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Changes in *Poa annua* Populations in Response to Herbicides and Plant Growth Regulators

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Poa annua (annual bluegrass; ABG) is an invasive weedy species in turfgrass. Herbicides and plant growth regulators (PGRs) are often used for ABG control, providing limited or inconsistent results. Identifying shifts in ABG populations in response to these treatments would be beneficial for understanding inconsistent control. Our research employed amplified fragment length polymorphic (AFLP) markers with the objective to determine if there are changes in genetic structure of ABG populations after multiple-year season-long control programs in three states. Annual bluegrass was sampled after the second or third year of seven different season-long ABG treatments consisting of herbicide or PGR applications. The trials were conducted at three different locations (East Lansing, Michigan; West Lafayette, Indiana; Lincoln, Nebraska). In the ABG samples, AFLP markers were identified for each site and 649, 745, and 762 were produced for Michigan, Indiana and Nebraska, respectively. Population analysis was conducted in Structure and identified five distinct ABG populations in Michigan, seven in Indiana, and six in Nebraska. Season-long treatments of trinexapac-ethyl or bispyribac-sodium (bispyribac) at a low rate effected genetic structure of populations at all locations. However, ABG populations that were affected by an individual herbicide or PGR did not respond consistently among locations. Bispyribac treatments increased ABG population variability in Michigan, but decreased variability in Indiana and Nebraska. Trinexapac-ethyl treatments decreased ABG population variability in Michigan and Indiana, but increased variability in Nebraska. This study provides a

genetic basis in understanding how herbicides or PGRs impact ABG populations over the long term and our results may help explain inconsistencies in chemical control of ABG.

For the endless grace God has given by himself and through others

When your learning how to ride a bike, it doesn't matter if you fall, what matters is how long it takes you to get back up and ride again.

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ASSISTANTSHIP

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Chapter one

Origin, History and Distribution

Annual bluegrass (*Poa annua* L.) is an allotetroploid, originating from two diploid *Poa* species, *P. supina* and *P. infirma* (Darmency and Gasquez, 1997). *Poa supina* is a stoloniferous perennial that is native to the mountainous regions of central Europe and *P. infirma* is a bunch type annual that is found on the Mediterranean coast in Western Europe (Huff, 2008). Annual bluegrass (ABG) originated in the Mediterranean region of Europe and was first characterized by Carl Linneaus (Tutin, 1957). This species is adaptive and aggressive, growing in a range of environments including arable land, disturbed sites, arctic snowbeds, grasslands, and lawns (Heide, 2001). Annual bluegrass has been identified from the Arctic Circle to the arid regions of Africa and throughout all the continents of the world (Frenot et al., 1999), making ABG one of the five most distributed plants on Earth (Fenner, 1985).

Annual bluegrass is the most written-about weed affecting golf turf and has been since the 1920's (Zontec, 1987). Piper and Oakley (1921) discussed ABG throughout the 1920's, concluding that its control would be timely and costly. These ideas were based on Piper and Oakley's observations of ABG's ability to adapt to multiple climates and reproduce in large quantities. In North America ABG is a major component of irrigated, close cut sports turf north of the transition zone and is the primary grass of some of the most celebrated golf courses around the world (Huff, 1999).

Characteristics and Biology

The ability of ABG to flourish around the world is, in part, attributed to its genetic and physiological variability. Annual bluegrass life cycles range from annuals (*Poa annua* v. *annua*) to perennials (*Poa annua* v. *reptans*) with potentially many of these types in an individual stand

of turf. Therefore, ABG stands may consist of individuals with diverse lifecycles and also have tremendous variability in color, leaf texture, seed production, and density. Annual and perennial varieties reproduce by seed, short stolons, and tillers, with the annual types reproducing predominately by seed and the perennial types by short stolons and nodal rooting.

Perennial types of ABG tiller and produce short stolons throughout the growing season, usually with reduced growth during the summer, and increased growth in the fall (Cline et al., 1993). Perennial types are described by having a longer vegetative growth stage than the annual types (Johnson and White, 1997). Perennial ABG possesses a number of desirable turf attributes that contribute to a dense stand, including shortened stems, tillers and leaves, and a single branched inflorescence (Huff, 2004). The perennial types also demonstrate a more prostrate growth habit than the annual types and proliferate under closely-mown, well-fertilized conditions (Tutin, 1957).

Annual types of ABG germinate in late summer to early autumn once air temperatures drop below 21° C, go dormant or semi-dormant over the winter, produce most of their seed throughout the spring (Lush, 1988), and then senesce in the summer. Though most of the seed is produced in the spring, seed production of the annual type can occur season-long and has been observed producing between 150,000 and 650,000 seeds m^{-2} yr^{-1} (Lush, 1989), with individual plants producing up to 2,250 seeds yr^{-1} (Holm et al., 1997). Even though perennial and annual types of ABG have considerably different characteristics, they are typically found in the same stand.

Annual bluegrass populations vary in response to environment and herbicide pressure (Warwick, 1979; Goss et al., 2005). Varying herbicide pressures and environments make ABG control difficult because a mixture of ABG types express many different phenotypes (McElroy et al., 2002), each with potentially different tolerances to chemical controls, cultural practices, and

climate (Cline et al., 1993). Studying ABG's variability and how it responds among different climatic regions and herbicides is valuable for improved management and control (Poole et al., 2005).

Variability in control has led to the common discovery of multiple ABG phenotypes growing within individual golf greens (Cline et al., 1993; Lush, 1988). The survival of such stands is attributed to each population's ability to produce tillers, stolons and seed (Carson et al., 2005). Perennial and annual types found in the same stand express different phenotypes and may survive throughout all seasons. This is due to the prolific seed production of the annual types coinciding with a reduction in tillers of the perennial types (Lush, 1988) or a reduction of another species in the same stand. If spaces are created on golf course greens due to individuals dying and thus creating voids, annual types of ABG will increase their population compared to perennial types. Annual type dominance is attributed to the large number of seed from annual types populating the soil seedbank (Lush, 1989). Conversely, if voids are not created, perennial types tend to dominate. Perennial type dominance is due to common cultural practices implemented for greens maintenance, such as more frequent irrigation, higher amounts of nitrogen, and lower mowing heights (Slavens et al., 2011; Engel, 1974; Carson, 2005). As ABG perennial and annual life cycles overlap and compete for stand dominance, both types are responsible for producing enduring populations (LaMantia and Huff, 2011). Heide (2001) reported that such different types of ABG growing within a stand demonstrate different photoperiodic and vernalization responses, thus increasing their chances for survival depending on the time of year or from changing climatic conditions from one season to the next.

Once established, ABG can grow at greens heights of cut (3-4mm) or under unmown conditions (Carson et al., 2005). Annual bluegrass has been documented to grow without sunlight, giving it the ability to grow in the most dense turfgrass stands (McElroy et al., 2004). Stand

density and self-generation give ABG some positive turf attributes. However, ABG is often less tolerant than other turfgrass species to environmental stresses like heat and drought, often leading to death in the absence of regular irrigation (Lush, 1989). Additionally, desirable perennial ABG varieties have unstable phenotypes; therefore many desirable traits that a given cultivar may possess will potentially be lost from one generation to the next (LaMantia and Huff, 2011).

Other factors leading to ABG summer thinning or death is the allocation of resources for seed production. This makes ABG more vulnerable to disease, and drought stress (Lush, 1989). Anthracnose (*Colletotrichum cereale*), brown patch (*Rhizoctonia solani*), dollar spot (*Sclerotinia homoeocarpa*), summer patch (*Magnaporthe poae*), waitea patch (*Rhizoctonia zeae*), pink snow mold (*Microdochium nivale*), and grey snow molds (*Typhula incarnate*), are common ABG diseases. Additionally, ABG is susceptible to insect damage by the annual bluegrass weevil (*Listronotus maculicollis*), the most damaging pest to turfgrass in the Northeastern United States (Vittum et al, 1999). Biotic and abiotic stresses are among the major influences of evolutionary biology (Nevo, 2001).

Genetics

Little is known about the genetic evolution and composition of ABG. However, Darmency and Gasquez (1997) provided evidence that current allotetraploid ABG (2n=4x=28) populations originated from an annual species, *P. infirma* (2n=2x=14), and a perennial species, *P. supina* (2n=2x=14), by showing that each displayed similar morphological and isozyme patterns. Huff and Mao (2012) provided similar results using phylogenetic analysis with two nuclear and two chloroplast gene sequences, also confirming Tutin (1957) and Nannfeldt's (1937) morphology and cytology studies that provided evidence for ABG's origin. In contrast, based on karyotypic comparisons, Koshy (1968) concluded that major chromosomal rearrangements had occurred in

the evolution of ABG. All theories of origin maintain that ABG is an allotetraploid, thus having both genomes of *P. infirma* and *P. supina.* This discovery is important to understanding ABG's ability to fluctuate between annual and perennial types.

Other findings show that true annual types of ABG may convert to perennial types in successive generations (Huff, 2004). Huff (2004) found that ABG perennial traits were linked together and inherited as a single trait. Genetic studies investigating the adaptability of allotetraploid grasses show that the most important mechanism involved in producing competitive species is the inheritance of perennial traits through an epistatic combination of alleles (Allard, 1997). Epistasis takes place when a gene or a combination of genes modify the effects of another gene, limiting the heritability of the same combination of genes during reproduction from one generation to the next.

Reproduction of ABG typically takes place by self-pollination (Ellis et al., 1973). However, the likelihood of outcrossing has been shown to occur up to 29% in densely populated stands of ABG (Mengistu et al., 2000a). Outcrossing recombines new combinations of genes, which likely influences variability in ABG populations. Greens maintenance such as mowing practices, irrigation, and traffic can move pollen throughout a green (Mengistu et al., 2000a) Further variation of ABG is due to its ability to flower under closely mown conditions, increasing outcrossing on greens and producing a wide range of genetically-based morphological differences within an ABG population. Through selection, intercrossing of superior ABG types have produced genotypes that provide superior adaptability and survival to a wide range of environmental conditions (Allard, 1997). However, while selecting for superior traits like those displayed by some perennial types is possible, they are often not passed on in successive generations (LaMantia and Huff, 2011).

Following reproduction, stands of ABG have been found to evolve from the wild type annuals (*Poa annua* v. *annua*) into perennial types (*Poa annua* v. *reptans*) under highly managed conditions (Carson et al., 2005) and transform from perennials to annuals when left unmowed (LaMantia and Huff, 2011). Perennial traits (short culms, short tillers, and leaves, reduced number of spikelets, and single-branched inflorescences) seem to be linked together and inherited as a single trait under stressful conditions like those involved with greens maintenance (Huff, 2004).

Before molecular markers were used to identify variability of ABG species, researchers used morphological characteristics and traits such as color, leaf texture, or seedhead formation and production. Within ABG populations, variation in inflorescence length and branching complexity, seedhead number, leaf area index and life cycle has been observed (Ellis et al., 1970; Lush, 1988).

Variability of ABG has more recently been analyzed by implementing the use of molecular markers. Sweeney and Danneberger (1995) used random amplified polymorphic DNA markers to show that greens height ABG was distinct from fairway types and that gene flow between these types was limited. Chwedorzewska (2008) used amplified fragment length polymorphic (AFLP) markers to characterize ABG individuals collected from Argentina, King George Island, and Poland and discovered high variation between all populations. Amplified fragment length polymorphic techniques have advantages over other molecular marker tools because they are reproducible, robust, relatively easy to implement, and produce large numbers of markers.

Cultural Controls

Prolific seedhead production by ABG annual types in the spring on golf greens inhibits ball roll and ABG susceptibility to summer stress make ABG an undesirable turfgrass species.

Attempts to control ABG populations produce inconsistent results (Reicher and Gaussoin, 2013). Such inconsistencies in control have led to managing ABG as a desirable turf by some managers where climatic conditions are conducive for ABG survival. However, whether managing for ABG survival or control, several cultural practices must be implemented.

Annual bluegrass proliferates at low mowing heights (3-4 mm) by producing stolons and seed. Seed production at such low mowing heights results in a persistent population of viable ABG seed within the seedbank (Lush, 1989; Mengistu et al., 2000a). Management practices for reducing populations include raising mowing heights, thus allowing desired species to better compete with ABG (Carson et al., 2005), whereas lowering mowing heights will encourage ABG (LaMantia and Huff, 2011). A consequence of ABG producing seed at low mowing heights is that it produces a shallow root system (Vargas and Turgeon, 2004). Plants producing seed at mowing heights of 3-4 mm or lower do not reserve sufficient carbohydrates to produce an abundant root system. Annual types of ABG persist in greater populations when frequent irrigation is applied (Rossi, 2001). Therefore, infrequent, deep irrigation is recommended to discourage ABG and shallow and frequent irrigation is recommended to encourage ABG (Vargas and Turgeon, 2004). Once established, ABG vegetative growth is decreased and inflorescence development increased with more frequent irrigation (Slavens et al., 2011). Conversely, decreasing the volume of irrigation will increase lateral spread of ABG and decrease seedhead production in annual types (Slavens et al., 2011).

Other common management strategies include hollow and solid-tine aerification in spring or fall, but this is suspected to encourage ABG by bringing seed to the surfacefor germination and growth. To limit ABG germination, aerification is recommended in the summer, when ABG does not typically germinate and seed production is low (Rieke and Murphy, 1989). Other strategies to reduce ABG populations include clipping removal following mowing. Returned ABG clippings

equates to unintentional overseeding of ABG (Gaussoin and Branham, 1989). Three years of clipping removal and overseeding with creeping bentgrass (CBG) decreased ABG above-ground populations by 28% and the ABG soil seedbank by 60% (Gaussoin and Branham, 1989). However, ABG can dominate CBG stands and seedbanks after years of management regardless of cultural practices (Huff, 1999; Lush, 1989).

Herbicide Control

Controlling ABG in cultivated turf stands can be attempted with numerous pre- and postemergence herbicides. However, consistent control of ABG is difficult, as no one chemical is effective for all locations and environments (Branham, 1991). Furthermore, ABG control can be affected by the changing weather over the course of a growing season, further complicating the efficacy and consistency of products used (Mengistu et al., 2000b). Adequate preemergence control has been found with prodiamine (Dernoeden, 1998), bensulide (Callahan and McDonald, 1992), and dithiopyr (Reicher et al., 2000), but in all cases acceptable control only occurred if applications were made prior to the major germination period for ABG in late summer or fall. Additionally, control is more localized in regions where summer and winter kill assist in controlling ABG. Follow-up preemergence applications may be required if the germination period for ABG is extended beyond the normal late summer/early fall window (Reicher and Gaussoin, 2013). All three of these herbicides have long soil residual and may cause injury to desired turf species (Smith and Callahan 1969; Dernoeden, 1998; Callahan and McDonald 1992; Reicher et al., 2000).

Bispyribac-sodium (bispyribac) is a synthetic postemergence herbicide belonging to the pyrimidinyl carboxy herbicide family (Shimizu et al., 2002) used for controlling ABG in CBG stands. It has been shown to occasionally cause unacceptable levels of discoloration, chlorosis and

other phytotoxins, even leading to death of CBG when applied to mixed stands, depending on application rate and conditions during application (Lycan and Hart, 2006). Additionally, inconsistent control throughout the spring and fall was observed (Lycan and Hart, 2006). Temperatures below 30 C° may reduce the metabolism of bispyribac by CBG resulting in elevated levels of injury (McCullough & Hart, 2006). Studies have been conducted to alleviate these negative effects by adjusting application rate, timing and by combining applications with chelated Fe + N products (McDonald et al., 2006). When applied to fairway height CBG stands, sequential applications of bispyribac have resulted in better turf quality than single applications (Teuton et al., 2007). Furthermore, applications in the summer have shown less stress to CBG and better control of ABG than treatments in the fall or spring (Lycan and Hart, 2006). Weekly applications of bispyribac beginning in the spring through the fall have provided ≥ 98% control of ABG on greens height stands of CBG (Teuton et al., 2007;McCarty and Estes, 2005). Inconsistency in control of ABG with bispyribac has often been attributed to seasonal or temperature effects on growth (Lycan and Hart, 2006), but these evaluations have made no mention of the potential changes of ABG populations in relation to control.

Plant Growth Regulators

Plant growth regulators (PGRs) applied to ABG stands suppress growth and/or spring seedhead production. Plant growth regulators are divided into two groups, Type 1 and Type 2. Type 1 PGRs inhibit cell division and differentiation in meristematic regions and are primarily taken up through plant foliage (Murphy et al., 2001). This leads to Type 1 PGRs effectively reducing cell elongation and seedhead production. Type 2 PGRs suppress cell elongation by interfering with the gibberellic acid synthesis pathway (Watschke and DiPaola, 1995). When

attempting to inhibit seedhead production with PGRs, timing is of utmost importance. Suppression of seedheads may not result if applications are made after seeding onset in the spring. For best control of seedheads, applications before seedhead formation are recommended (Murphy et al., 2001).

In spite of PGRs suppressing the growth of ABG and CBG, CBG stolons may continue their proliferation into voids created by decreased ABG populations, thus increasing CBG within a stand (Bigelow et al., 2007). Although PGRs have been shown to control ABG expansion within a stand, several objectionable effects are associated with them, including phytotoxicity and reduced recuperative capabilities (Baldwin and Brede, 2011).

Paclobutrazol (Pacl) is a Type 2 PGR that selectively suppresses ABG growth (Murphy et al., 2001). Paclobutrazol applications are made to manage ABG growth, therefore reducing the mowing requirement and increasing turf density (Woosley et al., 2003). Several negative effects have been observed with Pacl applications, including reduced turf quality, foliar discoloration and thinning of the turf stand (Koski, 1997).

Paclobutrazol treatments have shown acceptable control of ABG in recent trials, but a combination treatment of ethofumesate, a herbicide that selectively controls ABG applied in the autumn-winter, followed by Pacl in the spring-summer provided the best control of ABG in Kentucky (Woosley et al., 2003). Paclobutrazol applications and other PGRs are needed every two to three weeks throughout the growing season for the most consistent control. Stands with significant populations of ABG, may decrease in turf quality when Pacl is applied during long periods of high temperatures and stands must be monitored methodically to insure their survival (Bigelow et al., 2007).

Flurprimidol (Flur) is also a Type 2 PGR. Recent studies have been conducted to evaluate its effects when applied alone and in combination with other Type 2 PGRs, such as trinexapac-

ethyl (TE). Gradual reductions in ABG have resulted from Flur applications in stands with >30% ABG (Bigelow et al., 2007). However, Flur has been found to suppress seedhead production with varying results (McCarty, 2008).

Trinexapac-ethyl (TE) may be the most common used PGR in the turfgrass industry, because of the many beneficial attributes it has on ABG following applications (Ervin and Koski, 1998). Some of these positive attributes include chlorophyll enhancement (Ervin and Koski, 2001) and improved stress tolerance (McCann and Huang, 2008), while minimizing phytotoxicity. It has also been shown to increase plant vigor and the playability of ABG/CBG greens (McCullough et al., 2005). Seedhead suppression with TE requires multiple applications for control within a season (Kaminski, 2006). However, after multiple applications of TE, seedhead reduction may not be observed and loss in turfgrass color and quality can occur following applications. Attempting to control ABG with TE applications alone has been shown to have an antagonistic effect on control (Rossi, 2001).

Herbicide resistance is the ability of a plant to survive or reproduce following exposure to a dose of herbicide that would normally be lethal (Prather et al., 2000). Herbicide resistance was first identified around 1968 in a conifer nursery, when treatments of simazine failed to control common groundsel (Ryan, 1970). Applications of simazine were made repeatedly over several years and resistance was identified as the result of a mutation in a particular simazine-binding gene (Radosevich and Appleby, 1973). Currently, the International Survey of Herbicide-Resistant Weeds collects and reports on herbicide-resistant biotypes throughout the world (Heap, 2013).

Throughout the 1970s and 1980s more cases of herbicide resistance were confirmed and attributed to selection pressure caused by the repeated use of the same herbicide (Bandeen et al., 1982). During this time, many new modes of action were introduced into the market (Holt, 1992).

Each herbicide usually targets a single metabolic site, which is often determined by just one or a few genes and is conducive to the development of resistance in plants (Holt and Lebaron, 1990).

To date, 24 species and 169 populations of weeds have been confirmed resistant to glyphosate (Heap, 2013). Since the first discovery in 1998 (Powles et al., 1998), resistance to glyphosate has increased every year in intensity and number of species (Heap and LeBaron, 2001). Resistance prediction models proposed by Maxwell and Mortimer (1994) suggest herbicide resistance can develop in as little as six years within diverse weed populations.

The numerous types and genomic diversity of ABG, with the added selection pressure of a single herbicide mode of action applied to populations repeatedly, may contribute to the potential for ABG resistance to herbicides (Heap, 1995;Gressel and Segal 1978). Currently, 25 ABG populations have been documented resistant to a variety of individual herbicides (Binkholder et al., 2011; Brosnan et al., 2012; Darmency and Gasquez, 1983; Gressel and Segal, 1978; Hanson and Mallory-Smith, 2000; Heap, 2013; Isgrigg et al., 2002). The first confirmation of ABG glyphosate resistance occurred in 2007 after 10 years of annual applications to ABG in a zoysiagrass turf stand in Missouri (Binkholder et al., 2011). An ABG stand in Tennessee was also discovered in 2009 to be glyphosate-resistant, and was observed after 19 years of annual glyphosate applications (Brosnan et al., 2012).

Other ABG populations have been observed resistant to dinitroanilines (Isgrigg et al., 2002) or the phototsystem II inhibitors atrazine (Darmency and Gasquez, 1981), simazine (Gressel and Segal, 1978), or diuron (Hanson and Mallory-Smith, 2000). All occurrences were attributed to the repeated use of the same family of herbicide. Potential ABG resistance to PGRs has never been evaluated, even though it is recommended that they be applied every two to three weeks during the growing season (Woosley et al., 2003).

Resistance of ABG to glyphosate and other herbicides can be discouraged by using different products with different modes-of-action (Binkholder et al., 2011). Herbicides used in rotation restrict selection pressure during the growing season for which that herbicide is applied (Jasieniuk et al., 1996).

Evaluating herbicide resistance can be accomplished by measuring the loss of weed density or biomass in response to varying herbicide dosages (Jasieniuk et al., 1996). If species are resistant they can survive and reproduce when applications of herbicide rates are applied above recommended levels. Seed assessment at the conclusion of the growing season is another tool for resistance estimates. This is done by collecting seed from susceptible populations, re-growing their progeny and re-assessing for resistance with applications of herbicides. Assessing seed populations addresses the influence of susceptible individuals that miss herbicide treatment and contribute resistant progeny to the next generation (Gressel and Segal, 1978).

Objectives

The objectives of this study were to: (1) develop molecular markers that effectively characterize ABG populations, (2) help explain ABG population dynamics and reasons for inconsistencies in chemical control strategies.

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Chapter Two

Changes in *Poa annua* Populations in Response to Herbicides and Plant Growth Regulators

Poa annua (annual bluegrass; ABG) is an invasive weedy species in turfgrass. Herbicides and plant growth regulators (PGRs) are often used for ABG control, providing limited or inconsistent results. Identifying shifts in ABG populations in response to these treatments would be beneficial for understanding inconsistent control. Our research employed amplified fragment length polymorphic (AFLP) markers with the objective to determine if there are changes in genetic structure of ABG populations after multiple-year season-long control programs in three states. Annual bluegrass was sampled after the second or third year of seven different season-long ABG treatments consisting of herbicide or PGR applications. The trials were conducted at three different locations (East Lansing, Michigan; West Lafayette, Indiana; Lincoln, Nebraska). In the ABG samples, AFLP markers were identified for each site and 649, 745, and 762 were produced for Michigan, Indiana and Nebraska, respectively. Population analysis was conducted in Structure and identified five distinct ABG populations in Michigan, seven in Indiana, and six in Nebraska. Season-long treatments of trinexapac-ethyl or bispyribac-sodium (bispyribac) at a low rate effected genetic structure of populations at all locations. However, ABG populations that were affected by an individual herbicide or PGR did not respond consistently among locations. Bispyribac treatments increased ABG population variability in Michigan, but decreased variability in Indiana and Nebraska. Trinexapac-ethyl treatments decreased ABG population variability in Michigan and Indiana, but increased variability in Nebraska. This study provides a genetic basis in understanding how herbicides or PGRs impact ABG populations over the long term and our results may help explain inconsistencies in chemical control of ABG.

Introduction

Annual bluegrass is an allotetroploid, originating from two diploid *Poa* species, *Poa. supina* and *Poa infirma* (Darmency and Gasquez, 1997). *Poa supina* is a stoloniferous perennial that is native to the mountainous regions of central Europe and *P*. *infirma* is a bunch type annual that persists on the Mediterranean coast in Western Europe (Huff, 2008). Annual bluegrass originated in the Mediterranean region of Europe and was first characterized by Carl Linneaus (Tutin, 1957). Annual bluegrass has been identified from the Arctic Circle to the arid regions of Africa and on every continent in the world (Frenot et al., 1999), making ABG one of the five most widely distributed plants on Earth (Fenner, 1985).

The ability of ABG to flourish around the world is attributed to its genetic and physiological variability. Annual bluegrass life cycles range from annuals to perennials. Both annual and perennial varieties can reproduce by seed, stolons, and tillers, with the annual types reproducing predominately by seed and the perennial types by stolons and nodal rooting. Even though perennial and annual types of ABG have considerably different characteristics, they are often found in the same stand.

Annual bluegrass populations have been found to shift from wild type annuals (*Poa annua* v. *annua*) into perennial types (*Poa annua* v. *reptans*) under highly managed conditions (Carson et al., 2005), but from perennials to annuals when left unmowed (LaMantia and Huff, 2011). Sweeney and Danneberger (1995) found that gene flow between these types of differing populations (from fairways to greens or vice versa) was limited, suggesting that most variation occurs from within a stand. This variation is attributed to outcrossing in densely populated ABG stands, and has been documented to occur up to 29% (Mengistu et al., 2000). Through this type of natural selection, intercrossings of superior ABG types produce genotypes more adaptable to a wide range of environmental conditions (Allard, 1997). The adaptable nature of ABG enables it to

thrive in a wide range of environments and contributes to the difficulties and inconsistencies in controlling this weed.

Piper and Oakley (1921) concluded that controlling ABG would be timely and costly based on its ability to adapt to multiple climates and reproduce in large quantities. Annual bluegrass (*Poa annua* L.; ABG) is one of the most pervasive and challenging weeds facing turfgrass managers. In North America, ABG can be a major component of irrigated, close cut sports turf north of the transition zone and is the primary grass of some of the most celebrated golf courses around the world (Huff, 1999). However, ABG is often less tolerant than other turfgrass species to environmental stresses like heat and drought, often leading to death in the absence of regular irrigation (Lush, 1989). Additionally, desirable perennial ABG stands are unstable; therefore many desirable traits possessed by these stands can potentially be lost from one generation to the next (LaMantia and Huff, 2011).

Few consistent controls exist for ABG and varying populations is thought to be the reason for this inconsistency. Control failure from a particular chemistry, or reduced control over time is typically attributed to improper application, the product itself, cultural practices, weather and other environmental conditions. For instance, Lycan and Hart (2006) reported inconsistencies when summer applications of bispyribac provided better control than applications during the spring or fall. Variable control between seasons is often attributed to seasonal or temperature effects on the growth and physiology of ABG.

Genetic diversity of ABG combined with the added selection pressure of a single herbicide mode of action applied repeatedly can contribute to the potential for ABG resistance to herbicides or possibly PGRs (Heap, 1995;Gressel and Segal, 1978). Currently, 25 ABG populations have shown resistance to herbicides (Binkholder et al., 2011; Brosnan et al., 2012; Darmency and Gasquez, 1983; Gressel and Segal, 1978; Hanson and Mallory-Smith, 2000; Heap,

2013; Isgrigg et al., 2002). The first confirmation of glyphosate resistant ABG occurred in 2007 after 10 years of annual applications to ABG in a zoysiagrass turf stand in Missouri (Binkholder et al., 2011). An ABG stand in Tennessee was also discovered in 2009 to be glyphosate-resistant after 19 years of annual glyphosate applications (Brosnan et al., 2012).

Other ABG populations have been observed to be resistant to dinitroanilines (Isgrigg et al., 2002) or the phototsystem II inhibitors atrazine (Darmency and Gasquez, 1981), simazine (Gressel and Segal, 1978), or diuron (Hanson and Mallory-Smith, 2000). All occurrences were attributed to the repeated use of the same family of herbicide. Resistance of ABG to glyphosate and other herbicides can be limited by using products with different modes-of-action (Binkholder et al., 2011). Herbicides used in rotation restrict selection pressure during the growing season for which that herbicide is applied (Jasieniuk et al., 1996). Potential ABG resistance to PGRs has never been evaluated, even though it is recommended that they be applied every two to three weeks during the growing season (Woosley et al., 2003).

If variation in ABG populations has a role in affecting ABG control, ABG samples could be analyzed on a molecular level after herbicide treatments are applied. Previously shifts in ABG populations were determined by visually inspecting individual differences in color, leaf texture, and seedhead production (Ellis et al., 1973 Lush, 1988; Lush, 1989). Darmency and Gasquez (1981) studied the impact of triazine sensitive or resistant ABG on population dynamics and discovered both types contained a significant amount of polymorphism. Population diversity studies have more recently been analyzed using molecular markers. One marker technique is random amplified polymorphic DNA (RAPD), and these types of studies have continued to show ABG variability within and between ABG populations (Mengistu et al., 2000; Sweeney and Danneberger, 1995). Chwedorzewska (2008) used amplified fragment length polymorphic (AFLP) markers to characterize ABG populations and discovered that populations from

Argentina, King George Island, and Poland were also variable. Amplified fragment length polymorphisms have advantages over other marker techniques when analyzing population dynamics, because no upfront sequence information is necessary, they produce a high number of markers, and results are reproducible (Vos et al., 1995). Amplified fragment length polymorphism and RAPD fingerprinting techniques are used to detect subtle differences between individuals within a species (infra-species level) that other marker systems cannot. The objective of this study was to evaluate how ABG populations are impacted following treatments of PGRs or herbicides.

Materials and Methods:

Plant Material

Annual bluegrass was collected from established putting greens located in West Lafayette, IN; Lincoln, NE; and East Lansing, MI. Greens in Michigan and Nebraska were established in 1996 and the green in Indiana was established in 1991. All greens consisted of a mixture of ABG and creeping bentgrass (CBG), were mowed daily at 0.32 cm and sand-topdressed monthly. Greens received between 122-146 kg/ha of nitrogen annually. Seven season-long treatments of herbicides or PGRs were applied to control ABG on putting greens at all three sites. The same active ingredient(s) were applied according to label recommendations up to twelve times per year to the same plot for two or three years prior to our ABG sampling and treatments are listed in Table 1. Treatments were applied with 815 L/ha of water. Treatments were made from 2009-2011 in Indiana and Michigan, and from 2010-2011 in Nebraska. The design of the field experiments at each site was a randomized complete block design with three replications. Plots were 1.2 x 1.8 m in Indiana and Michigan and 1.5 x 1.5 m in Nebraska.

Collections of ABG were made April 11-17, 2012 at all three states and 15 ABG plants

were collected from each plot at each location (1,080 samples), inserted into 5.7 x 8.9 cm coin envelopes and immediately placed on dry ice in a plastic cooler. The plant material was then transported back to the University of Nebraska-Lincoln and stored at -80 C until it was later lyophilized. All plant material was lyophilized (VirTis Freezemobile, Gardiner, NY) for 48 hours and then stored at room temperature in zip top bags with silica gel.

DNA Isolation

Total DNA was extracted from lyophilized annual bluegrass tissue using the Qiagen Puregene Core Kit A (Qiagen Inc., Valencia, California) with minor modifications to the manufacturer's protocol as described below. Lyophilized plant tissue (10-20 mg) was transferred to Lysing Matrix A tubes (MP Biomedicals, Solon, OH) consisting of one 6.35 mm ceramic sphere and 0.56-0.7 mm garnet matrix (MP Biomedicals). Samples were processed in a FastPrep-24 (MP Biomedicals) for two cycles of 20 sec at 16 MHz to grind samples to a fine powder. Following visual inspection samples were ground further as needed. Cell Lysis Solution (750 µl) from Core Kit A (Qiagen Inc.) was added to sample tubes and mixed using a 60 Hz Vortex Genie 2 (Scientific Industries, Bohemia, New York) at 2,000 rpm's for 20 sec. Samples were then manually inverted, placed on a Clinical Rotator (Fisher Scientific, Hampton, New Hampshire) in an incubator set at 90 oscillations/min and incubated at 65° C for 60 min. After this initial incubation, 3.75 µl RNaseA was added to each sample, inverted 25 times for mixing and incubated again for an additional 20 min at 37° C. Samples were then cooled to room temperature for 20 min and 250 µl of Protein Precipitation Solution from Core Kit A was added to each sample, vortexed with a 60 Hz Vortex Genie 2 at 2,000 rpm's for 20 sec and placed on ice for 30 min. Samples were then centrifuged with an Accuspin Micro 17 (Fisher Scientific) at 6,000 rpm for 10 min. The aqueous phase (supernatant) was transferred to a two ml Eppendorf (Hamburg,

Germany) tube containing 750 µl isopropanol at room temperature and manually inverted 50 times for mixing. In order to precipitate the DNA from the supernatant, samples were again centrifuged using an Accuspin Micro 17 at 6,000 rpm for five min. The supernatant was then discarded and 750 µl of 70% ethanol was added to tubes containing the DNA pellet and manually inverted 20 times to wash the DNA. Samples were then centrifuged using an Accuspin Micro 17 5,000 rpm for five min. Excess ethanol was discarded and the remaining DNA pellets were air dried in a fume hood for 5-10 min and 50 µl Hydration Solution from Core Kit A was added. Samples were stored in a freezer at -15°C.

AFLP Analysis

All AFLP reactions were performed according to Vos et al. (1995) except for modifications as described below. The AFLP Core Reagent Kit (Invitrogen, Carlsebad, California) was used for the restriction and ligation steps. Restriction reactions consisted of 5 µl 5X reaction buffer (Invitrogen Core Reagent Kit), 2 µl EcoRI/ MseI (1.25 units/µl) endonuclease Mix (Invitrogen Core Reagent Kit), 0.5 µl BSA (1.0 mg/ml), 500 ng genomic DNA, and enough nuclease free water (Integrated DNA Technologies) to bring the final volume to 25 µl. The restriction reaction was incubated in a Mastercycler pro S (Eppendorf) thermocycler at 37° C for three hours, and then incubated at 70° C for 15 min to inactivate the restriction endonucleases, and finally held at 4° C. The ligation reactions were performed by adding 24 µl of adapter ligation mix (Invitrogen Core Reagent Kit) and 1.0 µl T4 DNA ligase (Invitrogen Core Reagent Kit) to 25 µl of the restriction reaction. The ligation reactions were incubated in a thermocycler at 20° C for three hours and held at 4° C overnight. The ligation reactions were diluted 1:10 in Tris/EDTA buffer (TE buffer: Tris 10mM / EDTA 1mM) pH 8. Pre-selective amplification reactions were

carried out using 5 µl diluted ligation reactions, 5 µl 10X PCR buffer minus $MgCl_2$ (Invitrogen, part# 52724), 1.125µl 5 µM EcoRI pre-selective primer (Table 2), 1.125 µl 5 µM MseI pre-selective primer (Table 2), 2.0 µl of 1.25 mM of each dNTP (dATP, dCTP, dGTP, dTTP; Invitrogen, part# 10297), 1.0 µl recombinant Taq polymerase (5U/µl, Invitrogen, part# 10342), 1.5 µl 50 mM $MgCl_2$ (Invitrogen, part# 52723), and 33.25 µl nuclease free water. Reactions were carried out with an initial incubation of 72° C for two min, followed by incubation at 94° C for two min, followed by 30 cycles at 94° C for 30 sec, 60° C for 1 min, and 72° C for 2 min, followed by an incubation at 72° C for 10 min and finally held at 4° C. Pre-selective amplification reactions were diluted 1:50 in Tris/EDTA buffer. The selective amplification reactions were carried out using 5 µl diluted pre-selective reaction, 2 µl 10X PCR buffer minus $MgCl_2$ (Invitrogen, part# 52724), 1 µl 5 µM EcoRI+3 selective primer (Table 2), 1 µl 5 µM Msel+3 selective primer (Table 2), 1.6 µl of 1.25 mM of each dNTP, 0.1 µl recombinant Taq polymerase (5U/µl, Invitrogen, part#10342), 0.6 µl 50 mM $MgCl_2$ (Invitrogen, part# 52723), and 8.7 µl nuclease free water (Integrated DNA Technologies). Selective amplification PCR consisted of an initial incubation of 72° C for two min, followed by 13 cycles at 94° C for 30 sec, 65° C for 30 sec, 72° C for 1 min, followed by 23 cycles 94° C for 30 sec, 56° C for 30 sec, 72° C for 1 min, and held at 4° C. The EcoRI selective PCR primers were labeled at the 5' end with 6-carboxyfluorescein (6-FAM) to enable detection on an Applied Biosystems 3730 Genetic Analyzer (Life Technologies, Carlsbad, California) at the University of Nebraska Medical Center, DNA Sequencing and Microarray Core Facility.

Data Analysis

GeneMapper 4.1 software (Life Technologies) was used to visualize AFLP reactions. All

samples from each site were used to create bin sets for each primer combination (Table 2), for a total of six bin sets. Bins were defined using a 50-500bp range with minimum peak detection set at 2,000 reflective fluorescent units. Markers for each sample were then scored based on the bin sets from their respective primer combination and site. GeneMapper software was then used to automatically score peaks as present (one) or absent (zero) based on the programs default criteria (Amundsen and Warnke, 2011).

Binary data from control samples was imported into Structure 2.3.4 (Falush et al., 2003; Pritchard et al., 2000) to assess the probability of samples falling into each *k* population. Data sets of all untreated samples from each specific site were processed in Structure using 10,000 burn-in iterations followed by 10,000 iterations. Ten replications at each *k* population from 2 to 30 were tested. The most likely *k* value was chosen for each site following the Δk method described by Evanno et al. (2005), which is based on the rate in change of the log probability of data between successive *k* values. Samples were sorted based on their probability of fitting into a certain *k*. Treatment samples were then analyzed in Structure based on the most likely *k* value at each site produced by the untreated samples. Samples of all untreated individuals from a site were included with all individuals from each treatment to produce a Structure plot for each treatment. Structure plots were produced by using data sets of samples pertaining to a specific treatment from a specific site using 10,000 burn-in iterations followed by 10,000 iterations and ten replications at the specified *k* value. Further analysis presented in the results and discussion for assigning an individual to a certain designated population was based on that individual having at least a 0.40 probability of being in one population. Substructure was also tested among groups of individuals when more than five individuals had greater than 0.40 probability of being assigned to the same population.

Using GenAlEx v. 6.4 (Peakall and Smouse, 2006), a distance matrix was generated from

the binary data in order to conduct an analysis of molecular variance (AMOVA), comparing the genetic diversity within and between treatments. An unweighted pair-group method with arithmetic mean (UPGMA) cluster analysis of the samples based on the genetic similarity (GS) matrix was produced using the sequential agglomerative hierarchical nested (SAHN) cluster analysis function in NTSYSpc v. 2.21o. The results of this analysis produced a dendrogram, representing the hierarchical clustering of individuals.

Results

Two AFLP selective primer combinations (Table 2: EcoRI+ACT/MseI+CAC, EcoRI+ACC/MseI+CTA) produced 656, 745, and 762 markers in Michigan, Indiana, and Nebraska, respectively. Each of the 720 ABG AFLP fingerprints was unique. Based on AMOVA, there were differences between treatments at each location (Tables 3, 4, and 5). The amount of genetic variation among populations was 1% in Michigan and Indiana and 2% in Nebraska.

An AMOVA comparing the treated plots to the checks revealed that bispyribac-low (Bis-low) and trinexapac-ethyl (TE) applications impacted ABG populations at all three sites. Flurprimidol/TE and Pacl impacted populations in Indiana and Nebraska, and Bis-high impacted ABG populations in Nebraska (Table 6). The remaining treatments did not impact ABG populations and were not used in downstream analysis.

Pairwise GS values between ABG individuals within a treatment and all individuals at each location were compared to evaluate genetic diversity at all locations (Table 7). The least similar individuals within a treatment at Michigan were from the TE treatment and have a mean GS value of 0.35. Michigan Bis-low and untreated individuals both have the highest mean GS within treatments (GS = 0.38). When comparing all individuals from Michigan the range in GS was 0.09-0.98 and the mean GS was 0.37. At Indiana, the Bis-low, TE, and untreated individuals

within treatments have mean GS values of 0.45, 0.52, and 0.52, respectively, representing the lowest and highest mean GS values in Indiana. Treatments of paclobutrazol (Pacl) and flurprimidol/TE (Flur/TE) in Indiana have a mean GS of 0.47 and 0.49, respectively. When comparing all individuals from Indiana the range in GS is from 0.15-0.73 and the mean GS is 0.47. Nebraska individuals show the highest range in mean GS of all sites when comparing individuals within treatments, with untreated and Pacl individuals representing the lowest (GS = 0.41) and Flur/TE the highest (GS = 0.49). Individuals within treatments representing Bis-low and TE in Nebraska have a mean GS = 0.44 and bispyribac-high (Bis-high) GS = 0.46. All individuals from Nebraska have a GS range from 0.14-0.90 and a mean GS of 0.43.

The Evanno et al. (2005) method of structure analysis demonstrated that individuals collected in Michigan, Indiana, and Nebraska consisted of five, seven, and six ABG populations respectively (Figures 1A-C). Michigan TE shows a loss of population three when compared to the check plot (Figure 2). Similarly, Indiana TE treatments show a loss of population five (Figure 3). Indiana TE treatments also show Structure population two clustering based on UPGMA with GS $\geq$ 0.64 (Figure 4). The Structure plot representing TE treated individuals in Nebraska shows a gain of population two (Figure 5).

Treatments of Bis-low also affected ABG populations at all three sites. Treatments of Bis-low in Michigan show a gain in population four (Figure 6). Treatments of Bis-low in Indiana show a loss of population six (Figure 7) and in Nebraska a loss of population four (Figure 8). In addition, Bis-low treatments in Nebraska do show individuals assigned to population four in Structure clustering based on UPGMA with GS $\geq$ 0.55 (Figure 9). The cluster also contains Bis-low treatments 102-1, 204-4, 305-3, 102-2, 305-1, 102-7, and check sample 306-6. Since 306-6 shows a high level of admixture, substructure on these 14 individuals was tested and is observed at k=2 (Figure 10).

Structure plots representing Pacl treatments in Indiana show the appearance of population two (Figure 11). Based on UPGMA, the individuals of Structure population two are genetically diverse (Figure 12). Treatments of Pacl in Nebraska represented in Structure show an addition of population two (Figure 13).

Indiana and Nebraska ABG populations were also affected by treatments of Flur/TE. Structure plot representing Flur/TE individuals in Indiana shows a loss of population one, but a gain in population five (Figure 14). Treatments of Flur/TE in Nebraska display a loss in the individuals assigned to population three as well as a gain of individuals assigned to population one (Figure 15). Treatments of Flur/TE in Nebraska show population six from Structure clustering based on UPGMA with GS $\geq$ 0.58 (Figure 16).

Discussion

Genetic markers are a useful tool for characterizing genetic diversity within and among populations. Each of the 720 genotyped individuals in this study possessed a unique fingerprint based on AFLP molecular markers, providing support that each of the ABG individuals in this study are genetically unique. Structure analysis of individuals collected from all three sites revealed that each location contained multiple ABG populations. These data together provide support that the ABG individuals in this study are genetically diverse. Variation in ABG populations has been observed using AFLPs, when comparing ABG from different regions and climates (Chwedorzewska, 2008). Additionally, morphological variation of ABG individuals collected from the same green is common within a site on ABG/CBG greens (Lush, 1988). Our study further supports such variation across geographic regions as well as within a location.

In the control study, variable control of ABG (data not shown) was observed and no one specific treatment provided the best control at all sites. Control of ABG did not correlate with an

effect on the population structure of the ABG population at any site. Our AMOVA shows that 11 of 21 treatments affected ABG population diversity, but the treatments impacting ABG population diversity at one location did not necessarily impact ABG diversity at another. Reports of site dependent ABG control are common. Environment and climate strongly determine which ABG types are present within a population (Frenot et al., 1999; Koshy, 1969; Poole et al., 2005) and control throughout the season is often variable (Reicher et al., 2012). Specifically, collections in the summer may consist of more perennial types and collections in the fall may be newly-emerging seed from annual types. The ABG individuals in our study were collected in April and ABG variability at this time of year may be limited to those individuals that survive the winter. Based on AFLP a significant amount of genetic variability was detected in these individuals, however the impact treatments have on ABG populations may change throughout the season. To address this point, additional collections were made during the summer and the fall, but the analysis of these samples was beyond the scope of this project.

Diversity of ABG populations changed due to Bis-low and TE treatments at all three sites, by increasing, decreasing, and/or shifting populations depending on location. Mengistu et al. (2000) found that adding the selection pressure of herbicides to control ABG populations in Oregon seed fields decreased ABG's variability. In their study, ABG that received more treatments displayed less population diversity. Conversely, triazine-resistant types of ABG have displayed similar population variability as susceptible populations (Darmency and Gasquez, 1981). In our study, treatments that provided higher control of ABG produced ABG populations with diversity similar to that in our untreated check plots based on Structure analysis.

It is interesting to note that Bis-low and Bis-high treatments impacted ABG populations differently. For example, Bis-low impacted ABG populations at all three sites, by the addition of populations at Michigan and the loss of a population at Nebraska and Indiana. The Bis-high

treatment led to an additional population at Nebraska, but had no effect at Michigan or Indiana. Herbicide applications made more often at lower rates are a key element in influencing populations towards resistance and reducing control (Neve and Powles, 2005). Additionally, TE impacting ABG at all three sites may be attributed to the beneficial traits observed after TE treatments (Ervin and Koski, 1998) and the antagonistic effect TE often has on control (Rossi, 2001). Our data suggest that lower rates of bispyribac had a greater impact on ABG populations than higher application rates.

Sweeney and Danneberger (1995) concluded that gene flow is limited between ABG populations on golf course greens and fairways. The high level of ABG genetic diversity in our study is more consistent with highly outcrossing species than with self-fertile species, however we did not test pollen flow or sexual compatibility of the ABG. If the level of ABG diversity is changing in the confines of our study, it is likely from ABG already present within the stand or through mechanisms of genomic instability often associated with ABG cultivars (LaMantia and Huff, 2011). For example, emerging seeds of ABG can fill spaces created by effective treatments that remove susceptible individuals, creating an opportunity for population structure to be altered.

Variation of individuals in our study may be limited due to the sampling method. Collections were made by hand and only samples that were visible on a specific collection date were harvested. Therefore, a majority of the ABG sampled in the spring contained seedheads and may be biased towards annual types. Perennial types do not form seedheads as prolifically as annual types and their growth habit and color is more similar to that of CBG (Lush, 1988), making our identification and collection of perennial types more difficult within CBG greens.

Detecting differences in individuals from variable species such as ABG within a site has previously been difficult based on morphological characteristics. Our study was successful in using AFLPs to detect genetic differences among a collection of ABG. These AFLP markers were

valuable for detecting population structural changes in response to herbicides or PGRs. Additionally, our data shows significant genetic diversity in ABG populations at all sites. This study confirms that inconsistent control may be due to differences in initial ABG populations and/or changes to ABG populations after two or three years of treatments with the same herbicide or PGR. This is the first report of such effects with PGRs. Differences in responses of ABG populations to treatments may also be due to environment. Therefore, further research is needed to evaluate the diversity of ABG throughout the growing season addressing if in addition to location; season may also impact ABG's response to herbicides or PGRs.

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Table 1. Herbicide and PGR treatments were made to control greens height annual bluegrass at three locations located in Indiana and Michigan from 2009-2011, and Nebraska from 2010-2011 before ABG samples were collected for DNA extraction.

Product	Rate	App. frequency	App. dates	Total apps yr^{-1}
	kg ai ha^{-1}	$weeks^{-1}$	--	--
Bispyribac (Bis-low)	0.012	2	May-Sep	8
Bispyribac (Bis-high)	0.025	2	Aug-Sep	4
Cumyluron	6.900	20	Apr, Aug	2
Paclobutrazol (Pacl)	0.140 0.280	2 2	Apr-May, Aug-Sep Jun-Jul	8 4
Flurprimidol (Flur)	0.035 0.070	2 2	Apr-May May-Aug	5 7
Flurprimidol/TE (Flur/TE)	0.099/0.033	2	Apr-Sep	12
Trinexapac-ethyl (TE)	0.096	2	Apr-Sep	12
Check	-	-	-	-

Table 2. AFLP pre-selective primers used for pre-amplification PCR (Integrated DNA Technologies, Coralville, IA). AFLP selective reaction primer pair sequences used for selective amplification PCR (Integrated DNA Technologies).

AFLP primers	**Sequence 5' – 3'**
Preselective reactions	
EcoRI pre-selective	gactgcgtaccaattcA
MseI pre-selective primer	gatgagtcctgagtaaC
Selective reactions (EcoRI+3/MseI+3)	
EcoRI+ACT/ MseI+CAC	gactgcgtaccaattcACT gatgagtcctgagtaaCAC
EcoRI+ACC/ MseI+CTA	gactgcgtaccaattcACC gatgagtcctgagtaaCTA

Table 3. Analysis of molecular variance (AMOVA) within- and among-group diversity of seven treatments of PGRs and herbicides and a check, treatments were applied to control ABG located in Michigan.

Source	df	SS	MS	Est. Var.	Variation	P-value
Among Trts	7	817.493	116.785	0.665	1%	0.002
Within Trts	227	22075.094	97.247	97.247	99%	
Total	234	22892.587		97.912	100%	

df, degrees freedom
SS, sums of squares
MS, Mean Squares
Est. Var., estimate of variation
P-value refers to the total genetic variation among individuals of a population

Table 4. Analysis of molecular variance (AMOVA) within- and among-group diversity of seven treatments of PGRs and herbicides and a check, treatments were applied to control ABG located in Indiana.

Source	df	SS	MS	Est. Var.	Variation	P-value
Among Trts	7	1073.424	153.346	0.960	1%	0.001
Within Trts	229	28605.551	124.915	124.915	99%	
Total	236	29678.975		125.875	100%	

df, degrees freedom
SS, sums of squares
MS, Mean Squares
Est. Var., estimate of variation
P-value refers to the total genetic variation among individuals of a population

Table 5. Analysis of molecular variance (AMOVA) within- and among-group diversity of seven treatments of PGRs and herbicides and a check, treatments were applied to control ABG located in Nebraska.

Source	df	SS	MS	Est. Var.	Variation	P-value
Among Trts	7	1292.520	184.646	2.127	2%	0.001
Within Trts	222	27420.371	123.515	123.515	98%	
Total	229	28712.891		125.642	100%	

df, degrees freedom
SS, sums of squares
MS, Mean Squares
Est. Var., estimate of variation
P-value refers to the total genetic variation among individuals of a population

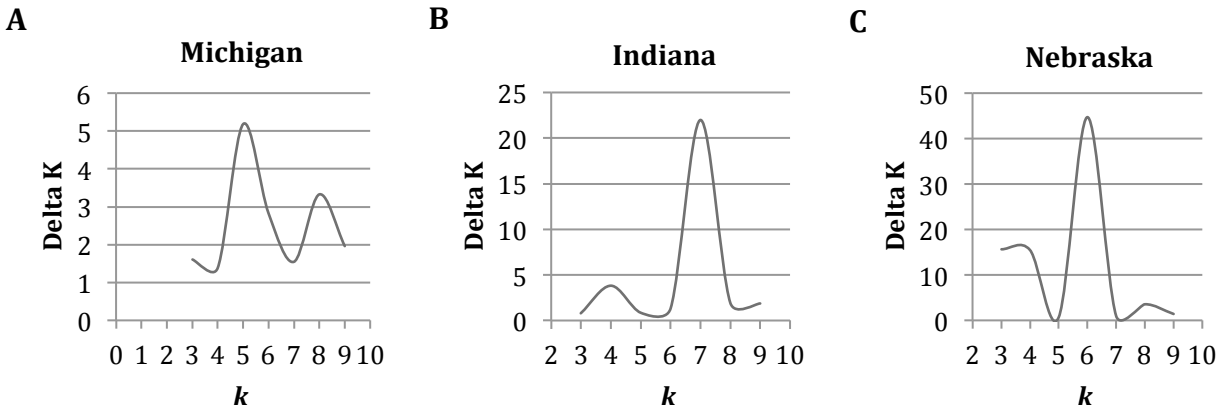
Table 6. Analysis of molecular variance (AMOVA) with the probability of populations within treated plots differing from populations in untreated plots. Analysis was conducted by GenAlEx v 6.4 (Peakall and Smouse, 2006 based on 999 permutations.

	Treatment						
Location	**Bis-low**	**Bis-high**	**Cumyluron**	**Pacl**	**Flur**	**Flur/TE**	**TE**
Michigan	0.044*	0.169	0.220	0.440	0.185	0.398	0.036*
Indiana	0.010*	0.256	0.178	0.037*	0.301	0.002*	0.001*
Nebraska	0.016*	0.032*	0.317	0.001*	0.373	0.012*	0.028*

*** Significant at P** $\leq .05$

Table 7. Mean, minimum**,** maximum and standard deviation of the Dice genetic similarity coefficient (GS) calculated from AFLPs produced at each specific site for treated by untreated pairwise comparisons and all treatments at each site.

Location	Treatment	Mean GS	Minimum GS	Maximum GS	Standard deviation
Michigan	All individuals	0.37	0.09	0.98	0.07
	Bis-low	0.38	0.23	0.98	0.07
	TE	0.35	0.17	0.63	0.06
	Untreated	0.38	0.23	0.98	0.07
Indiana	All individuals	0.47	0.15	0.73	0.10
	Bis-low	0.45	0.22	0.66	0.11
	TE	0.52	0.30	0.73	0.09
	Pacl	0.47	0.20	0.66	0.09
	Flur/TE	0.49	0.29	0.68	0.09
	Untreated	0.52	0.40	0.67	0.06
Nebraska	All individuals	0.43	0.14	0.90	0.10
	Bis-low	0.44	0.23	0.69	0.09
	TE	0.44	0.22	0.75	0.10
	Pacl	0.41	0.21	0.67	0.07
	Flur/TE	0.49	0.23	0.67	0.09
	Bis-high	0.46	0.23	0.82	0.10
	Untreated	0.41	0.23	0.69	0.09



Figures 1A-1C. Population estimates of annual bluegrass untreated control samples collected at each site based on methods from Evanno et al. (2005). Apex discerns population (*k*) level, where Delta *k* is based on the rate of change in the log probability of data between successive *k* values.

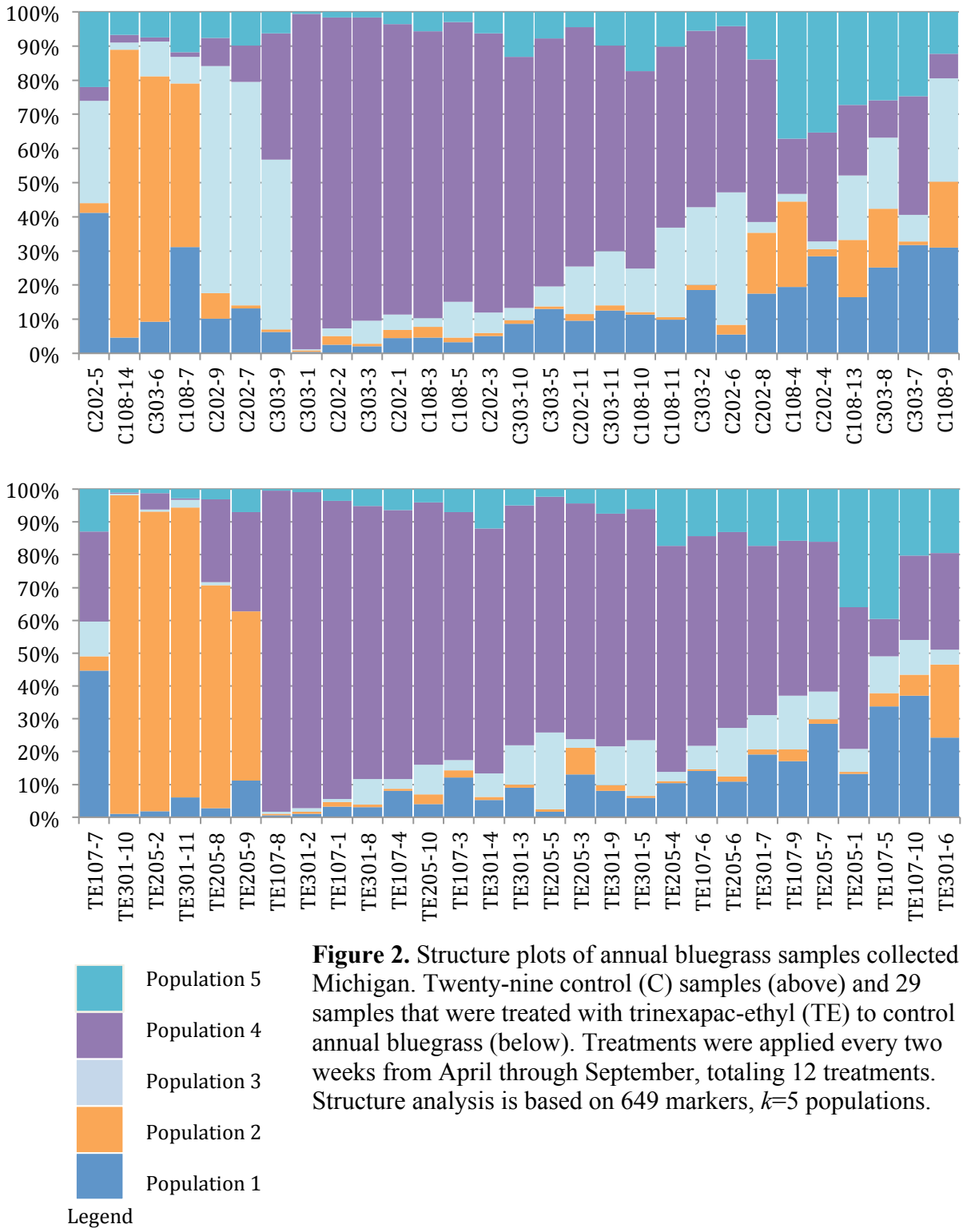


Figure 2. Structure plots of annual bluegrass samples collected in Michigan. Twenty-nine control (C) samples (above) and 29 samples that were treated with trinexapac-ethyl (TE) to control annual bluegrass (below). Treatments were applied every two weeks from April through September, totaling 12 treatments. Structure analysis is based on 649 markers, *k*=5 populations.

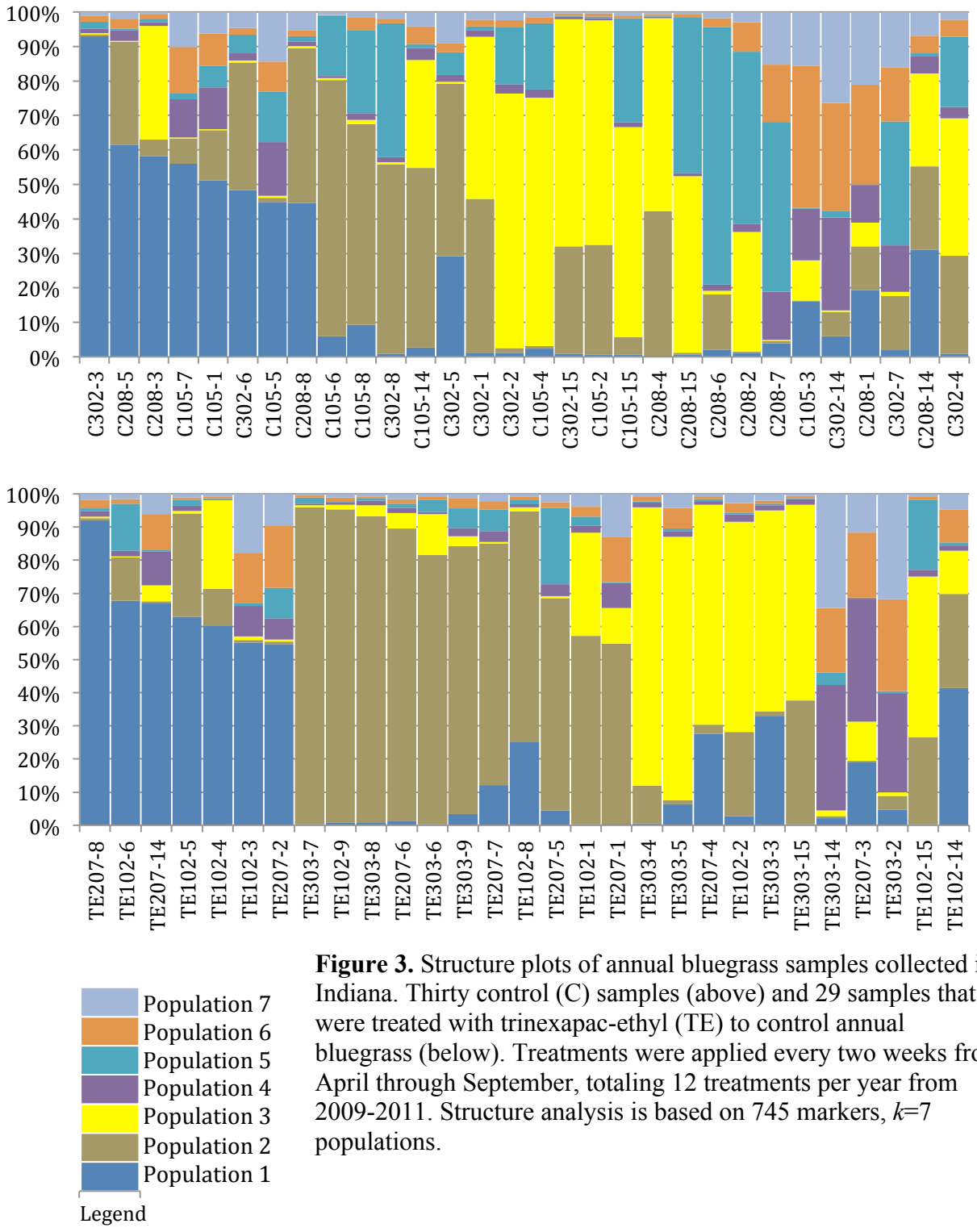


Figure 3. Structure plots of annual bluegrass samples collected in Indiana. Thirty control (C) samples (above) and 29 samples that were treated with trinexapac-ethyl (TE) to control annual bluegrass (below). Treatments were applied every two weeks from April through September, totaling 12 treatments per year from 2009-2011. Structure analysis is based on 745 markers, *k*=7 populations.

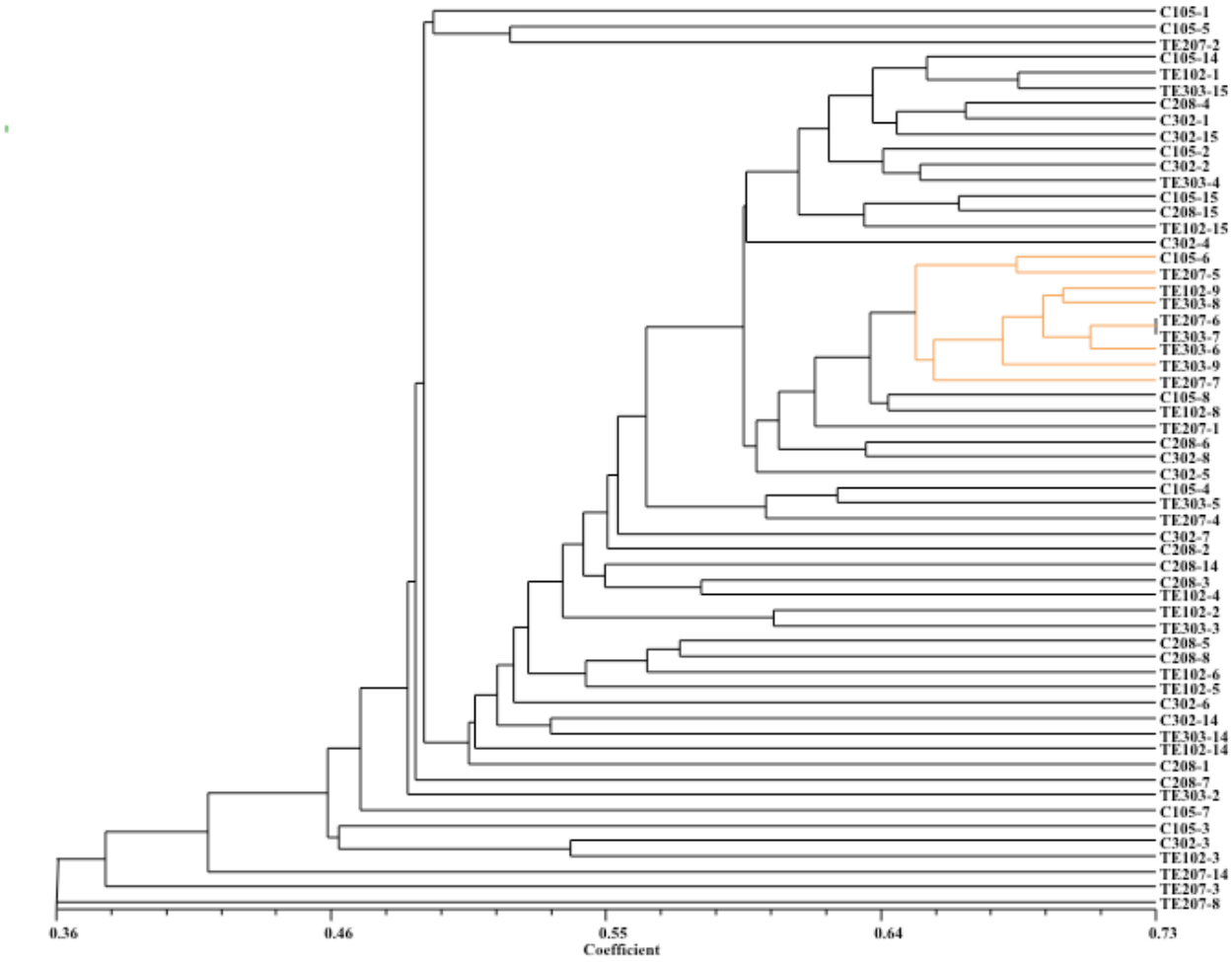


Figure 4. Unweighted pair-group method with arithmetic mean (UPGMA) analysis of samples based on the similarity matrix was employed using the sequential agglomerative hierarchical nested (SAHN) cluster analysis function using NTSYSpc v. 2.21o. Thirty control samples (C) collected in Indiana and 29 samples that were treated with trinexapac-ethyl (TE) to control annual bluegrass. Treatments were applied every two weeks from April through September, totaling 12 treatments per year from 2009-2011.

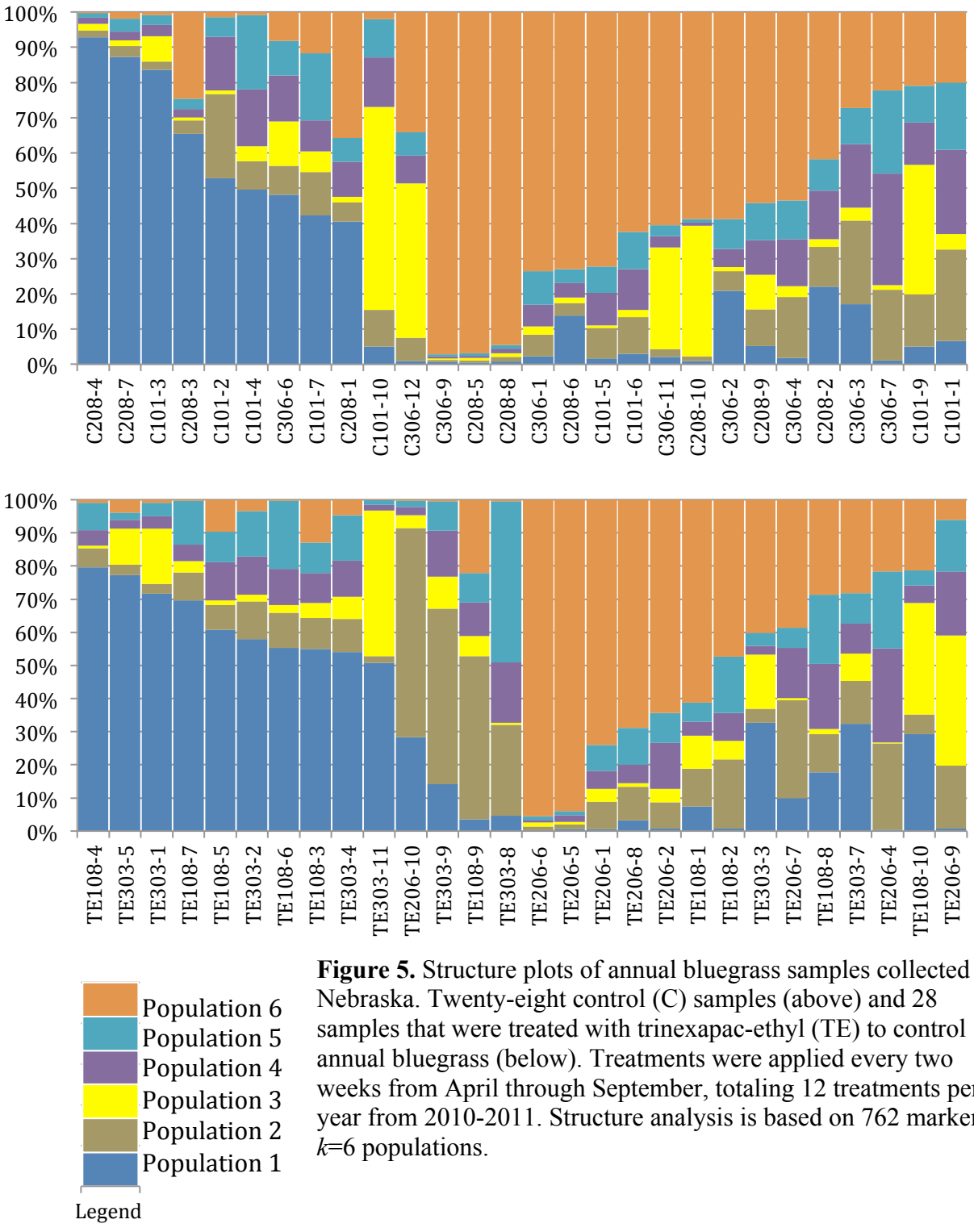


Figure 5. Structure plots of annual bluegrass samples collected in Nebraska. Twenty-eight control (C) samples (above) and 28 samples that were treated with trinexapac-ethyl (TE) to control annual bluegrass (below). Treatments were applied every two weeks from April through September, totaling 12 treatments per year from 2010-2011. Structure analysis is based on 762 markers, *k*=6 populations.

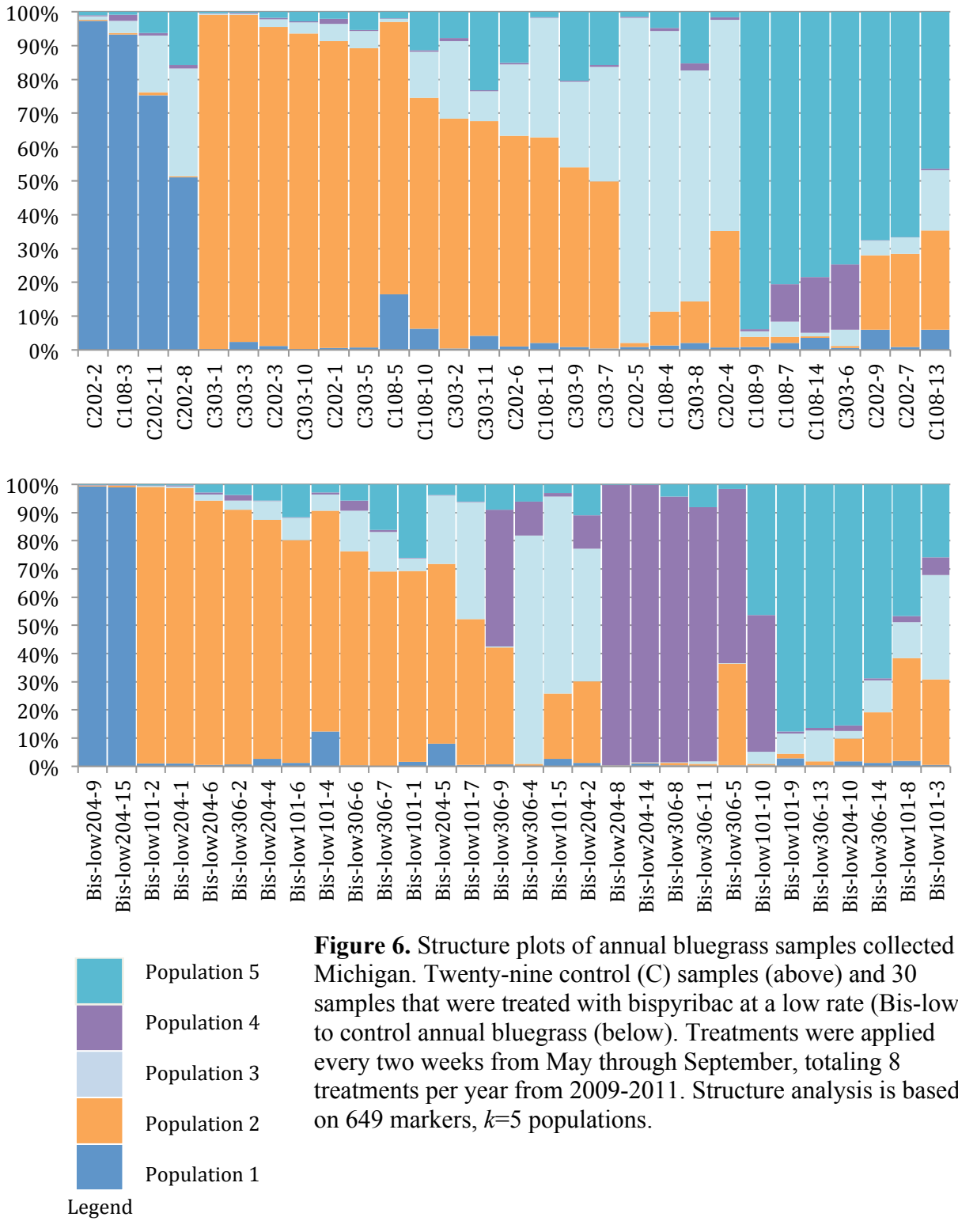


Figure 6. Structure plots of annual bluegrass samples collected in Michigan. Twenty-nine control (C) samples (above) and 30 samples that were treated with bispyribac at a low rate (Bis-low) to control annual bluegrass (below). Treatments were applied every two weeks from May through September, totaling 8 treatments per year from 2009-2011. Structure analysis is based on 649 markers, *k*=5 populations.

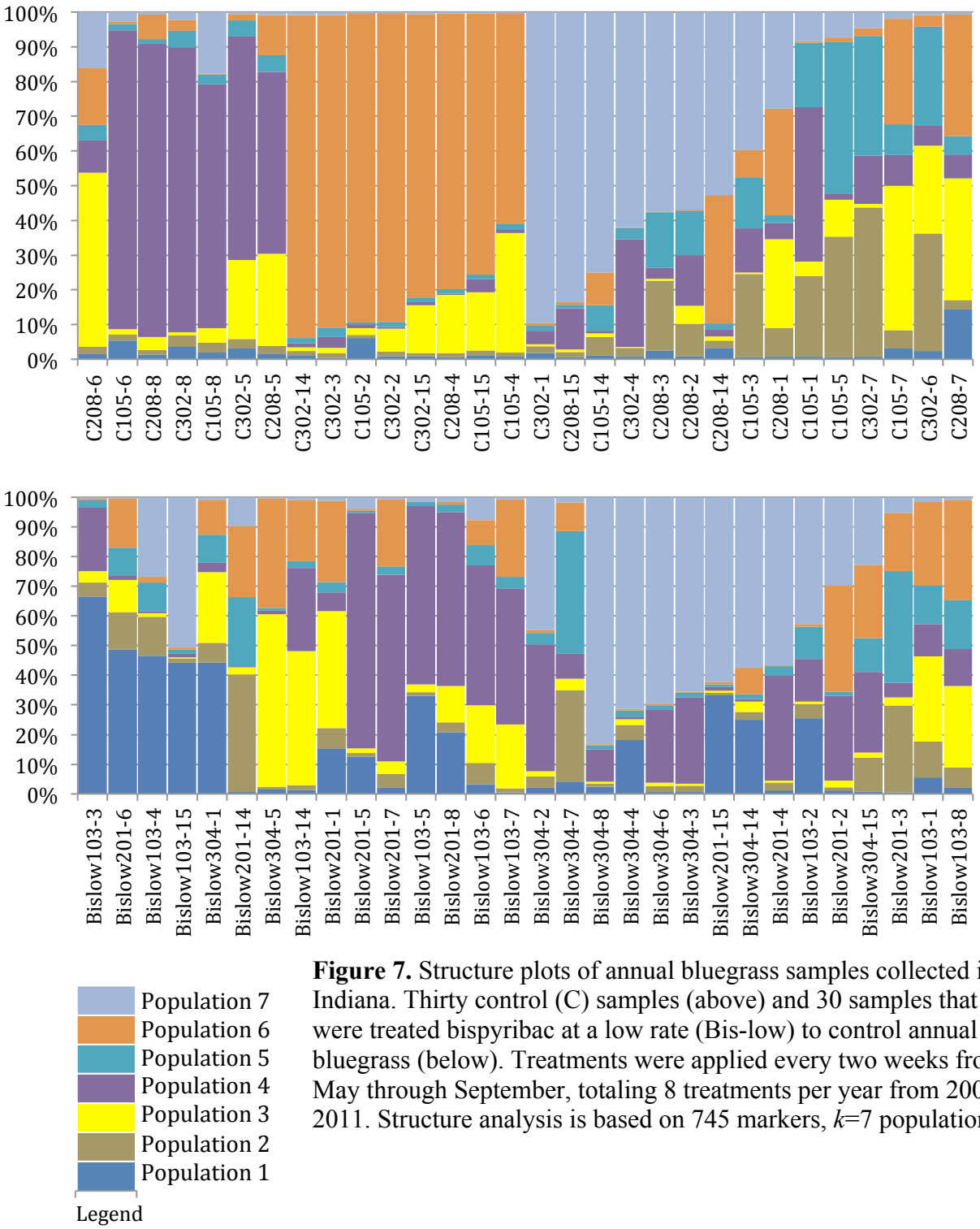


Figure 7. Structure plots of annual bluegrass samples collected in Indiana. Thirty control (C) samples (above) and 30 samples that were treated bispyribac at a low rate (Bis-low) to control annual bluegrass (below). Treatments were applied every two weeks from May through September, totaling 8 treatments per year from 2009-2011. Structure analysis is based on 745 markers, *k*=7 populations.

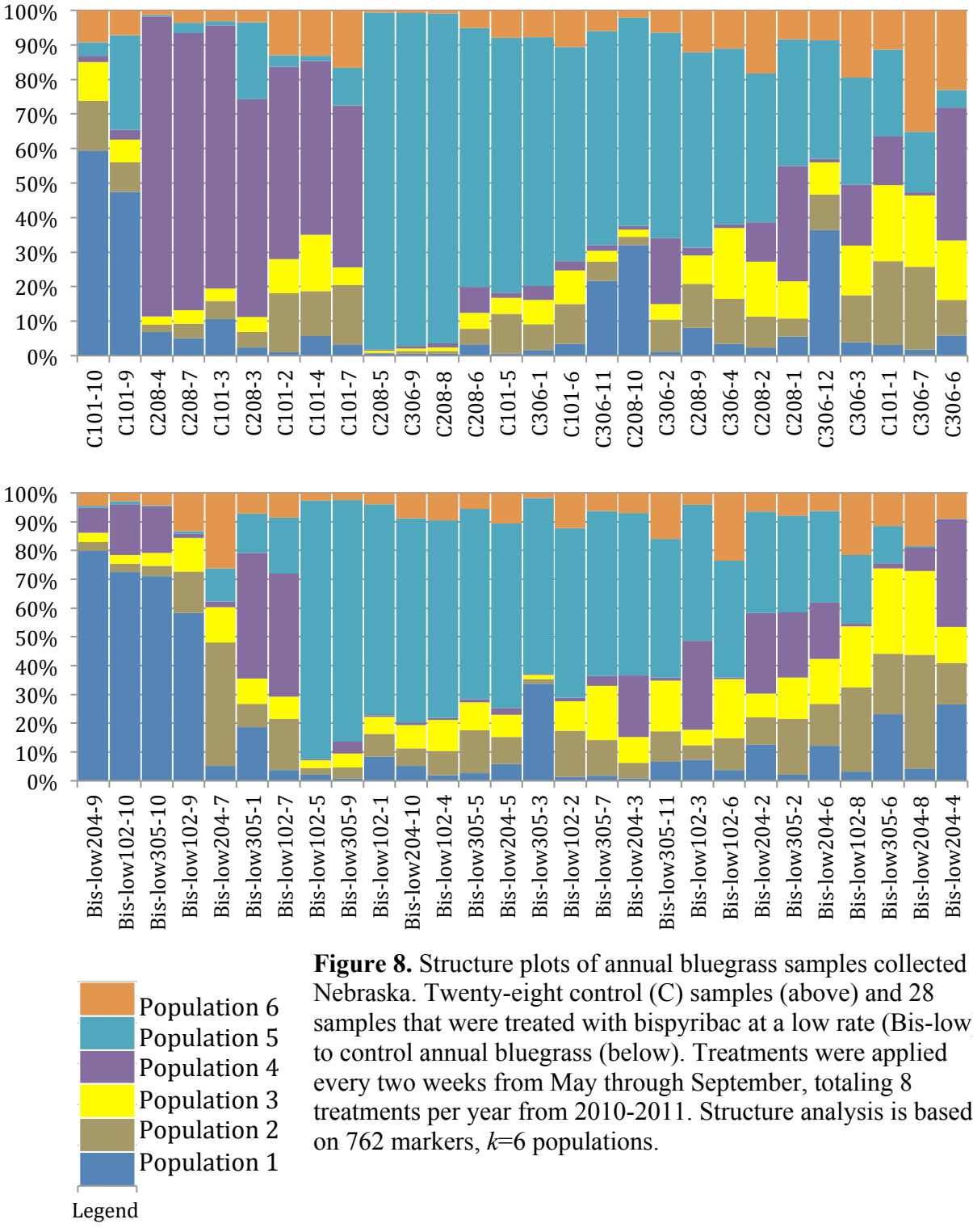


Figure 8. Structure plots of annual bluegrass samples collected in Nebraska. Twenty-eight control (C) samples (above) and 28 samples that were treated with bispyribac at a low rate (Bis-low) to control annual bluegrass (below). Treatments were applied every two weeks from May through September, totaling 8 treatments per year from 2010-2011. Structure analysis is based on 762 markers, *k*=6 populations.

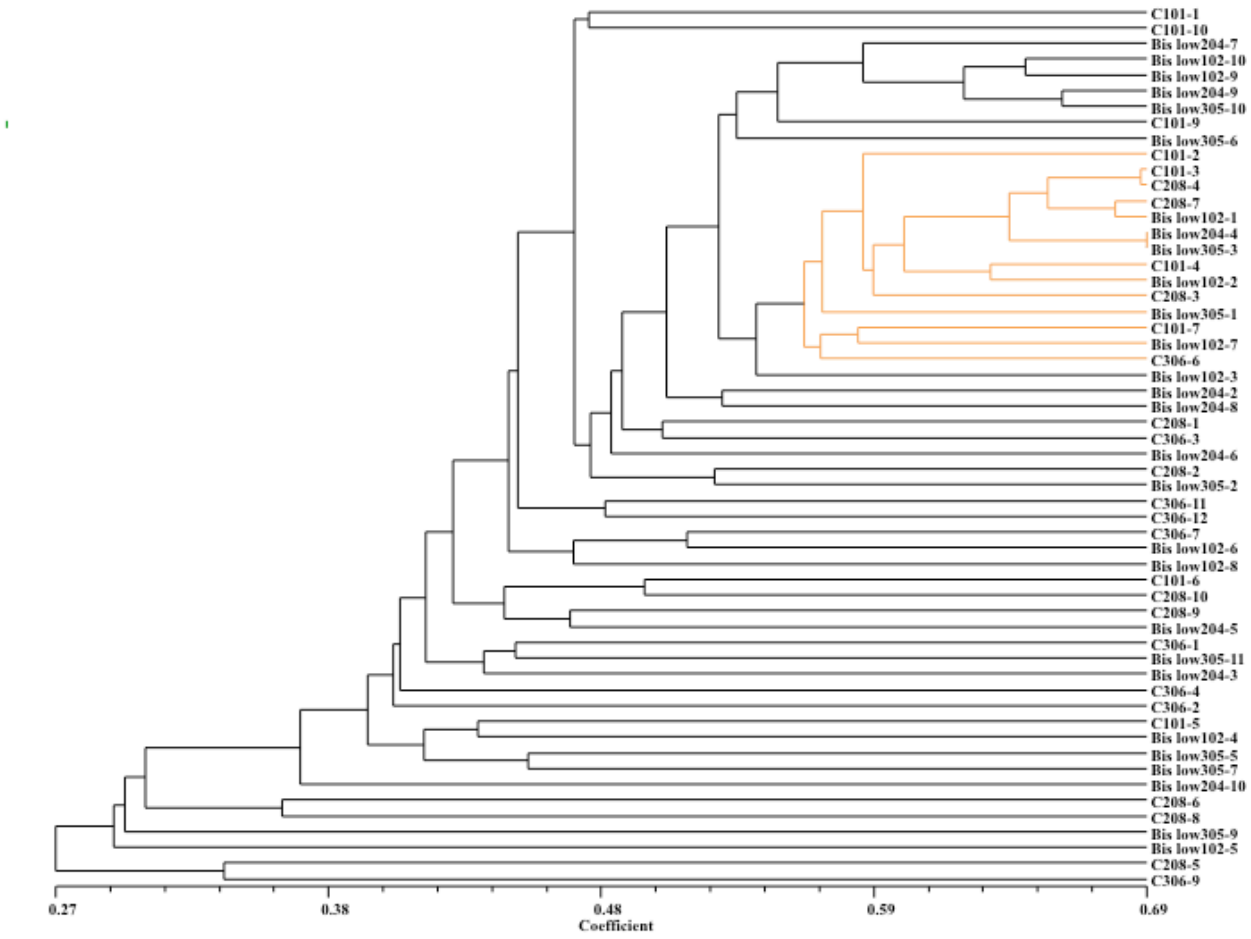


Figure 9. Unweighted pair-group method with arithmetic mean (UPGMA) analysis of samples based on the similarity matrix was employed using the sequential agglomerative hierarchical nested (SAHN) cluster analysis function using NTSYSpc v. 2.21o. Twenty-eight control samples (C) collected in Nebraska and 28 samples that were treated with bispyribac at a low rate (Bis-low) to control annual bluegrass. Treatments were applied every two weeks from May through September, totaling 8 treatments per year from 2010-2011.

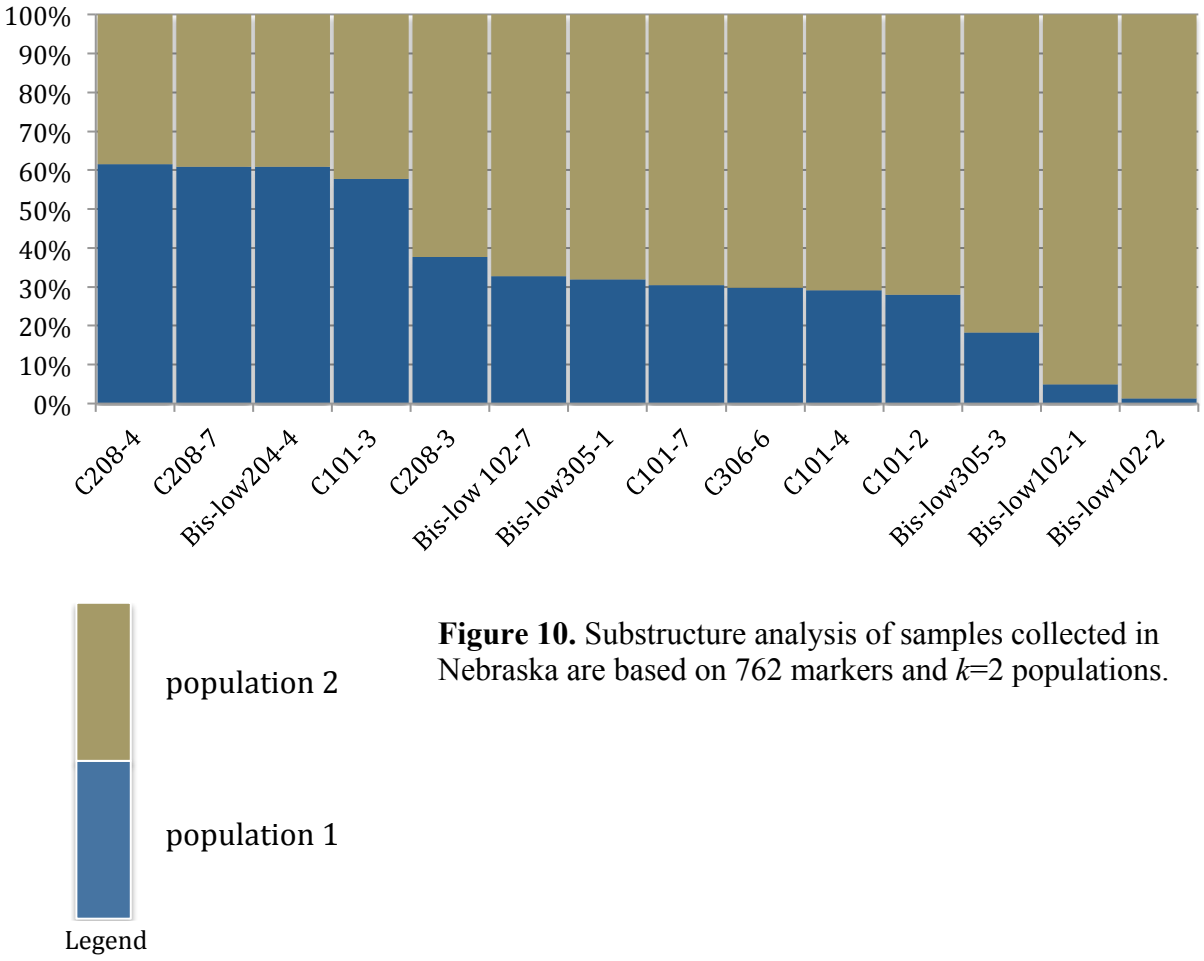


Figure 10. Substructure analysis of samples collected in Nebraska are based on 762 markers and *k*=2 populations.

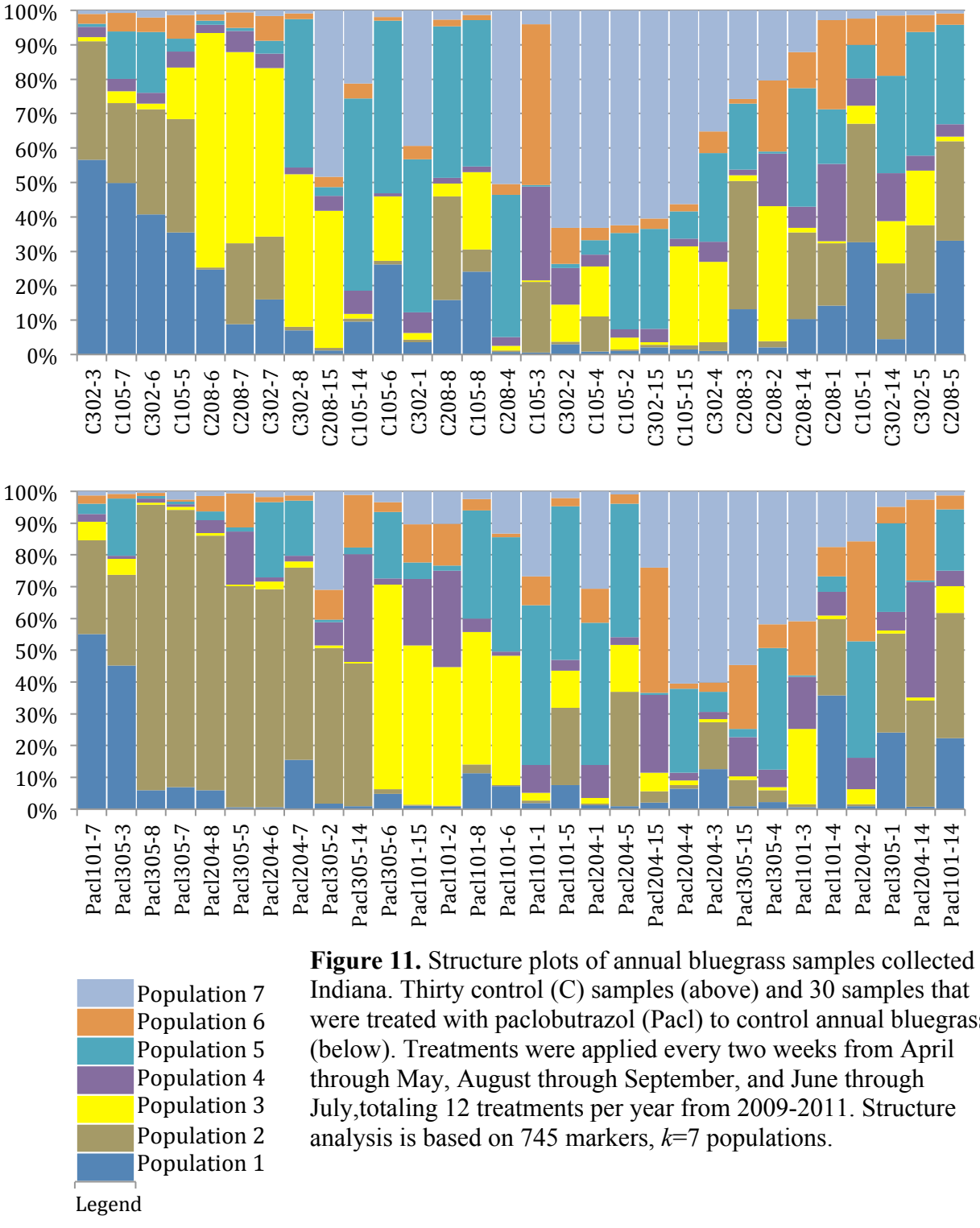


Figure 11. Structure plots of annual bluegrass samples collected in Indiana. Thirty control (C) samples (above) and 30 samples that were treated with paclobutrazol (Pacl) to control annual bluegrass (below). Treatments were applied every two weeks from April through May, August through September, and June through July,totaling 12 treatments per year from 2009-2011. Structure analysis is based on 745 markers, *k*=7 populations.

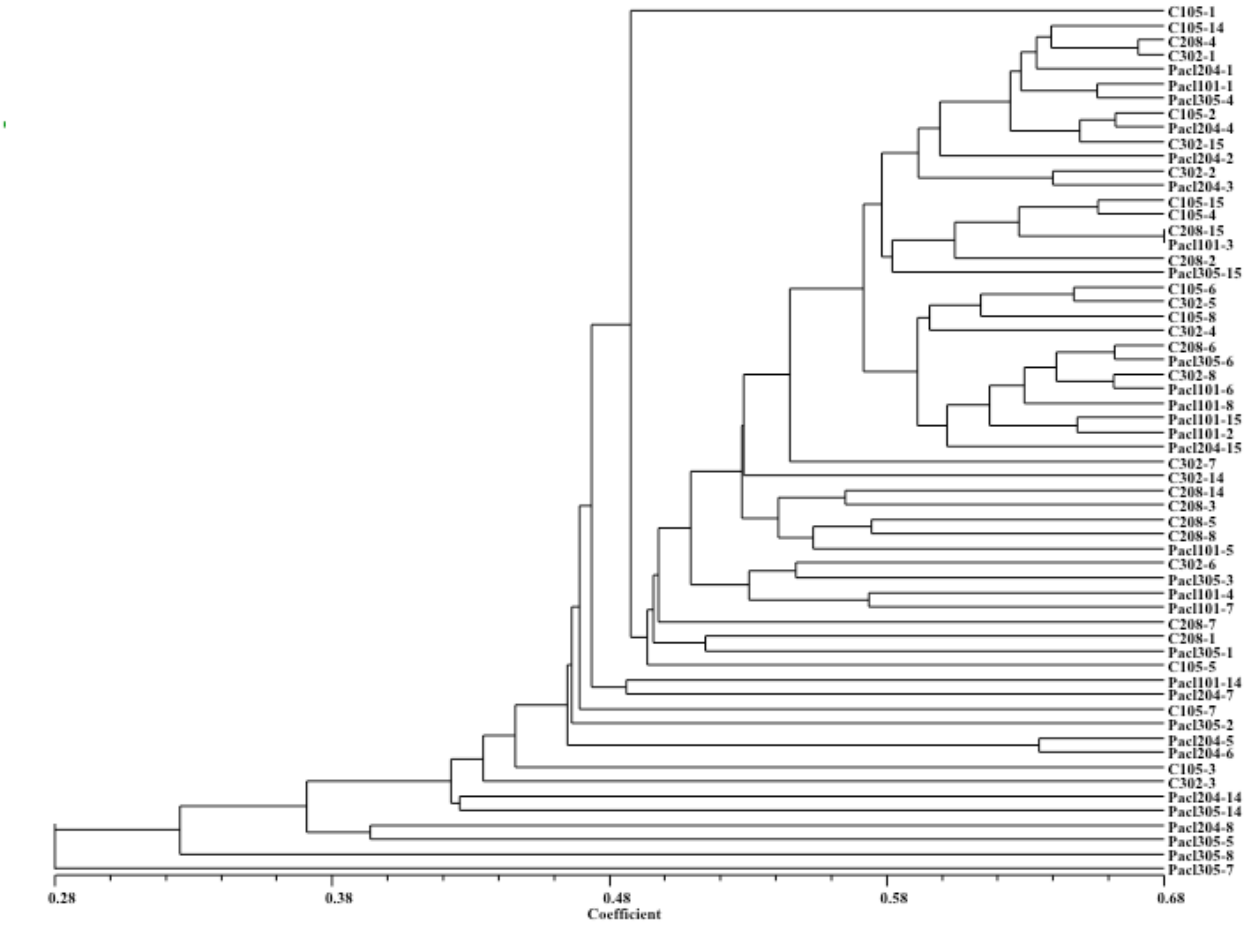


Figure 12. Unweighted pair-group method with arithmetic mean (UPGMA) analysis of samples based on the similarity matrix was employed using the sequential agglomerative hierarchical nested (SAHN) cluster analysis function using NTSYSpc v. 2.21o. Thirty control samples (C) collected in Indiana and 30 samples that were treated with paclobutrazol (Pacl) to control annual bluegrass. Treatments were applied every two weeks from April through May, August through September, and June through July, totaling 12 treatments per year from 2009-2011.

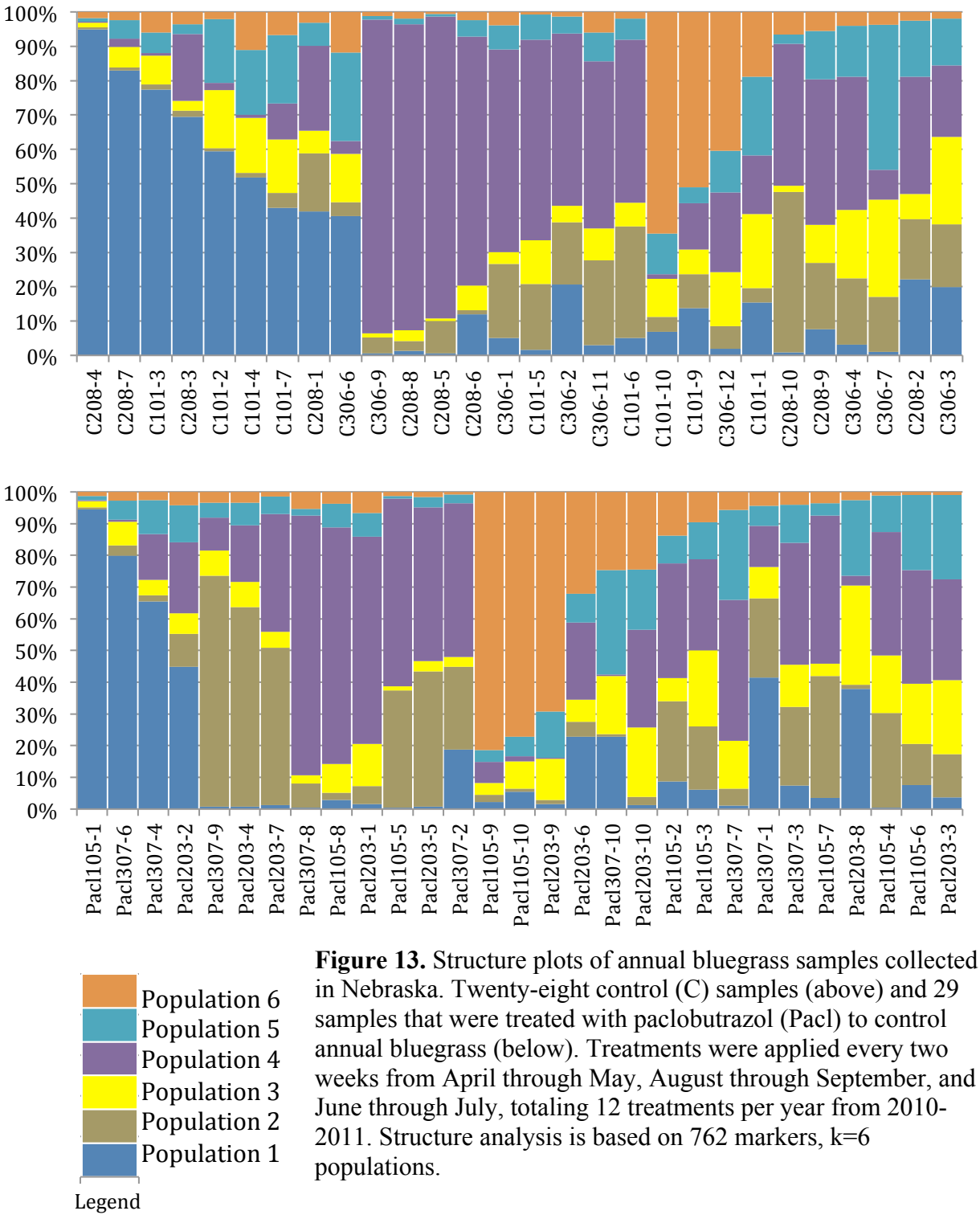


Figure 13. Structure plots of annual bluegrass samples collected in Nebraska. Twenty-eight control (C) samples (above) and 29 samples that were treated with paclobutrazol (Pacl) to control annual bluegrass (below). Treatments were applied every two weeks from April through May, August through September, and June through July, totaling 12 treatments per year from 2010-2011. Structure analysis is based on 762 markers, k=6 populations.

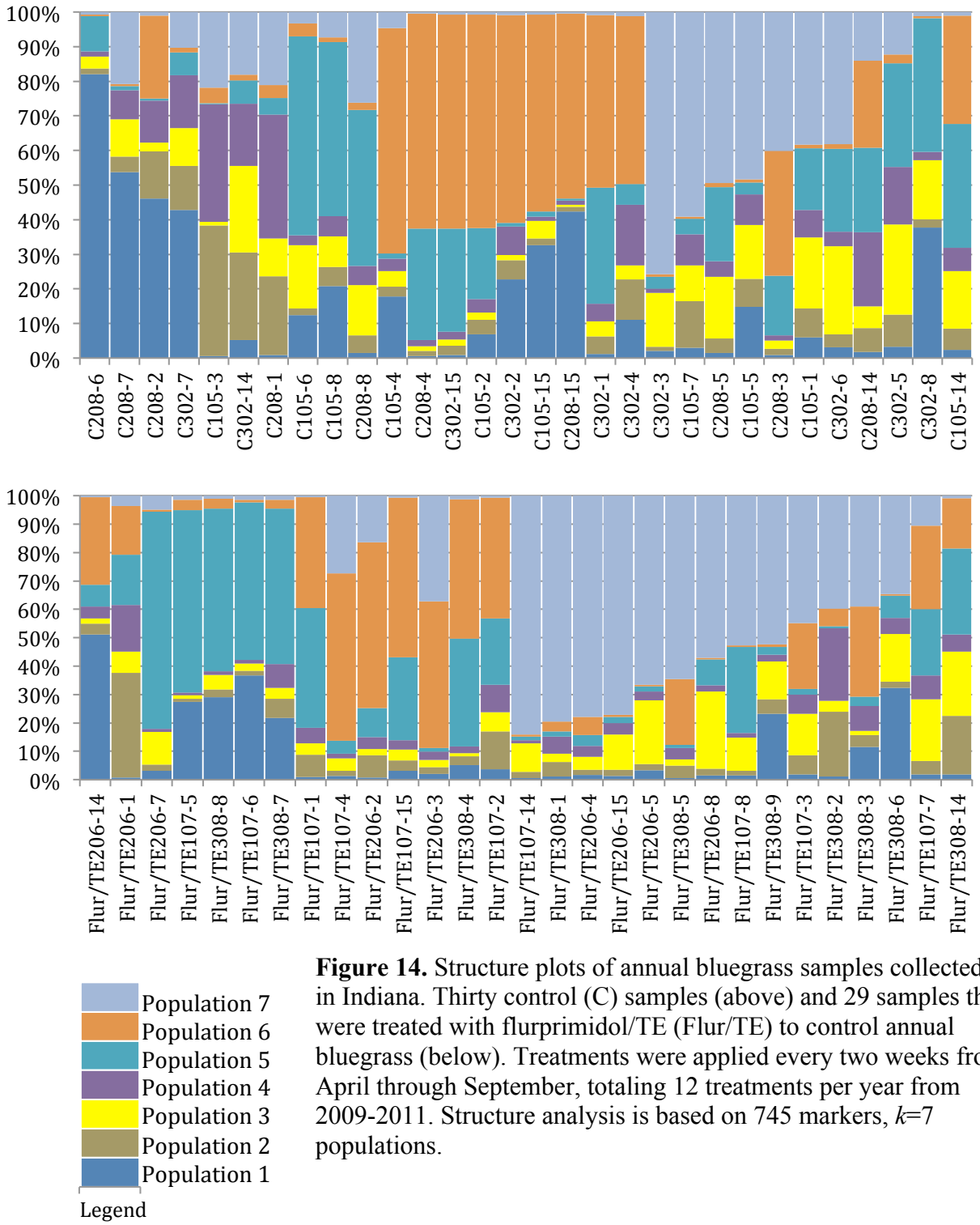


Figure 14. Structure plots of annual bluegrass samples collected in Indiana. Thirty control (C) samples (above) and 29 samples that were treated with flurprimidol/TE (Flur/TE) to control annual bluegrass (below). Treatments were applied every two weeks from April through September, totaling 12 treatments per year from 2009-2011. Structure analysis is based on 745 markers, *k*=7 populations.

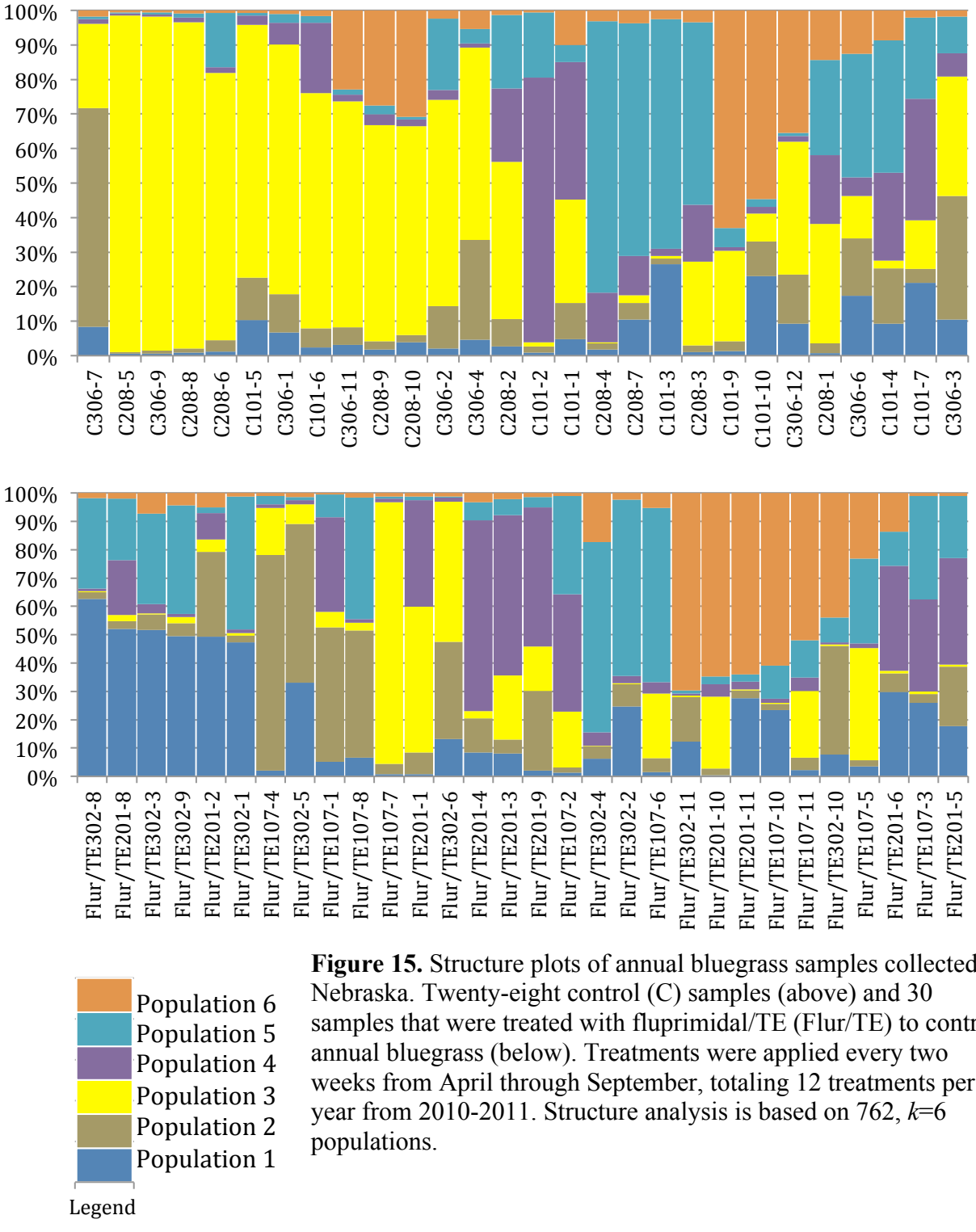


Figure 15. Structure plots of annual bluegrass samples collected in Nebraska. Twenty-eight control (C) samples (above) and 30 samples that were treated with fluprimidal/TE (Flur/TE) to control annual bluegrass (below). Treatments were applied every two weeks from April through September, totaling 12 treatments per year from 2010-2011. Structure analysis is based on 762, *k*=6 populations.

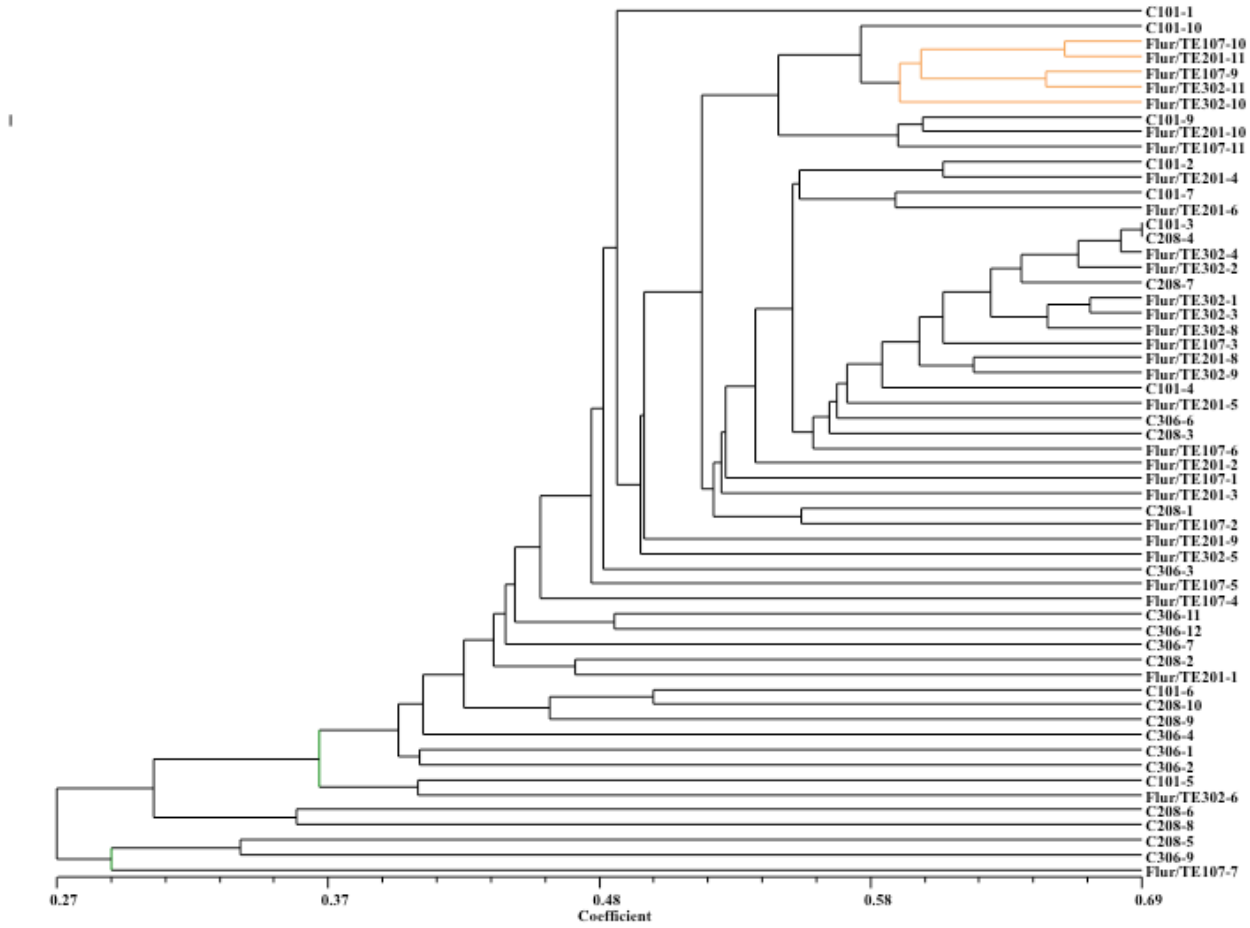


Figure 16. Unweighted pair-group method with arithmetic mean (UPGMA) analysis of samples based on the similarity matrix was employed using the sequential agglomerative hierarchical nested (SAHN) cluster analysis function using NTSYSpc v. 2.21o. Twenty-eight control samples (C) collected in Nebraska and 30 samples that were treated with fluprimidal/TE (Flur/TE) to control annual bluegrass. Treatments were applied every two weeks from April through September, totaling 12 treatments per year from 2010-2011.