 10 sites in grass seed growers of west Oregon.

Site	Number of samples collected	Current crop	Brief history of the site
McLagan Pugh Road	126 samples in fall, winter, and spring	Perennial ryegrass	*P. annua* herbicide resistance problem
Glaser perennial ryegrass	126 samples in fall, winter, and spring	Perennial ryegrass	*P. annua* herbicide resistance problem
Glaser meadowfoam	84 samples in fall and winter only. No collection in spring	Meadowfoam	Rotated to this crop due to serious problem of herbicide resistance in *P. annua*
McLagan Belle Plain Road	126 samples in fall, winter, and spring	Perennial ryegrass	*P. annua* herbicide resistance problem
Hyslop	72 samples in fall, winter and spring (36 samples per treatment for two residue treatments)	Perennial ryegrass	A replicated trial plots with 2 residue management treatments for 3 years
Bowers	540 samples in fall, winter, and spring (90 samples per treatment for 6 residue treatments)	Perennial ryegrass	A replicated trial plots with 6 residue management treatments for 3 years
Manning full straw chop	126 samples in fall, winter, and spring	Perennial ryegrass	*P. annua* herbicide resistance problem
Manning bale, flail rake	126 samples in fall, winter, and spring	Perennial ryegrass	*P. annua* herbicide resistance problem
George Pugh perennial ryegrass	126 samples in fall, winter, and spring	Perennial ryegrass	*P. annua* herbicide resistance serious problem
George Pugh tall fescue	126 samples in fall, winter, and spring	Tall fescue	*P. annua* herbicide resistance problem
Total samples collected from ten sites = 1578			

post- harvest residue management treatments imposed annually since 1992, creating a total of 16 sampling sites. Permanent plots were marked in the fields, and young seedlings were collected from the same general area at each site in all three collection periods (early fall 1994, early winter 1994/95, and early spring 1995). Seedlings were

transferred to the greenhouse and grown in 'cone-tainers' until fresh leaves were harvested for DNA extraction.

DNA extraction

DNA was extracted using the rapid, one-step extraction method (ROSE) (Steiner et al., 1995) buffer [10mM Tris-HCl (Ph 8), 312.5 mM EDTA (pH 8), 1% lauryl sarkocyl, 1% PVPP per 50 ml final volume, and 0.2% β-mercaptoethanol]. Three to five leaves were harvested per plant, cut into 1-cm pieces, and freeze-dried in sterile 2-ml microfuge tubes. Adding 4 to 6 sterile glass beads to each tube and shaking at a high speed 40 to 50 min ground the desiccated samples. The ROSE buffer (400 µl) was added to each ground sample and then incubated for 20 min. at 90 C with constant gentle shaking to mix. The samples were placed on ice to cool and then centrifuged (12000 rpm) for 1 min. to pellet plant cell debris. A 10 µl aliquot of the supernatant was transferred to a sterile microfuge tube, diluted with 1.7 ml of sterile, double deionized water, and kept at –20 C until used for PCR.

PCR conditions and DNA amplifications

PCR was performed in 12-µl reaction volume under conditions optimized for our extraction of *P. annua* leaf DNA. Using micropipettes, 8.4 µl of reaction mix [1x Stoffel buffer, 3.75 mM $MgCl_2$, 50 µm of each dNTP, 0.5 pico mole primer (Operon B-08 primer sequence 5'GTCCACACGG3'), and 1 unit Taq Stoffel fragment] was first aliquotted into

a 96-well assay plate and then overlaid with 3 drops of mineral oil. Finally, 3.6 µl of the diluted DNA samples was added to the reaction mixture under the oil. The thermal cycling program started with denaturation for 7 min. at 94 C followed by 43 cycles of 30-sec. denaturation at 94 C, 1 min. annealing at 46 C, a 1 C per 3-sec. ramp, and extension at 72 C for 2 min. A final extension at 72 C for 5 min. concluded the DNA amplification. After amplification the samples were kept at 4 C for a short period (< 24 h) or at –20 C for longer periods until electrophoresis.

Electrophoresis and silver staining

Amplification products were separated by polyacrylamide gel electrophoresis and stained with silver. Gels were made from Tris-HCl (pH 8.8), acrylamide (7.5% final), ammonium persulfate, and TEMED (N, N, N ', N '- tetramethylethylenediamine). Following polymerization of the gel, wells were loaded with 6 µl of each amplification product mixed with 9 µl of loading buffer. Three standard markers were included in every gel, and gels were run at 180 volts for 45 min. The staining protocol included incubation with silver nitrate solution (50 ml/gel) for 10 min., developing with sodium carbonate solution (50 ml/gel), and fixing with 7.5% acetic acid. The gels were dried between sheets of gel-drying film (Promega) and scanned into a computer for molecular weight and band density analysis (Molecular Analyst by BioRad). The original gels were scored by hand for presence or absence of 18 repeatable bands (Table 2).

Table 2. *Poa annua* RAPD bands scored for primer B8

Band ID #	Molecular weight (base pair)	Band ID #	Molecular weight (base pair)
14	872	8	450
13b	690	7	440
13	650	6	400
12	640	5b	360
11	630	5	310
10	600	4	270
9b	550	3	260
9	500	2	245
8b	470	1	230

Data analysis

A total of 1357 of the original 1578 samples produced reliable banding patterns. The 221 samples that amplified poorly and could not be reliably scored for some or all of the 18 band positions were dropped from further analysis. Because the ROSE technique normally does not include precise DNA quantification, some samples probably contained too little or too much DNA to amplify well under our PCR conditions. The basic data structure consisted of a (0, 1) matrix of 1357 rows, with one column identifying the individual and 18 columns describing the presence or absence of each of the 18 bands.

Analysis was performed on gene diversity and population differentiation using POPGENE. To do this, first the 1357 individual plant RAPD data were grouped into 47 populations based on the site and time of collection. Then the 47 populations were again regrouped based on three germination periods as fall, winter and spring groups or as 16 groups based on their respective collection sites within the grass seed growing region of western Oregon. Additional grouping was performed into those only from the Bowers farm and those not from Bowers. Based on time of germination, the fall, winter and spring collections consisted of 16, 16, and 15 populations with sample sizes of 488, 439 and 430 individuals, respectively. Since POPGENE is designed to analyze only up to two hierarchical levels our structuring required separate analysis to be made for every grouping of the populations. For example, the output for the time of germination would give only the among three seasons component and the within groups component as opposed to AMOVA that entertains more than two levels of hierarchies. We found POPGENE to be very convenient for analysis of our data because we were able to specify the populations being analyzed were in Hardy-Weinberg disequilibrium. We have added an inbreeding coefficient of 0.64 in all the POPGENE analyses assuming that the species has 22% outcrossing as suggested by Darmency et al. (1992).

Data were also statistically analyzed using ARLEQUIN, the updated version of AMOVA (Excoffier et al., 1992; Huff et al., 1993), on the Euclidean square distances between haplotypes (Table 7). ARLEQUIN conducts a nested multiple analysis of variance, and tests the significance of differences between populations by comparing

sums of squares of the chosen data structure to those of a large number of random permutations of the matrix elements. Our general structure for ARLEQUIN was sites, collection dates within sites, and within collection dates. Simple changes in the group structure data file allowed ARLEQUIN to analyze the data in alternative perspectives. In particular, we analyzed the data from the six residue management treatments at Bowers by dropping the 10 other sites from the data structure, and similarly analyzed the data from the two residue management treatments at Hyslop. The eight remaining sites were grass seed production fields where growers had reported problems controlling *P. annua*, including one perennial ryegrass field plowed after harvest in 1994, planted to meadowfoam, and treated with clethodim between the second and third collection periods. We also performed a non-random subdivision of our population into the most commonly occurring types (those present as more than one individual across all sites) and the unique types. There were 1045 individuals with the 184 most common haplotypes, and 312 unique individuals. We also conducted separate analyses by site, including all individuals at each site and testing only among and within collection dates. Haplotypic frequencies were also characterized by simple descriptive statistics, and the effect of sample size on the stability of these statistics was evaluated by random subdivision of the 450 individuals at the Bowers farm.

NTSYS-pc (Rohlf, 1993) was used to plot the data in several formats, including 3-D eigenvectors (principal components analysis, PCA), minimum spanning trees, and unweighted pair group method with arithmetic averaging (UPGMA) trees. Dendrograms were made based on Nei's (1978) genetic distance: method = UPGMA modified from

NEIGHBOR procedure of PHYLIP version 3.5 and redrawn also using either TREEVIEW or NTSYS-pc.

RESULTS

Gene diversity statistics of *Poa annua* populations using POPGENE analysis

POPGENE was used to analyze RAPD data from 1357 *P. annua* plants assigned into 47 populations. Sample size of populations varied from 8 to 40 where 15 sites consisted of 3 seasons of collections each and one site with only two collections. Of the total 18 loci analyzed, populations showed variations in number and percent polymorphic loci ranging from 8 out of 18 or 44% to 16 out of 18 or 88.9% polymorphic loci (Table 3). The 47 populations overall mean observed number of alleles (n_a) was 2.0 while the mean per population varied from 1.4 to 1.9. The mean effective number of alleles (n_e) [Kimura and Crow, 1964; Hartl and Clark, 1989] for all populations was 1.4 and a standard deviation (S.D.) of 0.37 while population means of n_e varied from 1.18 up to 1.48. The mean Nei's (1973) gene diversity (h) for the 47 populations was 0.241 with a S.D. of 0.185 and mean (h) of the populations varied from 0.12 to 0.29. The mean Shannon's (1949) information index (I) [Lewontin (1972)], which is also a genetic diversity estimate, was 0.375 and with a S.D. of 0.244, while values of the 47 ranged from 0.19 up to 0.44. The among populations diversity for all 47 populations resulted in (G_{ST}) = 0.146. Almost all the above estimates (n_a, n_e, h, I, G_{ST}, number and percent polymorphic loci) measure the genetic diversity although (h) is the most commonly used

Table 3. Distribution of Nei gene diversity statistics across 47 populations of *P. annua* from Western Oregon grass seed farms based on 18 RAPD loci.

Pop ID	Population	Sample size	Mean n_a	Mean n_e	h	I	# P. loci	Polymorphic loci (%)
01	Bowers, bale-S	27	1.67	1.32	0.19	0.29	12	66.7
02	Bowers, bale-F	27	1.78	1.40	0.25	0.38	14	77.8
03	Bowers, bale-W	21	1.67	1.36	0.22	0.34	12	66.7
04	Bowers, bale/flail/-S	26	1.72	1.32	0.20	0.31	13	72.2
05	Bowers, bale/flail/-F	28	1.83	1.43	0.26	0.40	15	83.3
06	Bowers, bale/flail/-W	23	1.83	1.44	0.26	0.39	15	83.3
07	Bowers, bale/flail/r/-S	28	1.83	1.40	0.24	0.38	15	83.3
08	Bowers, bale/flail./r/-F	28	1.83	1.48	0.29	0.44	15	83.3
09	Bowers, bale/flail./r/W	20	1.72	1.47	0.27	0.40	13	72.2
10	Bowers,bale/prop./-S	26	1.67	1.28	0.18	0.28	12	66.7
11	Bowers, bale/prop./-F	29	1.67	1.40	0.24	0.36	12	66.7
12	Bowers, bale/prop./-W	25	1.67	1.42	0.24	0.36	12	66.7
13	Bowers, bale/vac./-S	25	1.72	1.36	0.22	0.34	13	72.2
14	Bowers, bale/vac./-F	30	1.78	1.39	0.23	0.36	14	77.8
15	Bowers, bale/vac./-W	21	1.72	1.46	0.26	0.39	13	72.2
16	Bowers, full straw-S	20	1.89	1.43	0.26	0.40	16	88.9
17	Bowers, full straw -F	27	1.89	1.45	0.28	0.43	16	88.9
18	Bowers, full straw -W	19	1.67	1.39	0.24	0.36	12	66.7
19	Glaser-meadowfoamW	40	1.72	1.27	0.17	0.27	13	72.2
20	Glaser-meadowfoamF	40	1.56	1.21	0.14	0.22	10	55.6
21	Glaser-per.rye.-F	40	1.67	1.33	0.20	0.30	12	66.7
22	Glaser-per.rye.-S	39	1.78	1.32	0.19	0.30	14	77.8
23	Glaser-per.rye.-W	35	1.72	1.32	0.20	0.30	13	72.2
24	George Pugh per.ryeW	32	1.67	1.29	0.18	0.28	12	66.7
25	George Pugh per.rye.S	34	1.61	1.29	0.17	0.27	11	61.1
26	George Pugh per.rye.F	31	1.67	1.36	0.22	0.33	12	66.7
27	George Pugh tall fesW	40	1.67	1.25	0.16	0.26	12	66.7
28	George Pugh tall fes.S	34	1.83	1.32	0.20	0.32	15	83.3
29	George Pugh tall fes.F	36	1.83	1.39	0.24	0.38	15	83.3
30	Hyslop, b/f/r/- S	10	1.67	1.31	0.20	0.31	12	66.7
31	Hyslop, b/f/r/-F	10	1.67	1.36	0.22	0.33	12	66.7
32	Hyslop, b/f/r/-W	8	1.44	1.22	0.14	0.22	8	44.4
33	Hyslop, full straw -S	10	1.61	1.29	0.19	0.29	11	61.1
34	Hyslop, full straw -F	10	1.56	1.32	0.19	0.29	10	55.6
35	Hyslop, full straw -W	8	1.67	1.36	0.22	0.34	12	66.7
36	McLagan Bele Plain S	41	1.72	1.32	0.19	0.29	13	72.2
37	McLagan Bele Plain F	42	1.78	1.29	0.19	0.30	14	77.7
38	McLagan Bele PlainW	37	1.78	1.37	0.23	0.36	14	77.8
39	McLagan Pugh Rd.-S	41	1.72	1.35	0.20	0.31	13	72.2
40	McLagan Pugh Rd.-F	42	1.72	1.31	0.19	0.29	13	72.2
41	McLagan Pugh Rd.W	36	1.83	1.44	0.25	0.37	15	83.3
42	Manning, B/F/R/-S	33	1.67	1.27	0.17	0.27	12	66.7
43	Manning, B/F/R/-F	35	1.72	1.37	0.21	0.32	13	72.2
44	Manning, B/F/R/-W	40	1.61	1.25	0.16	0.25	11	61.1
45	Manning, full straw-S	36	1.78	1.30	0.19	0.30	14	77.7
46	Manning, full straw -F	33	1.72	1.27	0.17	0.28	13	72.2
47	Manning, full straw W	34	1.50	1.19	0.12	0.19	9	50.0
One-way ANOVA for 47 means			P < 0.05	P < 0.05	P < 0.05	P < 0.05	P < 0.05	
Mean of all 47 populations		1357	2.0	1.40	0.24	0.38	H_T = 0.245,	
Standard deviation (S.D.)			0.0	0.37	0.19	0.24	H_S = 0.209	
							G_{ST} = 0.146	

Table 3. (Continued)

F, W, S = fall, winter and spring collections; b = bale only; r = bale then rake; vac. = vacuum sweep; f = flail chop; prop. = Propane flame; n_a = Observed number of alleles; n_e = Effective number of alleles [Kimura and Crow (1964)]; h = Nei's (1973) gene diversity; I = Shannon's information index [Lewontin (1972)]; #P. loci = Number of polymorphic loci; H_T = Total diversity; H_S = Diversity within population; G_{ST} = Diversity among populations. * = P< 0. 05 (significance level for one way-ANOVA).

parameter. The Bowers populations produced the maximum values for n_e, h, and I while the Manning full straw chop population was the lowest.

Schoen and Brown (1991) survey of published isozyme data for eight self-fertilizing species resulted in mean (h) = 0.125, minimum (h) = 0.008, maximum (h) = 0.294, and range of (h) = 0.286 while for 9 outcrossing species mean (h) = 0.257, minimum (h) = 0.174, maximum (h) = 0.328 and range (h) = 0.154. The Nei's (1973) gene diversity statistics of *P. annua* from this RAPD analysis resulted in mean (h) = 0.241, minimum (h) = 0.12, maximum (h) = 0.29 and range (h) = 0.17. When we compare our results with those of Schoen and Brown for self-fertilized species, Nei's gene diversity maximum values are almost the same, the mean and the minimum (h) for *P. annua* are greater while the range (h) of *P. annua* is smaller. The gene diversity statistics of *P. annua* better resemble those of outcrossing species than of self-fertilizing species.

Distribution of Nei gene diversity and population differentiation of the 47 populations of *Poa annua* as structured into three germination groups.

The same 47 populations of *P. annua* were grouped into fall, winter, and spring collections consisting of 16, 16 and 15 populations with sample sizes of 488, 439 and 430 respectively, and were analyzed using POPGENE. Table 4 lists the following parameters for fall, winter, and spring populations of *P. annua*, respectively: a) mean observed number of alleles (n_a) 2.0,1.94, and 1.94, b) mean effective number of alleles (n_e) [Kimura and Crow, 1964; Hartl and Clark, 1989] 1.41, 1.4, and 1.37, c) mean Nei's (1973) gene diversity (h) 0.248, 0.236 and 0.225. The total diversity (H_T) for fall, winter, and spring populations were 0.25, 0.242 and 0.23 while the within group diversity (H_S) were 0.22, 0.21, and 0.20 respectively.

The among population diversity (G_{ST}) for fall, winter, and spring collections was 0.121, 0.142, and 0.133, respectively. The gene flow estimates from G_{ST} based on Slatkin and Barton (1989) as $N_m = 0.25(1- G_{ST})/ G_{ST}$ for fall, winter, and spring populations were 1.82, 1.51 and 1.64, respectively. When considering all 47 populations the total diversity (H_T) for all populations was 0.245 while the diversity within season (H_S) was 0.241 and diversity within the populations within seasons (H_C) was 0.209. The mean diversity among the population within the season (G_{CS}) was 0.132 while the diversity among the three seasons (G_{ST}) was only 0.017 (Table 4).

Genetic diversity and differentiation of 47 populations of *Poa annua* based on 16 sites of collections using POPGENE analysis

As we had structured for the three seasons, we also structured the 47 populations of the *P. annua* RAPD data into 16 groups based on site of collections and herbicide histories, and then analyzed for gene diversity and differentiation parameters using POPGENE. The 15 sites consisted of each three populations resulted from three season's

Table 4. Distribution of Nei gene diversity and population differentiation (18 RAPD loci) for 3 groups based on time of germination and 47 populations of *Poa annua* from Western Oregon grass seed farms.

Group by Season	# of population	Sample size	n_a	n_e	h	I	H_T	H_S	G_{ST}	N_m	% P loci
Fall	16	488	2.0	1.41	0.25	0.39	0.25	0.220	0.121	1.82	100
		S.D.	0.0	0.36	0.18	0.23	0.03	0.022			
Winter	16	439	1.9	1.40	0.24	0.37	0.242	0.206	0.142	1.51	94.4
		S.D.	0.2	0.38	0.19	0.26	0.038	0.026			
Spring	15	430	1.9	1.37	0.23	0.35	0.230	0.199	0.133	1.64	94.4
		S.D.	0.2	0.35	0.18	0.25	0.034	0.024			
All	47	1357	2.0	1.40	0.24	0.38	0.245	0.241	0.017		100
		S.D.	0.0	0.37	0.19	0.24	0.034	0.033			
								H_c/ S.D.	G_{CS}	Nm (G_{ST})	Nm (G_{CS})
								0.209/ 0.023	0.132	14.2	1.65
One-way ANOVA for the 3 mean values			*	*	*	*	*	*	*	*	*

n_a = Observed number of allele; n_e = Effective number of alleles [Kimura and Crow (1964)] ;h = Nei's (1973) gene diversity; I = Shannon's information index [Lewontin (1972)];#P. *Loci* = Number of polymorphic loci; H_T = Total diversity ;Hc = Diversity within populations within the group; H_S = Diversity within the species; G_{ST} = Diversity among groups ; G_{CS} = Diversity among populations within group;Nm = Gene flow based on N_m = 0.25(1- G_{ST})/ G_{ST} (or G_{CS} depending on which is used) (Slatkin and Barton, 1989);S.D. = Standard Deviation.
* = $P < 0.05$ for one-way ANOVA of F value probability.

samplings with sample size ranges of 28 up to 120 (Table 5), while the Glaser meadowfoam site consisted of only two populations, one from fall and one from winter. As depicted in Table 5, the overall mean n_a, n_e, h, and I remain the same for 47 populations as when we analyzed these same populations as three groups. Although the total diversity (H_T) remained the same (0.245), the (H_T) of the sites varied from 0.155 at Glaser meadowfoam to 0.286 at Bowers bale and flail + rake populations. The mean within site diversity (H_S) for all populations was 0.224, but individual populations varied from 0.15 to 0.269 at the same two sites, respectively. The within group within plants diversity for all populations remained the same (H_C) = 0.209. The between sites mean for all populations G_{ST} was 0.087, while the between population diversity within sites varied from G_{ST} = 0.016 at George Pugh perennial ryegrass to G_{ST} = 0.125 at Bowers bale and G_{ST} = 0.113 at Hyslop bale then flail + rake populations. The G_{CS} or mean genetic diversity between populations within sites for all groups was 0.065. (Table 5). The gene flow rate estimate (N_m) between sites for all populations was 2.6 while the gene flow between populations within sites was 3.55. Populations (N_m) within sites varied from N_m = 1.53 at Bowers bale to N_m = 15.2 at George Pugh perennial ryegrass. When all populations were considered in the analysis for all 18 loci analyzed the percent polymorphic loci was 100% while populations at different sites varied from 72.2% up to 88.9% polymorphic loci per site. Dendrograms from POPGENE analysis for 47 populations resulted in the topology shown in Figure 2. Our general interpretation of this tree is that the *P. annua* populations possessed substantial genetic diversity, a fraction of which might be potentially attributed to sites or collection dates. For example, the six residue management treatments of the 18 Bowers populations perfectly clustered into

three groups based on time of germination, with the six fall, the six winter, and the six spring populations clustering together to form three distinct groups. For the Bowers populations time of germination is clearly a major factor differentiating populations. Other cluster groups with consistency in time of germination are found in Hyslop populations where the pattern of clustering was based on time of germination for fall and winter, but not spring. Examination of trees for samples collected from other individual fields suggested the presence of a small, although probably significant effect of collection

Table 5. Distribution of Nei gene diversity and population differentiation (18 RAPD loci) for 16 groups based on site of collection and 47 populations of *Poa annua* from western Oregon grass seed farms.

No. and site names	# pop	*Sample size*	n_a	n_e	h	I	H_T	H_S	G_{ST}	N_m	*% Poly. Loci*	*N_m ratio
1. Bowers, bale	3	75	1.9	1.42	0.25	0.39	0.25	0.22	0.125	1.5	88.9	0.10
2. Bowers, bale/flail	3	77	1.9	1.44	0.27	0.41	0.26	0.24	0.092	2.5	94.4	0.16
3. Bowers, bale/flail/rake	3	76	1.9	1.48	0.29	0.43	0.29	0.27	0.058	4.1	94.4	0.27
4. Bowers, bale/propane	3	80	1.9	1.40	0.24	0.37	0.24	0.22	0.083	2.8	88.9	0.18
5. Bowers, bale/vacuum	3	76	1.9	1.44	0.26	0.34	0.26	0.24	0.090	2.6	88.9	0.17
6. Bowers, full straw	3	66	1.9	1.47	0.28	0.43	0.28	0.26	0.072	3.2	94.4	0.21
7. Glaser, meadowfoam	2	80	1.7	1.24	0.16	0.25	0.16	0.15	0.021	11.9	72.2	0.78
8. Glaser, per. rye.	3	114	1.9	1.33	0.20	0.31	0.20	0.19	0.025	9.8	88.9	0.64
9. George Pugh, per. rye.	3	97	1.8	1.31	0.19	0.30	0.19	0.19	0.016	15.2	77.8	1.00
10. George Pugh, tall fescue	3	110	1.9	1.32	0.21	0.33	0.21	0.20	0.040	6.1	88.9	0.40
11. Hyslop, bale/flail/rake	3	28	1.7	1.35	0.21	0.33	0.21	0.19	0.113	1.9	72.2	0.13
12. Hyslop, full straw	3	28	1.7	1.33	0.21	0.33	0.22	0.20	0.069	3.4	72.2	0.22
13. McLagan Belle Plain Rd.	3	120	1.8	1.34	0.21	0.33	0.21	0.20	0.051	4.7	77.8	0.31
14. McLagan Pugh Rd.	3	119	1.9	1.37	0.22	0.34	0.22	0.21	0.044	5.5	88.9	0.36
15. Manning, bale/flail/rake	3	108	1.8	1.31	0.191	0.30	0.19	0.18	0.048	4.9	83.3	0.33
16. Manning, full straw	3	103	1.8	1.26	0.17	0.27	0.17	0.16	0.030	8.1	77.8	0.53
All sites and all accessions	47	1357	2.0	1.4	0.24	0.38	0.25	0.22	0.087	2.6	100	0.17
	S.D.		0.0	0.37	0.19	0.24	0.03	0.03				
				H_C	G_{CS}	N_m (G_{CS})= 3.55						
	Mean			.209	0.065							
	S.D.			0.02								
One-way ANOVA for the 16 means	F-test P <			0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	

*N_m ratio = ratio of the N_m of every site to the maximum N_m at George Pugh perennial ryegrass.

dates within site. A substantial amount of diversity was also observed due to site of collection. The six treatments at Bowers which had clustered together based on time of germination, were separated from all the 10 remaining sites except for Manning bale/flail/rake. Populations at the remaining nine sites generally clustered based on site of collection, with only a few populations clustering outside of their respected sites (Figure 2). The 47 populations when grouped into 16 sites resulted in 57.2% G_{ST} among sites while 42.8% of the variability was within site component (Table 6). The G_{ST} and G_{CS} values for the total 47 populations grouped into 3 germination seasons partitioned into $G_{ST} = 0.017$ and $G_{CS} = 0.132$. This means that the diversity among the three germination periods was only 11.4% while 88.6% of the variability was within season.

We split the total 47 populations into Bowers's six sites and other ten sites and analyzed separately for gene diversity and population differentiation using POPGENE. The most interesting result of the G_{ST} partitioning was obtained when we analyze the Bowers six residue sites separately from the rest 10 sites. The Bowers six residue management treatments G_{ST} level among treatments was only 8.5% while the within treatment portion was 91.5%. When these same site populations were grouped by 3 germination periods, the among season and within season G_{ST} and G_{CS} values partitioned to 70.2% and 29.8% respectively. This again indicates that the Bowers bluegrass populations G_{ST} variation is attributed more to the time of germination (70% of the diversity) than for any other populations studied. This large amount of variation partitioning due to time of germination was not found in other 10 sites populations since the among collection dates proportion was only 3.9% of the total, with the remaining

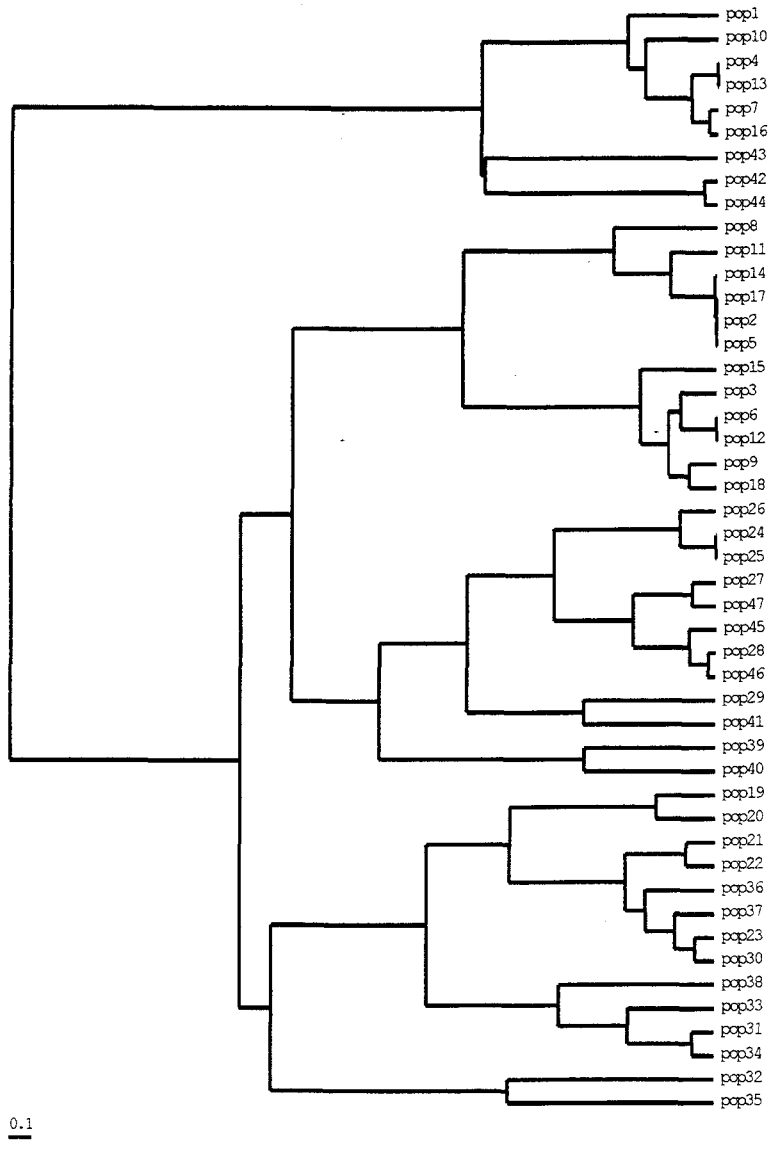


Fig.2. Dendrogram based on Nei's (1978) Genetic distance: Method = UPGMA modified from NEIGHBOR procedure of PHYLIP Version 3.5 for 47 populations of *Poa annua* of western Oregon grass seed farms. Eighteen RAPD loci of 1357 individuals were analyzed grouped as 47 populations using POPGENE. Distance metrics was made among populations based on Nei's Unbiased measures of genetic identity and genetic distance Nei (1978). The populations used were: 1, 2, 3 = S, F, W at Bowers, bale; 4, 5, 6 = S, F, W at Bowers, bale/flail; 7, 8, 9 =S, F, W at Bowers, bale/flail/r; 10, 11, 12 = S, F, W at Bowers, bale/prop.; 13, 14, 15 = S, F, W at Bowers, bale/vac.; 16, 17, 18 = S, F, W at Bowers, full straw; 19, 20 = W, F at Glaser-meadowfoam; 21,22,23 = F, S, W at Glaser-per.rye.; 24, 25, 26 = W, S, F at George Pugh per.rye.; 27, 28, 29 = W, S, F at George Pugh tall fes.; 30, 31, 32 = S, F, W at Hyslop, b/f/r; 33, 34, 35 = S, F, W at Hyslop, full straw; 36, 37, 38 = S, F, W at McLagan Belle Plain ; 39, 40, 41 = S, F, W at McLagan Pugh Rd.; 42, 43, 44 = S, F, W at Manning, b/f/r; 45, 46, 47 = S, F, W at Manning, full straw; F, W, and S indicate the fall, winter, and spring collections, respectively.

Table 6. Distribution of *Poa annua* RAPD differentiation (G_{ST} and G_{CS}) over several category groups of populations.

Group category	# of populations	Sample size	Mean G_{ST} among	Mean G_{CS} within	% among groups % G_{ST}	% within group % G_{CS}
1 group of all populations	47	1357	0.1466	.....	100	.....
3 groups by season	47	1357	0.017	0.132	11.4%	88.6%
16 groups by site	47	1357	0.087	0.065	57.2%	42.8%
3 Groups of Bowers/ season	18	450	0.0667	0.0283	70.2%	29.8%
6 groups of Bowers/ residue management	18	450	0.008	0.0858	8.5%	91.5%
3 Groups of other 10 sites /season	29	907	0.006	0.1446	3.9%	96.1%
10 groups of other 10 sites / site	29	907	0.107	0.0478	69.1%	30.9%

being within collections. However, the among sites component at the 10 other sites was elevated reaching values of G_{ST} = 0.107 and G_{CS} = 0.0478, indicating that nearly 69% of variation was among sites while the remaining 31% of diversity was within site (Table 6).

Genetic diversity and hierarchical analysis of molecular variance of *Poa annua* using (AMOVA)

Analysis of molecular variance conducted over all 16 sites revealed the presence of highly significant effects among sites, among collection dates within sites, and within collection dates (Table 7). However, nearly 88% of the total haplotypic variance fell within collections, quantifying our findings from the dendrograms that the diversity existing within collections was much greater than that associated with sites or collection dates within sites. The variance among sites was slightly larger than the variance among collections within sites. When data from the Bowers field were analyzed separately as we did in POPGENE, the six residue management treatments that had been imposed for three consecutive years prior to our sampling had no effect on the genetic structure of the *P. annua* population (Figure 2, and Table 7). The within collection dates and among collection dates with residue treatment components were highly significant. Indeed, the among collections within residue treatments component was much larger at Bowers, where it averaged 9% of the variance (Table 7) than at 10 other sites, where it averaged only 2% of the variance.

A simple analysis of the frequency of occurrence of more than one individual with the same haplotype at a site revealed some interesting differences among the populations from the 10 fields. At Hyslop, 82.1% of the individuals were needed to represent all of the types found there, and the single most common type only accounted

Table 7. Hierarchical analysis of molecular variance on distance metrics for various groupings of *Poa annua* haplotypes.

Data grouping	Variance component	Variance	% total	Prob.[a]	Φ-statistics
[1]All	Among sites	σ2a = 0.161	7.5	<0.001	Φ_{CT} =0.075
individuals	Among collections/sites	σ2b = 0.104	1.84	<0.001	Φ_{SC} = 0.052
	Within collections	σ2c = 1.887	87.64	<0.001	Φ_{ST} = 0.123
[2]Bowers	Among treatments	σ2a = -0.079	-3.37	1.00	Φ_{CT} = 0.034
All	Among collections/trts.	σ2b = 0.219	9.29	<=0.001	Φ_{SC} = 0.090
treatments	Within collections	σ2c = 2.214	94.08	<= 0.001	Φ_{ST} = 0.059
[3]Other	Among sites	σ2a = 0.208	10.52	<=0.001	Φ_{CT} = 0.105
10 sites	Among collections/sites	σ2b = 0.045	2.28	<= 0.001	Φ_{SC} = 0.025
	Within collections	σ2c = 1.726	87.20	<= 0.001	Φ_{ST} = 0.128
[4]Most	Among sites	σ2a = 0.187	10.17	<=0.001	Φ_{CT} = 0.102
common 184	Among collections/sites	σ2b = 0.084	4.58	<= 0.001	Φ_{SC} = 0.051
haplotypes	Within collections	σ2c = 1.567	85.25	<= 0.001	Φ_{ST} = 0.147
[5]Least	Among sites	σ2a = 0.103	3.51	<=0.003	Φ_{CT} = 0.035
common 312	Among collections/sites	σ2b = 0.134	4.58	<= 0.001	Φ_{SC} = 0.047
haplotypes	Within collections	σ2c = 2.698	91.91	<= 0.001	Φ_{ST} = 0.081

[1] 496 *P. annua* haplotypes, all sites. [2] 258 *P. annua* haplotypes at Bowers. [3] 324 *P. annua* haplotypes at all other sites. [4] 184 most common haplotypes (those occurring more than once in the whole populations of 1357 individuals with 1045 individuals possessing these 184 most common haplotypes. [5]Least common 312 haplotypes (those that occurred only once in the whole populations of 1357 individuals). [a]Probability of more extreme variance components and Φ-statistic than observed value by chance alone, based on 1000 random permutations. Φ_{CT}, Φ_{SC} and Φ_{ST} are F-statistic analogues for the correlations of molecular diversity within sites relative to the whole species, within collections relative to the site, and within collections relative to the whole species, respectively.

for 5.4% of the plants (Table 8). In contrast, at Manning's full straw load chop field, the proportion of plants needed to represent all of the banding patterns dropped to only 37.9%, while the single most common type accounted for 18.4% of the total population. *P. annua* in Glaser's meadowfoam field, like Manning's full straw field, was also less diverse than at Hyslop, and dominated by relatively few haplotypes. Genetic diversity at most of the other sites was intermediate between that at Hyslop and that at Manning's and Glaser's, although that at Bower's was slightly greater than that at Hyslop (Table 8).

Interesting results were obtained when we split the data in to the most commonly occurring haplotypes (those found in more than one individual in the whole population) and the least common types (Table 7). As might be expected, size of the within collections variance decreased for the most common types and increased for the least common types compared to the pooled analysis (Table 7). The among collections within sites component occupied 4.6% of the total variance for each group. However the variance among sites was much smaller in the least common group (3.5% of total) than in the most common group (10.2% of total).

Upon reflection, these results do make biological sense. The most common haplotypes would generally be the ones doing well as weeds in grass seed production fields. These most common types show highly significant effects among sites because each field has had a unique history of selection pressure and weed seed importation, creating its own unique mix of successful, dominant types. The significance within collections occurs as a result of the background diversity within this species, the

Table 8. *Poa annua* haplotype class distributions by site.

Site	Individuals collected per site	Unique haplotypes per site	Single most common haplotype	Most common 15% of haplotypes	Most common N types (N = √total no. of types)
	(number)	.....(% of each sites populations).....			
Bowers avg. residue trt.	75	84.9	4.4	27.2	24.0
Hyslop	56	82.1	5.4	28.2	27.8
McLagan Pugh Rd.	119	71.4	6.7	36.6	30.6
McLagan Belle Plain Rd.	120	61.7	5.8	38.6	32.3
Glaser per.rye.	114	61.4	6.1	38.2	32.5
Manning, bale/flail/rake	108	57.4	12.0	41.3	37.6
George Pugh, tall fescue	110	60.0	10	41.6	38.4
George Pugh, per. rye.	97	55.7	9.3	46.6	44.4
Glaser meadowfoam	80	50	17.5	51.2	55.0
Manning, full straw chop	103	37.9	18.4	56.8	58.0
Bowers bale/flail/rake	76	88.2	3.9	25.1	22.6
Bowers bale/flail	77	81.8	3.9	27.1	23.2
Bowers propane flame	80	86.2	5.0	26.7	23.3
Bowers vacuum sweep	76	84.2	3.9	27.9	23.6
Bowers full straw chop	66	87.9	4.5	25.3	23.7
Bowers bale-only	75	81.3	5.3	30.9	27.5
Bowers, pooled data	450	57.3	2.7	42.1	25.0
All sites pooled data	1357	36.6	3.6	58.2	34.3

selection pressure at each site, and some degree of genetic control over germination patterns. The small size of the among collections within sites variance may imply that among the traits of this highly successful weed are a tendency to maintain only relatively modest genetic barriers between fall, winter, and spring germinating types. The least common types may be those not faring quite as well under current grass seed production practices, and may represent the background population diversity in Willamette Valley *P. annua*, the gene pool from which the more successful types have arisen.

It is likely that the most abundant genotypes in commercial production fields are those that tolerate the herbicide and residue management treatments that growers have imposed in the recent past. However, because we collected our seedlings at the 2-leaf growth stage, we may have "rescued" some seedlings that would have otherwise succumbed to diuron present in the field soil. Our knowledge of the genetic diversity of Willamette Valley *P. annua* should help us formulate more effective strategies for managing this weed.

DISCUSSION

Dendrograms and PCA plots of the entire data set, or large subset of it, were extremely complex and difficult to concisely summarize individual plant data. Individuals from any particular site were scattered over a large portion of the trees or PCA plots. The UPGMA algorithm produced large numbers of tied trees, and tree nodes were often highly multifurcating. However, when individuals were structured as

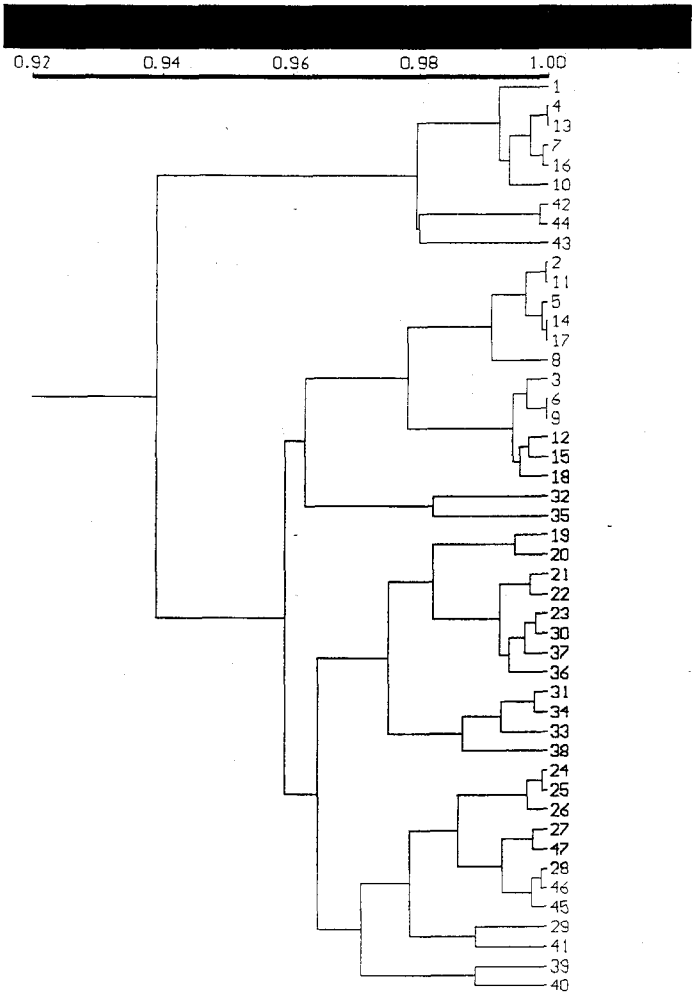


Figure 3. Dendrogram for 47 populations of *Poa annua*. The Nei 1978 distance matrix was converted to similarity and then UPGMA clustering was used to draw the tree using NTSYS-pc. The populations used were: 1, 2, 3 = S, F, W at Bowers, bale; 4, 5, 6 = S, F, W at Bowers, bale/flail; 7, 8, 9 =S, F, W at Bowers, bale/flail/r; 10, 11, 12 = S, F, W at Bowers, bale/prop.; 13, 14, 15 = S, F, W at Bowers, bale/vac.; 16, 17, 18 = S, F, W at Bowers, full straw; 19, 20 = W, F at Glaser-meadowfoam; 21,22,23 = F, S, W at Glaser-per.rye.; 24, 25, 26 = W, S,F at George Pugh per.rye.; 27, 28, 29 =W, S, F at George Pugh tall fes; 30, 31, 32 = S, F, W at Hyslop, b/f/r; 33, 34, 35 = S, F, W at Hyslop, full straw; 36, 37, 38 =S, F, W at McLagan Belle Plain ; 39, 49, 41 = S, F, W at McLagan Pugh Rd.; 42, 43, 44 = S, F, W at Manning, B/F/R; 45, 46, 47 = S, F, W at Manning, full straw. F, W, and S indicate the fall, winter, and spring collections, respectively.

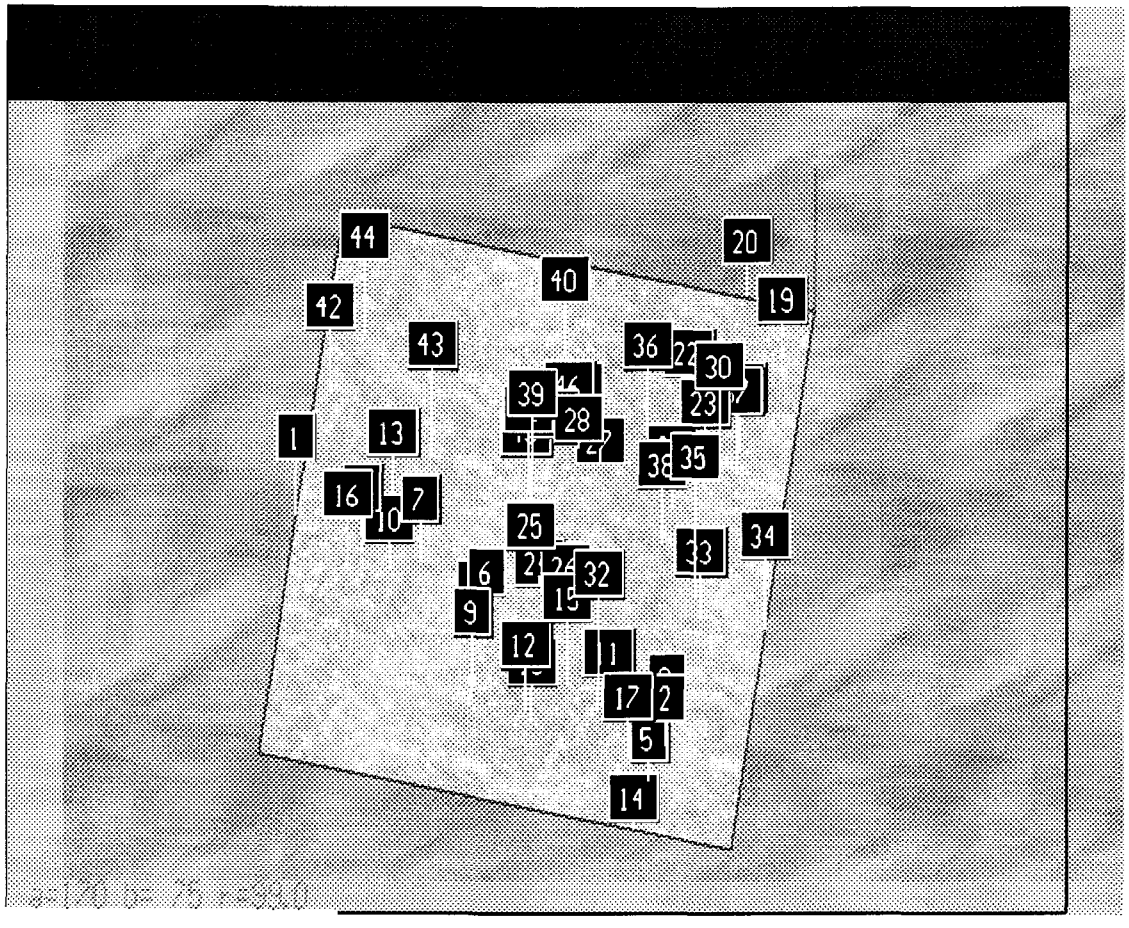


Figure 4. A principal component analysis for the similarity of the 47 populations of *Poa annua* where the first three components account for the 85% of the variation explained by three axes. **The populations used were: 1, 2, 3 = S, F, W at Bowers, bale; 4, 5, 6 = S, F, W at Bowers, bale/flail; 7, 8, 9 =S, F, W at Bowers, bale/flail/r; 10, 11, 12 = S, F, W at Bowers, bale/prop.; 13, 14, 15 = S, F, W at Bowers, bale/vac.; 16, 17, 18 = S, F, W at Bowers, full straw; 19, 20 = W, F at Glaser-meadowfoam; 21,22,23 = F, S, W at Glaser-per.rye.; 24, 25, 26 = W, S,F at George Pugh per.rye.; 27, 28, 29 =W, S, F at George Pugh tall fes; 30, 31, 32 = S, F, W at Hyslop, b/f/r; 33, 34, 35 = S, F, W at Hyslop, full straw; 36, 37, 38 =S, F, W at McLagan Belle Plain ; 39, 49, 41 = S, F, W at McLagan Pugh Rd.; 42, 43, 44 = S, F, W at Manning, B/F/R; 45, 46, 47 = S, F, W at Manning, full straw. F, W, and S indicate the fall, winter, and spring collections, respectively.**

populations based on collection sites and dates and analyzed either by POPGENE or AMOVA, the UPGMA algorithm resulted in trees with populations clustered based on their respective herbicide history sites and on season of germination. However, the exact

clustering details depend on the method used to display the relationship. The Nei 1978 distance matrix for 47 populations was converted to similarity matrix and then clustered using UPGMA of the NTSYS-pc. The tree obtained by this method was similar to that from the NEIGHBOR procedure of PHYLIP (Figure 2) in terms of clusters except that few topological changes were seen (Figure 3). Although the matrix comparison correlation coefficient was low (r = 0.63), the principal component analysis supports the relationships among populations exactly the same way as seen on the trees (Figure 4). A minimum spanning tree of the Hyslop data was examined for the tendency of individuals at the same or adjacent nodes to have come from the same collection date, and this was

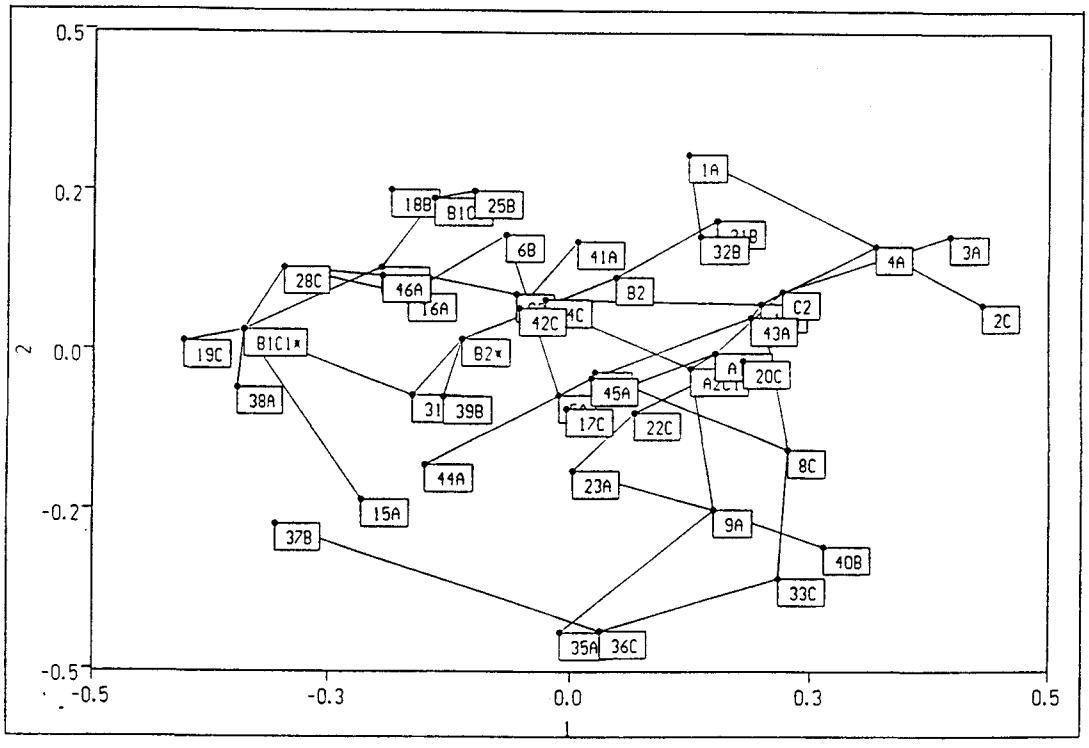


Figure 5. Principal coordinate analysis of Hyslop site *Poa annua* polymorphisms for 18 RAPD bands with minimum length spanning tree superimposed. A, B, and C denote plants collected in fall 1994, winter 1994-95, and early spring 1995.

found to be highly significant (Figure 5) and (Table 9). However, it was also obvious that the tendency of a given type to germinate in the fall, winter, or spring was seldom, if ever, absolute. Some genetic exchange would probably occur between types even if all of the individuals germinating in fall, winter, or spring could be prevented from cross pollinating with individuals germinating in other time periods by effective herbicide treatments, other cultural practices, or natural plant maturation and senescence.

The mean effective number of alleles (n_e) for all individuals and all loci was 1.4. According to Kimura and Crow (1964); Hartl and Clark (1989), the effective number of alleles (n_e) estimates the reciprocal of homozygosity. Based on this estimate the *P. annua* populations' homozygosity is about 71%. This homozygosity is close to what the literature implies for an outcrossing of 22% in *Poa annua.* The fact that progenies derived from seeds of single plants segregated for resistance (chapter 3) and the within population diversity of 91 to 98% support an estimate of up to 29% outcrossing in our populations. The genetic difference among 47 populations $G_{ST} = 0.146$ for *P. annua* of western Oregon in our study is far less for self pollinating species reported $F_{ST} = 0.51$. This value however is in close accord with $F_{ST} = 0.1 - 0.22$ for mixed mating and 0.10 - 0.20 for outcrossing species (Hamrick and Godt, 1989; and Hawksworth and Kalin-Aroyo, 1995). This might have been caused due to the high outcrossing rates in the populations that reduced the diversity among groups but increased the diversity within the species.

Table 9. Summary of Hyslop *Poa annua* minimum spanning tree. Number of times adjacent or identical nodes were from collections made in the fall (A), winter (B), or spring (C).

Identical nodes only

Node class	A	B	C
A	2	0	3
B		4	2
C			8
Too few cases for $\chi 2$ test.			

Adjacent nodes only

Node class	A	B	C
A	20	10	28
B		26	10
C			30
$\chi 2 = 22.39$, $P<0.001$			

Adjacent or identical nodes

Node class	A	B	C
A	22	10	31
B		30	12
C			38
$\chi 2 = 27.58$, $P<0.001$			

It is interesting to compare results from AMOVA (Table 7) with that of the Nei gene diversity analysis and differentiation parameters indicated on (Table 6). The G_{ST} among the 16 sites was 0.087 while the within site diversity G_{ST} was 0.065, indicating

population differentiation between sites to be higher than population differentiation within the site for 16 sites and 47 populations. The maximum G_{ST} values per site were at Bowers and at Hyslop with G_{ST} values of 0.1249 and 0.1134 respectively whereas the lowest G_{ST} values per sites were for George Pugh perennial ryegrass, Glaser meadowfoam, and Manning full straw chop fields, with G_{ST} values of 0.0162, 0.021 and 0.03 respectively (Table 5). Other diversity parameters like h, I, H_T, and H_S all showed similar pattern of diversity distribution across sites where the maximum values of diversity were found for Bowers sites followed by Hyslop and the minimum diversity values recorded for Glaser meadowfoam and the Manning full straw chop sites. The remaining site diversity statistics remain between these two extremes similarly as it was shown in the AMOVA analysis (Table 7).

Our results either using the AMOVA (Table 7) where $\Phi_{SC} = 0.09$ or POPGENE (Table 6) where [$G_{ST} = 0.008$ + $G_{CS} = 0.0858$] when grouped into six, and [$G_{ST} = 0.0667$ + $G_{CS} = 0.0283$] when grouped into three for Bowers are in close agreement. However, there was some discrepancy in the percentage partitioning, probably because we got a negative percentage for Φ_{CT} in AMOVA. Furthermore, the percentage in POPGENE is for only two hierarchies where either it is among residue treatments and within treatments or it is among collection dates and within collection dates, as opposed to AMOVA, which handles more than two levels of hierarchies in single analysis.

Further examination of the distribution of the G_{ST} values as presented in Table 5 or Figure 6 shows an interesting pattern in accord with the specific site herbicide histories

and selection pressures. For example, the 8 out of 16 sites that showed $G_{ST} \leq 0.05$ are those with low gene diversity statistics and dominated by herbicide resistant genotypes with low numbers of susceptible types. The 6 out of 16 sites with G_{ST} values between 0.05 to 0.1 seem to have experienced a moderate level of selection pressure. The 2 out of 16 sites that have G_{ST} values between 0.1 and 0.15 (Bowers and Hyslop) had experienced less herbicide selection pressure than the other ten sites. This indicates that genetic diversity of the populations was being shaped in response to herbicide selection pressures. We have confirmed this in the greenhouse test on progenies of these plants and we saw many genotypes of the following sites [the Bowers (sites 1-6) and the Hyslop (sites 11- 12)] to be susceptible to diuron (Table 12). We do not expect the existence of significant variation in mutation rates or drift across populations within the grass seed grower's farms of those different sites. However, we know that the herbicide selection pressure varied across sites even before we sampled these materials. The greater diversity statistics and differentiation values at Bowers and Hyslop and the lesser values at other sites are in agreement with the concept that populations of *P. annua* of western Oregon grass seed crops have been shaped by selection.

One more advantage of the POPGENE analysis is that it tests neutrality based on the overall Ewens-Waterson Test for Neutrality (Manly, 1985) for every locus. This help to determine whether mutation, migration, selection are in balance to assume neutral evolution of populations or whether selection prevails in shaping the population. When

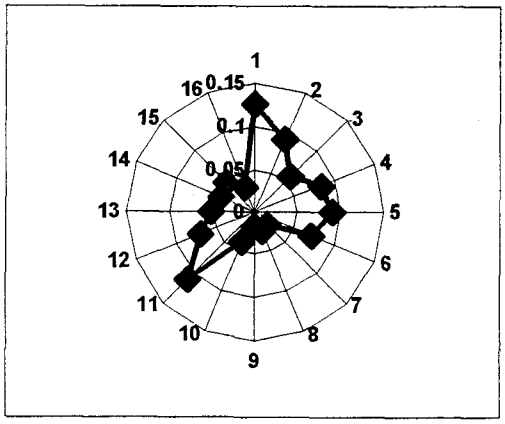


Figure 6. Distribution of *Poa annua* RAPD G_{ST} across 16 sites in the grass seed growers of Western Oregon. Numbers 1 – 16 refer to the population's ID numbers based on sites of collections as described on Table 5. The values 0.0, 0.05, 0.1 and 0.15 are the scale of G_{ST} for the graph.

considering the 1357 individuals and 47 populations as one component of the *Poa* population of the western Oregon grass seed crops all 18 loci showed the calculated and simulated F (based on $F = \sum_{i=1}^{k} n^2 i / n^2$ where n = sample size and k = number of alleles, Manly, 1985) to be within the boundary of the 95% neutrality (Table 10, and Figure 7). However, means for majority of the loci were near the maximum values where heterozygous types should be at a disadvantage. On the other hand, when considering the neutrality test per season or per site we found a few loci fixed (F = 1.0), which means only one allele was found per locus. These distributions have a similar trend to what we saw from the gene diversity and differentiation distributions. For example, there were no alleles lost in fall group, but one was lost both in winter and spring groups. More loci showed the maximum F = 1 values at Glaser meadowfoam fields than at Bowers. These parameters could be carefully used as indicators of selection.

When we examine the distribution of the calculated rate of gene flow N_m, the maximum N_m was for George Pugh perennial ryegrass field (N_m = 15.2) while the smallest for Bowers, bale (N_m = 1.53) (Table 5). Highest values of N_m occurred at sites of high selection pressure and low values at Hyslop and Bowers, with an average over all sites of N_m = 2.6 (Table 5), and average fall, winter and spring N_m = 1.82, 1.51 and 1.64 respectively (Table 4). These numbers can be used as indicators of selection because the trend matches the diuron use histories at these sites. The diversity among groups also matches this pattern, and reduced G_{ST} is also an indicator of selection. Taking the ratio of individual N_m values at the sites or seasons to the maximum N_m at the George Pugh site (15.2) produces indices of selection intensity. This gives indices of 0.12, 0.10 and 0.11 for fall, winter and spring populations, respectively. This is an expected trend because most of the herbicides are applied at fall and are very active on germinating young seedlings. Similarly we get indices from 0.1 at Bowers to 0.77 at Glaser meadowfoam, indicating that the variability for herbicide selection pressure from site to site is greater than from season to season. Minimum, maximum, and mean indices for each of the 16 sites were 0.1, 1.0 and 0.17 respectively (Table 5). This large gene flow rate may result from a substantial outcrossing combined with an ample supply of seed from the soil seed bank. Hence, these populations remain relatively diverse despite continuous use of herbicides although not as diverse as at the low selection pressure sites. However diversity was smaller in dominantly herbicide resistant populations than in dominantly

Table 10. The Overall Ewens-Watterson Test for Neutrality for the 18 RAPD loci of *Poa annua.* [Based on Manly (1985) 'The Statistics of Natural Selection' (p.272-282)]

Locus	n	k	Obs. F	Min F	Max F	Mean*	SE*	L95*	U95*
B8-1	1357	2	0.837	0.500	0.999	0.860	0.028	0.502	0.999
B8-2	1357	2	0.778	0.500	0.999	0.873	0.027	0.502	0.999
B8-3	1357	2	0.500	0.500	0.999	0.873	0.026	0.505	0.999
B8-4	1357	2	0.878	0.500	0.999	0.872	0.027	0.507	0.999
B8-5	1357	2	0.998	0.500	0.999	0.877	0.025	0.504	0.999
B8-6	1357	2	0.954	0.500	0.999	0.870	0.026	0.504	0.999
B8-7	1357	2	0.608	0.500	0.999	0.871	0.026	0.505	0.999
B8-8	1357	2	0.899	0.500	0.999	0.867	0.028	0.504	0.999
B8-9	1357	2	0.607	0.500	0.999	0.880	0.024	0.507	0.999
B8-10	1357	2	0.535	0.500	0.999	0.872	0.028	0.503	0.999
B8-11	1357	2	0.500	0.500	0.999	0.872	0.027	0.506	0.999
B8-12	1357	2	0.800	0.500	0.999	0.868	0.027	0.507	0.999
B8-13	1357	2	0.612	0.500	0.999	0.875	0.026	0.503	0.999
B8-14	1357	2	0.507	0.500	0.999	0.882	0.025	0.506	0.999
5B	1357	2	0.977	0.500	0.999	0.872	0.026	0.506	0.999
9B	1357	2	0.764	0.500	0.999	0.867	0.027	0.505	0.999
13B	1357	2	0.932	0.500	0.999	0.871	0.026	0.505	0.999
8B	1357	2	0.981	0.500	0.999	0.869	0.027	0.506	0.999

* These statistics were calculated using 1000 simulated samples.

Obs. F = Observed F
L95, U95 = Lower and upper limits of the 95% confidence intervals respectively.
n = number of observations
k = number of alleles

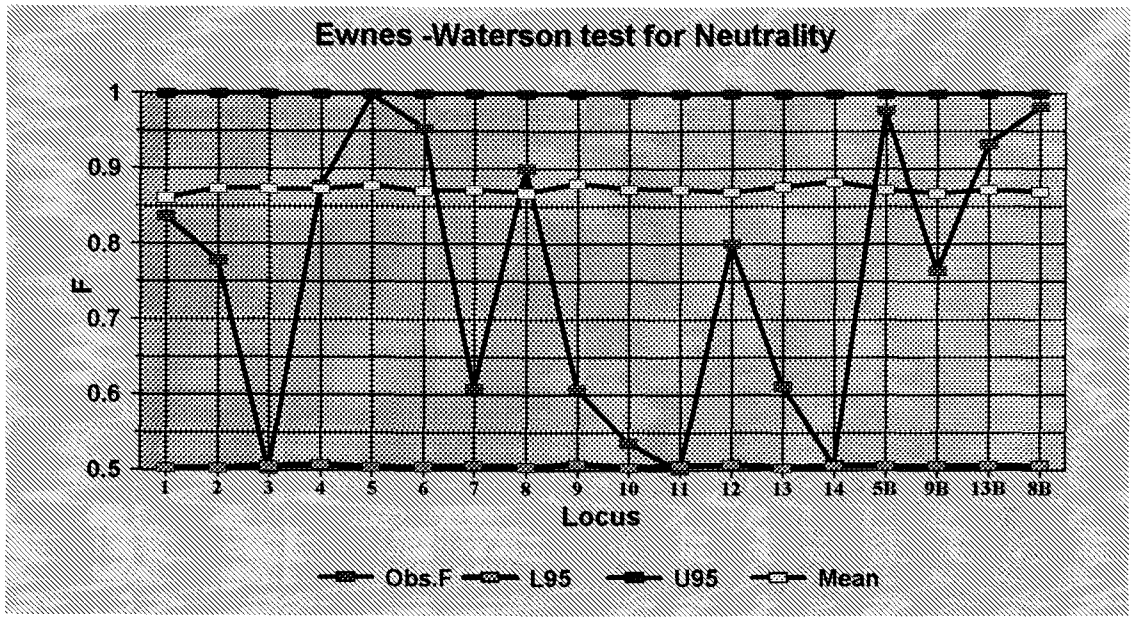


Figure 7. The Ewens-Waterson test for neutrality for 18 RAPD loci of *Poa annua* 1357 samples based on (Manly, 1985).

herbicide susceptible populations. This calculated rates of N_m also mean the amount of gene flow required for the population to show similarity with other populations. These findings agree with that of Darmency and Gasquez (1983) whose results indicated that herbicide-susceptible populations were more diverse than resistant populations. Warwick and Black (1993) found that the total diversity in triazine resistant *Brassica rapa* populations ($H_T = 0.174$) was smaller than in susceptible populations ($H_T = 0.191$), the same trend as we saw. However, the gene diversity among resistant populations ($G_{ST} = 0.121$), and among susceptible populations ($G_{ST} = 0.037$) of *Brassica rapa* is in

disagreement with our results, where we found greater diversity among susceptible populations (G_{ST} = 0.125) than among resistant populations (G_{ST} = 0.016). The founding populations of the western Oregon *P. annua* must have been rich in diversity with selection pressure narrowing this diversity somewhat at our sites. The absence of the significant correlations between sample size or population number on most of the gene diversity parameters implies that the observed rates of diversity are real (Table 11). Hamrick and Godt (1989) reported the associations among genetic parameters where they found significant positive correlation of (h) with (H_S) and (H_T) and negative correlation with (G_{ST}). (H_T) was also reported to have a positive correlation with (H_S) and weakly with G_{ST} while (H_S) values were negatively correlated with (G_{ST}). Our results in most but not all agree with the above results. We found a negative correlation for (N_m) gene flow estimate with all parameters except sample size (Table 11).

Table 11. Correlation Coefficients above diagonal and significance level below diagonal for Nei gene diversity and genetic differentiation parameters based on 16 sites and 47 population of *Poa annua* of western Oregon grass seed crops. (Data from 18 RAPD loci POPGENE analysis output).

	G_{ST}	h	H_S	H_T	I	n_a	n_e	N_m	# of populat-ions	%poly-morphic loci	Sample size
G_{ST}		0.63	0.47	0.62	0.59	0.22	0.63	-0.85	0.32	0.22	-0.61
H	**		0.98	0.98	0.96	0.75	0.99	-0.68	0.46	0.75	-0.29
H_S	NS	***		0.98	0.94	0.79	0.97	-0.57	0.47	0.80	-0.17
H_T	*	***	***		0.96	0.75	0.99	-0.67	0.46	0.75	-0.28
I	*	***	***	***		0.74	0.95	-0.65	0.46	0.75	-0.28
n_a	NS	***	***	***	***		0.76	-0.35	0.40	0.99	0.28
n_e	**	***	***	***	***	***		-0.66	0.45	0.77	-0.27
N_m	***	**	*	**	**	NS	**		-0.44	-0.36	0.43
# of populations	NS	NS	NS	NS	NS	NS	NS	NS		0.40	0.05
%polymorphic loci	NS	***	***	***	***	***	***	***	NS		0.27
Sample size	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	

* = P < 0.05
** = P< 0.01
*** = P < 0.001
NS = No significant correlation

CHAPTER 3
A *psb*A MUTATION (219 VALINE TO ISOLEUCINE) CAUSES RESISTANCE TO METRIBUZIN AND DIURON IN ANNUAL BLUEGRASS (*Poa annua* L.)

ABSTRACT

Biotypes of *Poa annua* with a Val to Ile substitution mutation at amino acid residue 219 resulted in resistance to diuron and metribuzin in the field. The herbicide-binding region of a *psb*A gene DNA fragment from metribuzin and diuron resistant and susceptible biotypes of *P. annua* was selectively amplified using PCR. Sequence analysis of the herbicide-binding region of six resistant biotypes of *P. annua* exhibited a substitution of valine to isoleucine at position 219 of the D1 protein of the *psb*A gene. This is the same mutation as reported for *Chlamydomonas* and *Synechococcus* through site-selected or site-directed mutagenesis and in cell cultures of *Chenopodium rubrum.* To our knowledge no higher plant has ever been reported to exhibit resistance in the field to photosystem II inhibitors due to any *psb*A mutations other than position 264 serine to glycine exchange. The six *P. annua* biotypes exhibiting a valine to isoleucine change at position 219 did not show any change at position 264 or at other position within the herbicide binding regions. The presence of other biotypes of *P. annua* resistant to diuron or metribuzin that do not exhibit any mutation of the *psb*A gene indicates that resistance to diuron and metribuzin can apparently be attained by other mechanisms. The existence of at least two mechanisms of resistance to the same herbicides by populations of *P.*

annua of western Oregon grass seed crops poses serious challenges to the seed industry and will require development of well thought out management strategies.

INTRODUCTION

A number of chemically different classes of herbicides block the photosynthetic electron transport chain on the reducing side of photosystem II (PS II). These include the s-triazines, substituted ureas, and the phenolic derivatives (see, Trebst, 1980; Arntzen et al., 1982, for review). The PS II herbicides act by displacing the secondary plastoquinone Q_B from its binding site in photosystem II (Velthuys, 1981). The protein target of the PS II herbicides has been identified as D1 polypeptide subunit of PS II also called the herbicide or Q_B binding protein (Pfister et al., 1981). The D1 polypeptide is encoded by the chloroplast *psb*A gene (Zurawski et al., 1982), and is highly conserved in plants, algae and cyanobacteria (see, Erickson et al., 1985, for review). In higher plants the chloroplast *psb*A gene is unique, located in the large single copy region close to the left junction of the inverted repeat (Driesel et al., 1980).

The first report of herbicide resistance involved a triazine herbicide (Ryan, 1970), and since then biotypes of more than 60 species have been documented to possess triazine resistance (LeBaron, 1991). The 1995/96 International Survey of Herbicide Resistant Weeds revealed 183 herbicide resistant weed biotypes (124 different species) in 42 countries, and 32% of these were triazine resistant (Heap, 1997). According to the survey, another 18% of weed biotypes were resistant to ALS inhibitors, 15% to bypyridiliums, 9% to phenyl ureas/amides, 8% to synthetic auxins, 7% to ACCase

inhibitors, 3% to dinitroanilines, and the remaining 8% were resistant to other herbicides. Resistance to triazine herbicides has been attributed either to a rapid metabolic detoxification of the herbicide (Shimabukuro, 1968), or to a modified site of action that prevents herbicide binding within the chloroplasts (Arntzen et al., 1982). Susceptible as well as resistant biotypes of some weed species can rapidly metabolize atrazine via hydroxylation reactions in the plant's metabolism (Ali and Machado, 1984). However, triazine resistant weeds degrade atrazine at a much lower rate than corn (Gressel, 1985). An extensive survey on atrazine metabolism by 53 grass species revealed *Panicoideae* to be more tolerant to triazine as a result of their ability to detoxify it (Jensen et al., 1977). However, LeBaron (1991) reported the absence of clear relationships between plant families and genera in their tendency to evolve resistance, with resistance usually developing in one, or at most, a few species in a weed community exposed to a herbicide. Triazine and substituted urea resistant biotypes of *Lolium rigidum* biotypes due to enhanced rates of metabolism have been reported (Burnet et al., 1993). Burnet suggested that the resistance be due to increased activity of Cyt P450 monoxygenase enzymes that have the capacity to either de-alkylate or ring-hydroxylate these herbicides. Two enzymes implicated in the development of resistance to PS II herbicides due to enhanced detoxification are Cytochrome P450 (Cyt P450) and Gluthatione –S-Transferase (GST) (see, Gronwald, 1994, for review). The Cyt P450 enhanced detoxification mechanism is suggested to be polygenic and under nuclear control, with cross-resistance to various herbicides and no fitness reductions for resistant biotypes (Gronwald, 1994).

With a few exceptions, triazine resistance is due to target site resistance resulting from a modification at the herbicide target site, the D1 protein of PS II (see, Gronwald, 1994, for review). The substituted urea herbicides, although chemically distinct from triazines, are also potent PS II inhibitors (Gronwald, 1994) and bind at overlapping but not identical sites with the triazines (Trebst, 1991). Biotypes highly resistant to triazine herbicides as a result of a modified D1 protein fail to show resistance to substituted urea herbicides (Gronwald, 1994) because mutation Ser_{264} to Gly providing resistance to triazine herbicides does not affect binding of substituted urea herbicides (Arntzen et al., 1982;Trebst, 1991). However, triazine resistant plants have been reported to show resistance to other PS II inhibitors such as triazinones, uracils and pyridazinones (Oettmeier et al., 1982; Fuerst et al., 1986).

The *psb*A gene sequences from several organisms have been determined, and quite frequently resistance by photosynthetic organisms to PS II herbicides, like triazinones, triazines, and DCMU (diuron), results from mutations in the *psb*A gene, leading to an amino acid exchange in the Q_B binding protein. In higher plants a mutation affecting amino acid 264 (serine) leads to resistance against triazine herbicides and in algae mutations in position 215, 219, 255, 256, and 275, are known to confer resistance to several classes of herbicides (see, Schwenger-Erger et al., 1993, for review).

Double and triple mutants of *Chenopodium rubrum* cell cultures were characterized by Schwenger-Erger et al. (1993) where a valine to isoleucine change at position 219 was decisive for metribuzin resistance although there were some other

changes between amino acid residues 209 and 291. For example, the metribuzin resistant *psb*A mutant cell cultures possessed mutations either at 219 and 251 in most cases, or at 219, 251, and 256 or 219, 229, and 270, or at 219, 220, and 270 or at 219, 251, and 272. All these changes resulted in resistance to metribuzin. None of the double or the triple mutant cell cultures possessed the classical field mutation at residue 264 of the *psb*A gene. Most of the mutations in the D1 protein leading to herbicide resistance in cyanobacteria and green algae were identified by randomly induced mutagenesis or by site directed gene technology methods for the *psb*A gene (see, Kless, 1994, for review).

A detailed model of the herbicide-binding region in PS II has been developed (Tietjen et al., 1991). Based on this model, 17 amino acids of the *psb*A gene are in contact with the Q_B binding site. These include: Phe_{211}, Met_{214}, His_{215}, Leu_{218}, Val_{219}, Thr_{237}, Ile_{248}, Ala_{251}, His_{252}, Phe_{255}, Gly_{256}, Ala_{263}, Ser_{264}, Phe_{265}, Asn_{266}, Ser_{268} and Leu_{275}. The eight site mutations including Val_{219} that have been reported so far to cause resistance to herbicides all involve residues that have contact with Q_B. Tietjen et al. (1991) further characterized the relative importance of each residue in herbicide binding. For example, mutation Ser_{264} had greatest impact on binding of most PS II inhibiting herbicides. Val_{219} and Leu_{275} were characterized as being located peripherally to the Q_B binding site, as compared to the other amino acids found nearer the center. Mutations to the more bulky amino acids Ile_{219} or Phe_{275} will only touch those inhibitors extending into this peripheral space. As a result, the Val_{219} to Ile mutant showed R/S I_{50} ratio of 20, 2, 2, and 3 for metribuzin, atrazine, s-triazine and s-thiazole, respectively (Tietjen et al., 1991).

High levels of resistance to atrazine have been correlated with a single amino acid substitution (Ser to Gly) in higher plants at position 264 in the chloroplast thylakoid membrane protein (Goloübinoff et al., 1984; Hirschberg and McIntosh, 1983; Hirschberg et al., 1984). A change of Ser to Ala at the same position in this protein is correlated with lower levels of resistance to atrazine and diuron in algal mutants (Erickson et al., 1984) and a cyanobacterial transformant (Golden and Heselkorn, 1985). In higher plants spontaneous herbicide resistance has been found in the field in various weed plants (Hirschberg and McIntosh, 1983; Van Oorschot, 1991; Rubin, 1991). In all reported cases it is only a Ser_{264} to Gly change that is responsible for the acquired resistance (Trebst, 1996). This mutation is also reported for dicot species such as *Amaranthus hybridus* (Hirschberg and McIntosh, 1983; McNally et al., 1987), *Solanum nigrum* (Goloübinoff et al., 1984), *Chenopodium album* (Gasquez et al., 1985; Bettini et al., 1986) and *Senecio vulgaris* (Trebst, 1996). The only monocot species with a *psb*A gene mutation at residue 264 showing resistance to atrazine is annual bluegrass *Poa annua* (Barros and Dyer, 1988). This mutation in higher plants is accompanied by a slowing down of electron transfer between the primary QA and secondary QB quinones of PS II reaction centers (Arntzen et al., 1979; Bowes et al., 1980; Ort et al., 1983) leading to an increased susceptibility to photoinhibition in the resistant biotypes (Sundby et al., 1993). The triazine resistant mutants display a marked decrease in growth rate at normal light intensities (Hart and Stemler, 1990) and a reduction in competitive fitness (see, Holt and Thill, 1994, for review). However, mutations of the D1 at positions 219 and 275 have been demonstrated to have no role in electron transfer system (Erickson et al., 1985). Erickson et al. (1985) studied the molecular basis of resistance to atrazine and cross-

resistance to diuron by using uniparental mutants of *Chlamydomonas reinhardi* resistant to diuron (strain Dr2) and to atrazine (strain Ar207) that possessed Val $_{219}$ to Ile and Phe$_{255}$ to Tyr respectively. The Dr2 showed a 2-fold resistance to atrazine and a 15-fold resistance to diuron while Ar207 a 15-fold resistance to atrazine and a 0.5- fold resistance (2-fold increased susceptibility) to diuron. The electron transport for these two mutants was normal as opposed to Ser$_{264}$ to Ala or Ser$_{264}$ to Gly mutations that alter the electron transport system (Erickson et al., 1985). An extended study on the mutant strain (Dr-18) of *C. reinhardi* by Lien et al. (1977) revealed that the mutant required 10-fold higher concentration of DCMU and CMU to show the same degree of inhibition as the wild type. This mutant was later sequenced, and determined to consist of a Val$_{219}$ to Ile exchange like the Dr2 in the *psb*A gene (Erickson et al., 1985).

The resistance to PS II inhibitors due to alteration of the *psb*A gene is maternally inherited. However, hybrids with resistance from paternal plastids were found at a frequency of 0.2 to 2% (Darmency, 1994). In *P. annua*, two maternally inherited resistant biotypes with similar whole plant response to atrazine but with differences in chlorophyll fluorescence transients and atrazine inhibition of chloroplast activities have been detected (Gasquez and Darmency, 1983). They characterized this new kind of triazine-resistance and named it M, which was maternally inherited, and equally resistant to their R type in seedling treatments and in ID$_{50}$ in chloroplast analysis. The M plants have longer fluorescence transient than R and S plants and have also a slightly displaced slope of atrazine inhibition compared to R chloroplasts. This biotype was not confirmed by sequence analysis but rather was distinguished by means of chlorophyll fluorescence

transient of whole leaves and by the slope of triazine inhibition curves of isolated chloroplasts.

Three levels of resistance to atrazine have also been found in *Chenopodium album*: low (S), intermediate (I), and highly resistant (R), where the I genotypes display a 10-fold increase of resistance at the whole plant level (Gasquez et al., 1985). These characters were inherited maternally. The I and R biotype sequences of their *psb*A gene possessed the same substitution of Ser to Gly at residue 264 (Bettini et al., 1987).

Poa annua is one of the worst weed problems confronting grass seed growers in the Willamette Valley. Its presence in harvested seeds complicates seed cleaning and reduces crop value if seed can not be cleaned to *Poa*-free status (Mueller-Warrant, 1996). Over 20 populations of *P. annua* in grass fields of Oregon has been shown to be ethofumesate-resistant, triazine-resistant and urea herbicides resistant (Heap, 1997). Several of the *P. annua* populations have shown multiple resistance to atrazine, diuron, terbacil, and ethofumesate (Heap, 1995; Gamroth et al., 1996; Mueller-Warrant, 1996) with greenhouse experiments indicating a 10-fold increase in resistance to both atrazine and diuron (Heap, 1995). Managing herbicide resistant weeds requires a good understanding of the genetics of resistance. Understanding the mechanisms of resistance involved in these populations of *P. annua* for various herbicides is vital. Wise management decisions require full understanding of the mechanisms of resistance, the gene or genes involved, inheritance of resistance, gene flow, as well as the details of the biology of the species. This study therefore was designed to fulfil the objective of

understanding the mechanism of resistance involved in populations of *P. annua* resistance to diuron and metribuzin in western Oregon grass seed crops.

MATERIALS AND METHODS

Seeds for these experiments were obtained from our greenhouse grown *P. annua* plants collected in 1994/5 from grass seed fields of western Oregon for the genetic diversity studies.

Greenhouse experiments

In the greenhouse experiments, we tested the tolerance to field rates of diuron and metribuzin using 225 accessions of *P. annua*. The 225 accessions of seeds from single plants were planted in rows using flats in the greenhouse. Every flat consisted of ten rows and each row was a single accession of *P. annua*. Eighty accessions out of 225 were also tested for their reaction to metribuzin in addition to diuron.

Every accession's seedlings were counted after emergence and recorded before any herbicide treatment. On May 30, 1997, seedlings in the 2-leaf stage were treated with diuron at 1.1 kg a.i. /ha for 225 accessions and metribuzin at 0.37 kg a.i./ha for 80 accessions. Two flats from the metribuzin treatments were divided into two halves, and one half was covered with plastic sheets while spraying the herbicide and used as untreated control accessions for reference. Other untreated checks from earlier plantings

were also grown in the greenhouse for comparisons. The treated seedlings were kept in the greenhouse at 12h supplemental light, 21 C day, 15 C night and watered daily.

Seedlings were counted again at two weeks and at three weeks after herbicide treatment and the progeny classes and accession classes were determined based on their reaction to the herbicides, as (R) resistant, (I) intermediate, and as (S) susceptible. Progeny were scored as resistant if uninjured by herbicide, intermediate if injured but alive, and susceptible if killed. Accessions were scored as resistant if any progeny were resistant, susceptible if all progeny were killed, and otherwise intermediate. After identifying the response of *P. annua* accessions to diuron and metribuzin, the mechanism of resistance was studied through DNA sequence analysis of the *psb*A gene.

DNA extraction

We extracted the total DNA using a modified procedure of the ROSE (Steiner et al., 1995) buffer [10mM Tris HCL (pH 8), 312 mM EDTA (pH 8), 1% lauryl sarkocyl, 1% PVPP per 50 ml final volume, and 0.2% β-mercaptoethanol]. Three to five leaves were harvested per plant, cut in to 1-cm pieces, freeze-dried in sterile microfuge tubes. Adding 4 to 6 sterile glass beads to each tube and shaking at a high speed 40 to 50 min ground the desiccated samples. The ROSE buffer (500 µl) was added to each ground sample and then incubated for 20 min. at 90 C with constant gentle shaking to mix. The samples were kept on ice to cool and then centrifuged (12000 rpm) for 5 min. to pellet plant cell debris.

The following steps were then performed to further clean DNA samples and to quantify the DNA yield. The supernatant was placed in clean tubes, 2/3 volume (330 µl) cold isopropanol was added, each sample was then well mixed and held at –20 C for 10 min. The samples were then centrifuged for 10 min. at 12000 rpm and pellets were carefully saved in the tube while we decanted the supernatant. The pellets were washed with 300 µl 80% ETOH (ethyl alcohol) by spinning for 5 min., then air-dried. The dried pellets were resuspended in 400 µl TE (Tris base +EDTA pH 8). We added 8 µl of 0.5 mg/ml of RNASE (final concentration = 10 µg/ml) to the samples and incubated for 30 min. at 37 C. The DNA was precipitated by rocking the tubes after adding 200 µl of 7.5 M ammonium acetate and 1.2 ml of absolute ETOH, and then held for 10 min. at –20 C. Samples were finally spun for 10 minutes, decanted, washed, and air-dried. The pellets were then resuspended in 80 µl of TE and kept in storage at –20 C until needed for amplification. DNA was quantified for every sample using a fluorometer, and the DNA samples were diluted to final concentrations of 10-ng/µl for the PCR use.

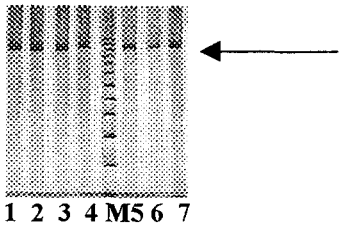
PCR conditions and DNA amplification

Two primers were designed and synthesized based on known sequences of the chloroplast *psb*A gene sequence from various species. These primers should amplify a 932 base pair fragment of the chloroplast *psb*A gene located in herbicide binding region of any plant species because it is highly conserved across many taxa. The primer sequence 5' GGA TGG TTT GGT GTT TTG 3' with 18 bases length correspond to the *psb*A 5' region of RNA-like sequence of the *psb*A gene from 91st base to108th base of the

total *psb*A gene. The second primer 5' TAG AGG GAA GTT GTG AGC 3' also with 18 bases correspond to the 3' region of the DNA sequence from 1006th to 1023rd base of RNA-like sequence of 5' GCT CAC AAC TTC CCT CTA 3'. Both primers were used to amplify the target region of our interest (932 base pair fragment) from total DNA using the PCR (Figure 8). The two primers were also used for sequencing the fragment but used as separate reactions and aligned during analysis. Another primer was synthesized based on the sequence we obtained from *P. annua* itself and this primer was used for only sequencing the fragment. The sequence of the third primer with 20 bases length was (5' CTC CTG TTG CAG CTG CTA CT 3') and it starts from the region of residue 150, and corresponds to bases 446 to 465 of the *psb*A sequence. This primer gives a clear sequence of the herbicide-binding region. PCR was performed in 100 µl reaction volume under conditions optimized to amplify the specific 932 base pair fragment of DNA of the chloroplast *psb*A gene sequence involved in herbicide binding. Using micropipettes 90 µl of reaction mix [1x Stoffel buffer, 3.75 mM $MgCl_2$, 50 µM of each dNTP, 1.0 pM of each primer, and 1 unit Taq Stoffel fragment] was first aliquotted in to a 96-well assay plate and then overlaid with 3 drops of mineral oil. Finally, 10 µl of 10ng/µl diluted DNA sample was added to the reaction mixture under the oil. The thermal cycling program started with denaturation for 7 min. at 94 C followed by 43 cycles of 30-sec. denaturation at 47 C, a 1 C per 3-sec. ramp, and extension at 72 C for 2 min. A final extension at 72 C for 5 min. concluded the DNA amplification. After amplification the samples were kept at 4 C for a short period (<24 h) or at –20 C for longer periods until electrophoresis.

Electrophoresis, DNA extraction from gels and purification

To determine whether the target sequence was amplified by the PCR we ran five µl of the 100 µl PCR product from every sample in TBE agarose gel electrophoresis. The remaining 95 µl amplified product was then run in TBE agarose gel electrophoresis using wider and two adjacent wells for every sample consisting each 47.5 µl of the PCR product at 180 volts for two hours. We added markers for every gel to give us the exact estimate of the 932 base fragment although in our case this is the only amplified product we found under these conditions (Figure 8).



The single band PCR product (932 base pairs)

Figure 8. A polyacrylamide gel electrophoresis indicating the amplified 932 base pair fragment DNA of the herbicide-binding region of the *psb*A gene from *Poa annua* accessions. The two primers used to amplify this DNA fragment were 5' GGA TGG TTT GGT GTT TTG 3' and 5' TAG AGG GAA GTT GTG AGC 3'. The PCR reaction conditions are explained in the text. Numbers (1- 7) samples identity used to amplify the product and M stands for the marker used to estimate the fragment sizes amplified.

Following the QIAquick gel extraction protocol and kit (buffers, tubes, filters), the DNA was extracted from the 932 base fragment and purified for the sequencing use. The purified product for each sample was kept in tubes labeled and submitted to the Gene Center of the Oregon State University laboratory for automatic sequencing. The primers

were submitted for sequencing, and in most cases only 2.5 to 3.5 µl of the purified samples (as determined by running only 5 µl in TBE agarose gel) were used to give clean sequences. Multiple sequences were made for most accessions to get a consensus sequence of the target region of the gene. Analysis of the sequences were made first by editing multiple sequences, and once we got consensus sequences, homology tests were made with known *psb*A sequences from various sources on the Internet and other published sources. Translation was made using Expasy, an Internet translation tool.

RESULTS

Poa annua accessions' response to diuron and metribuzin in greenhouse

Large number of *P. annua* seedlings were susceptible to diuron and metribuzin, as we killed between 46 and 94% of those treated with diuron, and 75 to 96% treated with metribuzin (Table 12). However, a large percentage of the accessions contained at least one individual (out of approximately 50 tested) highly resistant to diuron. Some individual accessions were relatively uniform in their response to diuron, while others possessed resistant, intermediate, and susceptible types, suggesting that many of these populations were still segregating for resistance to diuron. In general, these accessions were more sensitive to metribuzin than to diuron. However, three accessions from Bowers farm possessed individuals highly resistant to metribuzin by the second rating, and several accessions at two other sites were intermediate in their response to metribuzin. Only one accession (#145) out of 80 tested was resistant to both diuron and metribuzin. Its reaction seemed to be maternally inherited because all the progeny from

Table 12. Progeny testing herbicide tolerance classification

Accessions sources and type of herbicide used	Accessions tested	Progeny class[1] (% of progeny)			Accession class[2] (% of accessions)		
Test for diuron tolerance	(Number)	R	I	S	R	I	S
Bowers per. rye.	76	1.4	4.9	93.7	19.1	6.4	74.5
McLagan Pugh Rd.	33	6.4	35.6	57.9	66.7	15.2	18.2
McLagan Belle Plain	28	28.7	25.0	46.2	82.1	10.7	7.1
Glaser per. rye.	50	16.6	33.9	49.5	66.0	22.6	11.3
Glaser meadowfoam	33	6.8	28.2	65.0	51.5	12.1	36.4
George Pugh, per. rye.	1	5.1	41.0	53.8	100.0	0.0	0.0
Hyslop	4	1.9	14.7	83.3	25.0	25.0	50.0
Test for metribuzin tolerance							
Bowers per. rye.	28	4.4	2.6	93.0	10.7	3.6	85.7
McLagan Pugh Rd.	16	3.7	5.0	91.3	0.0	0.0	100.0
McLagan Belle Plain	10	15.8	8.7	75.5	0.0	30.0	70.0
Glaser per. rye.	5	0.9	3.1	96.0	0.0	40.0	60.0
Glaser meadowfoam	21	0.1	5.4	94.5	0.0	4.8	95.2

[1]Progeny were scored as resistant if uninjured by herbicide, intermediate if injured but alive, and susceptible if killed.
[2]Accessions were scored as resistant if any progeny were resistant, susceptible if all progeny were killed, and otherwise intermediate.
Data shown are from first rating for progeny class (2 weeks after treatment) and second rating for accession classes (3 weeks after treatment).

single plant parent showed uniform reaction to the herbicides. Since 225 of the accessions were tested for diuron but only 80 out of the 225 were also tested for metribuzin, we expect some of the diuron resistant accessions were also resistant to metribuzin. The uniformity of their response to diuron across the rows of the progenies was similar to that of accession #145 indicating that these other accessions were similar to accession # 145.

Sequence analysis

As displayed on Figure 9, six accessions including accession #145 out of the 20 accessions of *P. annua* that were sequenced exhibited a Val_{219} to Ile substitution in the *psb*A gene. Other accessions resistant to diuron or metribuzin but not exhibiting this same mutation were also found. The susceptible accession to diuron and metribuzin did not exhibit this mutation. None of the six mutants show the classical mutation of Ser_{264} to Gly, but rather remain wild type at that position (Fig 10). The six accessions that showed a mutation of Val_{219} to Ile came from two field sites: one accession from Glaser perennial ryegrass field and the remaining five from McLagan Belle Plain road perennial ryegrass fields. These fields are well known sites for herbicide resistant *P. annua*. We determined the existence of reduced rates of among population genetic variation resulting from herbicide selection pressure in those fields (see chapter 2).

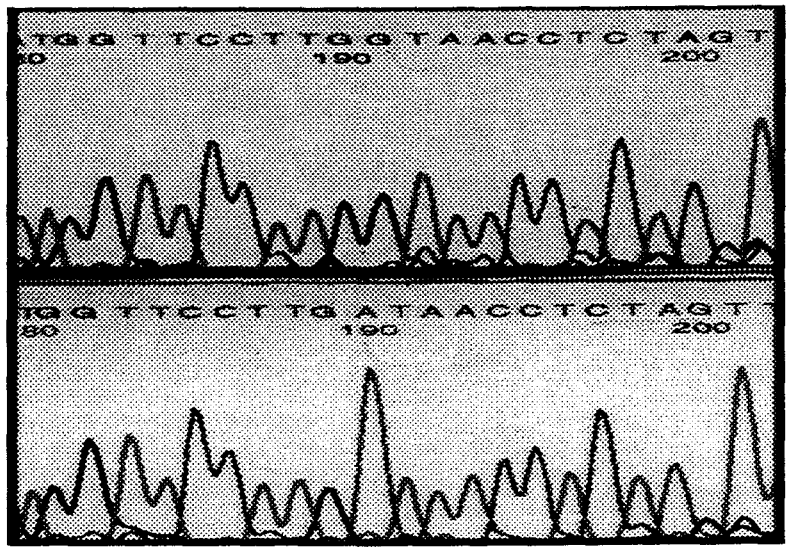


Figure 9. Portion of the *psb*A gene sequences from the herbicide-binding region of the wild type and mutant *Poa annua*. A point mutation at amino acid residue 219 (GTA to ATA) results in a change from Val to Ile in the metribuzin and diuron resistant accessions of *Poa annua*. The numbers of bases indicated on the figure do not correspond to the amino acid positions but to the position of bases for sequencing started by the primer used (5' CTC CTG TTG CAG CTG CTA CT 3').

atc tct ggt gct att att cct act tcg gcg gcg atc gga ttg cac ttt tac cca att tgg
I S G A I I P T S A A I G L H F Y P I W 97
gaa gct gca tct gtt gat gag tgg tta tac aat ggt ggt cct tat gag cta att gtt cta
E A A S V D E W L Y N G G P Y E L I V L 117
cac ttc tta ctt ggt gta gct tgt tat atg ggt cgt gag tgg gaa ctt agt ttc cgt ttg
H F L L G V A C Y M G R E W E L S F R L 137
ggt atg cgt cct tgg att gct gtt gca tac tca gct cct gtt gca gct gct act gct gtt
G M R P W I A V A Y S A P V A A A T A V 157
ttc ttg att tac cct att ggt caa gga agc ttc tct gat ggt atg cct tta gga ata tct
F L I Y P I G Q G S F S D G M P L G I S 177
ggt act ttc aac ttt atg att gta ttc cag gca gag cac aac atc ctt atg cat cca ttc
G T F N F M I V F Q A E H N I L M H P F 197
cac atg tta ggt gta gct ggt gta ttc ggc ggc tcc cta ttt agt gct atg cat ggt tcc
H M L G V A G V F G G S L F S A M H G S 217
ttg gta acc tct agt ttg atc agg gaa act act gaa aat gaa tct gct aat gag ggt tac
L V T S S L I R E T T E N E S A N E G Y 237
aaa ttt ggt caa gag gaa gaa act tat aat att gtg gct gct cat ggt tat ttt ggc cga
K F G Q E E E T Y N I V A A H G Y F G R 257
tta atc ttc caa tat gct agt ttc aac aac tct cgt tct tta cac ttc ttc ttg gct gct
L I F Q Y A S F N N S R S L H F F L A A 277
tgg cct gta gta ggg atc tgg ttc act gct tta ggt att agt act atg gct ttc aac cta
W P V V G I W F T A L G I S T M A F N L 297
aat ggt ttc aat ttc aac caa tct gta gtt gat agt caa ggt cgc gtt att aat act tgg
N G F N F N Q S V V D S Q G R V I N T W 317
gct gat atc atc aac cgt gct aat ctt ggt atg gaa gta atg cac gaa cgt aat gct cac
A D I I N R A N L G M E V M H E R N A H 337
aac ttc cct
N F P 340

Figure 10. The nucleotide sequence of the metribuzin and diuron susceptible P*oa annua psb*A herbicide binding region including the deduced amino acids at their specific residues. The metribuzin and diuron resistant mutant *psb*A sequence of *Poa annua* accessions possessed a change of Val_{219} to Ile at the underlined amino acid residue and nucleotide position as a result of a change from GTA to ATA. The numbers refer to the residue number of the amino acids of the entire *psb*A gene. The underlined bases correspond to the third primer we used for sequencing the fragment with the sequence (5'CTCCTGTTGCAGCTGCTACT 3'). Although we obtain larger sequence using the three primers we present here only the region that resulted in consistent sequences for most of the accessions, covering from amino acid residue 78 to amino acid residue 340 of the entire chloroplast *psb*A gene. Single letter amino acid code: A: alanine, C: cystiene, D: aspartate, E: glutamate, F: phenylalanine, G: glycine, H: histidine, I: isoleucine, K: lysine, L: leucine, M: methionine, N: asparagine, P: proline, Q: glutamine, R: argenyl, S: serine, T: threonine, V: valine, Y: tyrosine, W: tryptophan.

DISCUSSION

The summary of amino acid changes in herbicide tolerant mutants of higher plants or algae adapted from Trebst (1991, 1996) and including our results is presented in (Table 13). The mutation of the *psb*A gene at residue 219 from Val to Ile has been reported to result in resistance to metribuzin, DCMU (diuron), and ioxynil in *Chlamydomonas* and *Synechococcus* (for review, see Trebst, 1991 and 1996,). The same mutation in cell cultures of *Chenopodium* was reported to show resistance to metribuzin (Schwenger-Erger et al., 1993). We do not yet know whether the Val_{219} to Ile mutant accessions also possessed additional resistance from nuclear controlled mechanism of resistance present in other accessions. Selection in the field may have played a role in preserving combinations of resistant mechanisms in the same individuals.

Failure to find *psb*A mutants of higher plants in the field other than Ser_{264} to Gly has puzzled researchers for a long time. Our sequence analysis on diuron resistant and metribuzin resistant *P. annua* accessions revealed that there are biotypes of higher plants with site mutations other than residue 264, and these biotypes resist PS II inhibitors at field rates. Besides annual bluegrass' short life cycle and significant amount of outcrossing, herbicide rates in western Oregon grass seed crops must have been ideal for individuals to evolve and been selected. Grass seed growers have applied 1 to 1.5 kg/ha atrazine, 2.2 kg/ha simazine and 1.1 to 2.2 kg/ha diuron for approximately 30 years. These rates are limited by the tolerance of crops such as perennial ryegrass to these herbicides.

Table 13. Amino acid changes in herbicide tolerant mutants of higher plants or algae[1]

Amino acid change	Resistant to	Organism
Single Replacement		
Phe_{211} to Ser	Atrazine/DCMU	
Val_{219} to Ile	Metribuzin/DCMU/ioxynil	*Chlamydomonas,*
		Synechococcus
**Val_{219} to Ile	Metribuzin/diuron	*Poa annua* (currently added)
Thr_{220} to Ala		*Chenopodium* cell cultures
Ala_{251} to Val	Metribuzin	*Chlamydomonas*
Phe_{255} to Tyr		*Chlamydomonas*
Gly_{256} to Asp	Atrazine/DCMU/bromacil	*Chlamydomonas*
Ser_{264} to Gly	Atrazine	*Amaranthus, Senecio* etc
Ser_{264} to Ala	Metribuzin/atrazine	*Anacystis, Chlamydomonas*
	DCMU/ bromacil	*Euglena, Synechocystis*
	Atrazine/DCMU	*Synechococcus*
Ser_{264} to Thr	Triazine	*Nicotiana* cell culture, *Euglena*
Ser_{264} to Asn	Triazine	*Nicotiana* cell culture
Asn_{266} to Thr	Ioxynil	*Synechocystis*
Leu_{275} to Phe	Metribuzin/bromacil /DCMU	*Chlamydomonas*
Double Mutations		
Val_{219} to Ile + Ala_{251} to Thr		*Chenopodium* cell cultures
Val_{219} to Ile + Glu_{229} to Gly + Ser_{270} to Phe		*Chenopodium* cell cultures
Val_{219} to Ile + Ala_{251} to Thr + His_{272} to Arg		*Chenopodium* cell cultures
Deletions		
$Ser_{221/222}$		Synochocystis
$YKF_{237-239}$		*Synochocystis*
$GQ_{240-241}$		*Synochocystis*

[1]Adapted from Trebst (1991, and 1996)

** The change of Val_{219} Ile for *Poa annua* of our current report is included on this table.

The number of mutant accessions within the sequenced accessions is far higher than expected by mutation rates of resistance evolutions per generation, 10^{-5} in most cases. For example, out of 20 accessions of *P. annua* we have sequenced for the *psb*A gene, 3 were known susceptible to metribuzin and diuron, 14 were resistant to diuron but reaction to metribuzin unknown, and one was resistant to both metribuzin and diuron. Of the 14 diuron resistant biotypes of *P. annua* five exhibited a Val_{219} to Ile substitution in their *psb*A gene. The remaining 9 diuron resistant biotypes and the 2 metribuzin resistant biotypes sequences remain wild. The metribuzin and diuron resistant accession possessed the mutation at the position we have indicated. We expect that more mutants would have been found from diuron resistant accessions within the population of the *P. annua* had we sequenced all the resistant accessions. We conclude that the rate and pattern of herbicide use by western Oregon grass seed growers must have provided suitable selection pressure to select these mutants. Higher rates might have killed the mutants, while lower rates might have allowed the susceptible types to survive.

The existence of both maternally inherited site mutation single gene herbicide resistance and nuclear controlled resistance for the same herbicides within the populations of *P. annua* in western Oregon grass seed crops will complicate the problem of addressing herbicide resistance. Gressel et al. (1996) emphasized that "the tendency to cut dose rate is increasing resistance due to multiple-cumulative events (polygenic, amplification, or sequential mutations within a gene)". The dilemma here is whether rotation or rate of herbicide use should target the major gene or minor genes with cumulative effects, since both types exist in the same populations.

The mutation of the herbicide binding D1 protein at residue 264 has been characterized to cause inhibition of electron transport system leading the mutant to be less fit than the susceptible biotypes especially under no herbicide use. However, the Val_{219} to Ile we report now has been characterized to cause resistance to herbicides but no effect on electron transfer. Because the mutant may be equally fit as the wild types, use of no herbicide or low selection pressure of herbicide may fail to reduce the numbers of this kind of mutant from the populations. One other aspect of the mutation of Val_{219} to Ile is its capability of resisting more than one kind of herbicide as it is reported to result in resistance to metribuzin, atrazine, diuron and ioxynil in various organisms. Unlike the Ser_{264} to Gly change in other weed species that exhibit a negative cross resistance to diuron, the absence of this in Val_{219} to Ile mutants is a problem to growers, as it eliminates several alternate herbicides.

The degree of resistance by site mutation of Val_{219} to Ile has been reported to range from 10 to 20 fold resistance, which is close to the level of resistance found via detoxification of herbicides by enzyme action through polygenic effects. This would be an advantage if high doses of herbicides can be used to delay herbicide resistance due to minor genes with cumulative effects and mutants of the kind we found. However, the grass seed growers of western Oregon usually grow perennial ryegrass or tall fescue that would be susceptible to herbicides themselves should the rate used be increased from those used right now. One option may be to rotate crops like orchardgrass that better tolerate those herbicides, and use higher doses of herbicides to eliminate resistant weeds from their fields. Alternate crops like meadowfoam or clovers are another option,

allowing use of herbicides with different sites of action. Otherwise the increase in populations of *P. annua* biotypes with multiple herbicide resistance in western Oregon grass seed crops may affect seed producers and companies, leading to shifts in production, increased seed cleaning costs, and decreased profitability.

We believe that more populations of the *P. annua* must be studied to determine the level and type of resistance in the specific fields. Monitoring is needed to avoid further contamination of noninfected fields. The procedure we presented here could be further modified and simplified for quick and inexpensively test a large number of samples for specific mutations, such as Val_{219} to Ile.

CHAPTER 4
CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS

The major findings of this thesis are the following:

Poa annua found in western Oregon grass seed crops has high total genetic diversity and a greater proportion of the diversity is found within populations than between populations.

The diversity among *P. annua* populations (G_{ST} = 0.146) is generally lower than expected for self-fertilized species. The diversity among herbicide-susceptible populations is greater than among herbicide-resistant populations.

The gene diversity statistics and differentiation parameters indicate that populations of *P. annua* collected from fields with high herbicide selection pressure possessed less diversity than from low herbicide selection pressure fields. We conclude that selection is shaping the genetic diversity and differentiation of *P. annua* populations in western Oregon grass seed crops.

The *P. annua* genetic diversity is partially associated with time of germination as well as herbicide history sites. The association of time of germination on population

diversity was greater in dominantly herbicide susceptible populations than resistant populations. However, the site to site or herbicide history variation associated with the diversity is greater than germination time effect in fields and populations of *P. annua* that are predominantly resistant to herbicides.

In addition to increased proportion of the within population diversity (Hs), and reduced level of diversity among groups, we have observed that progenies derived from single plant seeds showed high segregation for herbicide resistance. We conclude that the gene flow within and among populations of *P. annua* of the western Oregon is substantial.

High gene flow rates coupled with a long-term supply of genotypes from the seed bank of *P. annua* must have been the main factors involved in maintaining the diversity of most populations despite the continuous use over many years of herbicides in these fields.

Resistance to diuron and metribuzin in *P. annua* is caused by site mutation at residue 219 a valine to isoleucine change of the D1 protein of the *psb*A gene.

Resistance to diuron or metribuzin in *P. annua* genotypes can also be due to other features such as enhanced metabolism.

This first reported case of field resistance to herbicides resulted from which site mutation of the D1 protein at residue 219 in *P. annua* confirms that higher plants also do possess mutations other than the serine to glycine change at residue 264, like in resistant mutants of *Cyanobacteria* and other lower plants that resist PS II inhibitors.

The substitution of a valine 219 to isoleucine has been characterized in other organisms to confer resistance up to 10 to 20 fold higher rates of herbicides than the susceptible wild biotypes. This character is almost the same to most herbicide resistance mechanisms due to metabolism. The selection pressure or herbicide use rates of western Oregon grass seed crops must have been ideal to select such mutants and also metabolically resistant biotypes of *P. annua* so that both mechanisms may be found in the same plant.

FUTURE DIRECTIONS

It is reasonable to assume that the existing amount of diversity in *P. annua* is a potential threat for rapid evolution of resistance to those herbicides commonly used and to new herbicides that may be used in the future. It also means that no single measure is adequate to control weeds with such diversity, but that various methods will have to be integrated. It is therefore necessary to bring back well known, basic principles of weed control practices into the monocultured grass seed production of western Oregon. Weed research and agronomic experiments should incorporate rotation of crops coupled with

tillage practices and renewing the existing stand of perennial crops with modified spacing that best fit the objective of suppression of weeds.

The distribution and proportions of *psb*A mutants and metabolism based resistant biotypes must be monitored within individual grass seed grower's farms. This would help to avoid the flow of genes from highly infested fields to other sites. Seed movement is the principal method of dispersal of chloroplast mutations, while pollen is the major carrier for nuclear controlled resistance. Further study must be initiated to confirm the estimated high outcrossing rates in *P. annua* populations of the western Oregon grass seed crops.

Fitness of the newly detected resistant mutants, biotypes resistant through other mechanisms, and susceptible biotypes must be studied under use of herbicides and in their absence. The new mutants we found in *P. annua* have never been characterized in higher plants under field conditions regarding their fitness. It is well known however, that a valine to isoleucine change at residue 219 in lower organisms does not result in a reduction of electron transport in photosystem II in the absence of herbicides. This may make those biotypes to be as fit as the susceptible ones. Unless the fitness of resistant biotypes is lower than that of susceptible biotypes in absence of herbicides, the usefulness of rotation of herbicides to delay the evolution of resistance is questionable. Therefore, the study of fitness is critical for the better understanding of the benefit of rotation of herbicides.

The selection pressure seems to favor both the selection for polygenes with small effects and a mutant of the sort reported here since we found both in the same fields. It is possible that some resistant genotypes may have both characters. We do not yet know whether the mutants also have a nuclear controlled resistance via metabolism. We need to investigate whether certain genotypes with both traits of resistance might even be the most difficult to control even at increased rate of herbicides.

The seed bank dynamics of *P. annua* of the western Oregon grass seed crops must be studied well because it supplies genotypes to existing populations and both stores and restores resistant populations over time.

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