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Genetic diversity within a population of *Microlaena stipoides* as revealed by AFLP markers

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Abstract

Microlaena stipoides (Labill.) R.Br. (microlaena), a C_3 perennial grass, is common within grazed native pastures in the high rainfall zone (> 550 mm average annual rainfall) of south-eastern Australia. It has the ability to spread via seed production or vegetatively using both rhizomes and stolons. This experiment aimed to determine how variable a microlaena population was within a single area, with the aim of determining if microlaena relied on seed or vegetative spread to sustain populations. Leaf samples of microlaena were collected from 85 locations, sampling two transects, within a pasture at Chiltern, in north-east Victoria (S36°12', E146°35'). The genetic diversity among samples was analysed using amplified fragment length polymorphism (AFLP) markers. We obtained 1,612 fragments using 10 primers combinations. Polymorphism for the markers ranged from 47 to 65%. These results indicated that the populations of microlaena that exist within the pasture at Chiltern are likely to have undergone some degree of outcrossing (F_{st} = 0.0219). It is likely that recruitment is occurring from sexual reproduction as well as via clonal spread within the microlaena population examined. This ability to use vegetative spread as well a both sexual and asexual reproduction may make populations of microlaena more resilient in the longer term.

Key words: Poaceae, native grass, genetic diversity

Introduction

Microlaena (*Microlaena stipoides* var. *stipoides* (Labill.) R.Br.) is a widely distributed native C_3 perennial grass that can be found throughout the high rainfall (> 550 mm annual average rainfall) zones from the mountains of Cape York Peninsula through New South Wales and Victoria to Tasmania, as well as in the wetter districts of South Australia and the south west of Western Australia (Sharp and Simon 2002). Microlaena is an important species in many grazed native pastures (Simpson and Langford 1996). It has been described as spreading vegetatively via both

rhizomes and stolons (Jacobs *et al.* 2008), where rhizomes can be defined as modified stems that remain underground and stolons are above ground, both developing roots at the nodes (Renvoize 2002). In a pasture situation, microlaena seed yields can be substantial (mean of 800 seeds m^{-2}); but, seed predation can be as high as 30% within a 24-h period (Mitchell *et al.* 2014). Microlaena is present at low levels in the seed bank and does recruit, albeit at modest levels (five seedlings m^{-2}) (Mitchell *et al.* 2014). This raises the question as to whether microlaena populations are maintained by recruitment of new individuals or vegetative spread.

Microlaena is a tetraploid grass with 2n=48 (Murray *et al.* 2005) and a genome size of approximately 869 mega base pairs (Nock *et al.* 2011). Three breeding systems operate in microlaena: chasmogamy (fertilisation occurs in open flowers), cleistogamy (fertilisation occurs within closed flowers) and cleistogenes (produced from the axillary buds at the lower nodes of the flowering culm) (Clifford 1962; Edgar and Connor 1998; Groves and Whalley 2002). Nevertheless, there has been relatively little research into the reproductive biology of microlaena. Due to the diversity and propagation strategies for microlaena, it is not known how much genetic diversity occurs within this species.

Only a limited amount of information is available about the genetic diversity of microlaena (Shapter *et al.* 2013). Genotypic variation among microlaena individuals within a pasture was revealed by visual assessments of DNA bands produced using the Random Amplified Polymorphic DNA (RAPD) technique (Magcale-Macandog and Whalley 2000). The RAPD technique that was used in this study is sensitive to changes in assay conditions and concerns about its repeatability and transferability between laboratories have been raised (Bryan 2006). Another study using whole-chloroplast genome sequences examined the genetic diversity of microlaena along a 240 km longitudinal transect in Victoria, with greatest diversity being recorded in drier environments (Fitzgerald *et al.* 2010). However, neither of these studies can assist in our understanding of the population biology of microlaena.

Species that propagate vegetatively are problematic for population analysis because identification of genetically individual plants can be difficult (Harper 1977). However, the advent of plant molecular genetics in the late 1980s allowed for the measurement and description of diversity by the analysis of genetic markers (Bryan 2006). Amplified fragment length polymorphism (AFLP) is a molecular genetic technique that is often used to assess genetic variability within a population (Vos *et al.* 1995). The AFLP technique is based on selective polymerase chain reaction (PCR) amplification of restriction fragments from a complete digest of genomic DNA (Vos *et al.* 1995). It produces a high number of polymorphic informative markers per primer pair from a small amount of DNA and has proved to be robust, reliable and reproducible (Vos *et al.* 1995; Hayashi *et al.* 2005). The AFLP technique is most useful for distinguishing populations within a species, rather than distinguishing species within a genus (Moncada *et al.* 2005). The AFLP method detects variation across the whole genome, spanning both coding and non-coding DNA regions and may therefore be more representative of overall genetic patterns (Koopman 2005; Meudt and Clarke 2007). Compared to other fingerprinting techniques AFLP shows increased reproducibility and does not require any prior knowledge of the analysed genomes (Turner *et al.* 2013). However, in some instances the AFLP may not be the best method, especially if fragments are non-homology and non-independence and problems in distinguishing heterozygote from homozygote bands (e.g. Koopman 2005; Meudt and Clarke 2007).

Within a number of grass species, the AFLP has been used to characterise variation (Mengistu *et al.* 2000; Hodkinson *et al.* 2002; Szczepaniak *et al.* 2002; Li *et al.* 2009), and in particular, genetic variability in species that spread vegetatively (Subudhi *et al.* 2005; Hol *et al.* 2008).

The detection of genetic diversity by using molecular techniques such as AFLP may assist in determining how much reliance microlaena has on asexual (vegetative spread and cleistogamous flowering) versus sexual (chasmogamous flowering) reproduction. The objective of this study was

to determine the level of polymorphism that exists within a population of microlaena. The approach taken was to intensively sample a microlaena-dominated native pasture.

Methods

Site descriptions

The experimental site was located at Chiltern, in north-east Victoria (S36°12’, E146°35’), within an existing native grass pasture, the dominant component of which is microlaena (19% basal cover and 40% of DM). A range of soil types exist across the site, including Red Kurosol (Isbell 1996) on the upper slopes, Brown Kandosol on the mid-slope and Grey Kurosol on the lower slopes. Soils across the site are acidic (pH_{CaCl2} 4.2) and low in phosphorus fertility (Colwell P 22.4 mg/kg). The profile has a moderate soil depth (weathered rock at approximately 0.8 m depth).

Sample collection

Two sites within a paddock at Chiltern were chosen and four transects laid out, which roughly aligned to the compass directions of north, south, east and west. Sampling was undertaken on 29 March 2010. Samples were collected at two sites, along transects (Figure 1). The distance between the centres of the sites was 270 m. At the closest point the transects were 140 m apart. For Site 1, these transects will subsequently be referred to as Site 1 north, Site 1 south, Site 1 east and Site 1 west. Site 2 follows the same nomenclature. A sample was collected in the centre and then at 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, 51.2, 102.4 m from the centre in each directions. Longitude and latitude were recorded using a global positioning system (GPS, Garmin eTrax Vista) for each sampling location.

Four 50-mm mid-section leaf segments were collected from microlaena plants. Leaves were selected for sampling based on being healthy, fully expanded and of comparable age. Young fresh leaf material was collected and placed in labelled zip lock plastic bags on ice. These samples were then stored at –80 °C.

Leaf width across the lamina at the widest point and colour (Munsell 1968) were recorded for all samples to provide some indication of the morphological variation that existed within the populations. Leaf width was measured using digital callipers (accurate to 0.01mm).

In some instances, a plant could not be found at the specified distance. A total of 85 of a possible 88 samples were collected.

(insert Figure 1)

DNA extraction

DNA extraction followed the method of Guidet *et al.* (1991): frozen leaf material was finely ground in liquid nitrogen, transferred to a 10-ml plastic tube and suspended in 2 ml of extraction buffer (200 mM Tris-HCl, pH 8.5; 250 mM NaCl; 25 mM EDTA). Then, an equal volume of phenol-chloroform was added. The tubes were centrifuged at 5,000 rpm for 12 min; the upper aqueous phase was removed into a new 10-ml tube and an equal volume of chloroform:isoamyl alcohol (24:1) was added to each sample. The samples were mixed by inversion to form an emulsion, and then centrifuged at 5,000 rpm for 12 min. The upper aqueous phase was removed to a new tube and ⅓ volume 3 M NaOAc and ⅔ volume of isopropanol was added to each sample, mixed by inversion, and then stored at –20 °C overnight. The samples were then centrifuged at 5,000 rpm for 15 min and the liquid was drained, taking care not to disturb the pellet. Two ml of 70% ethanol was added and mixed for 3 min by inversion. The samples were then centrifuged at 5,000 rpm for 8 min. A fine pipette tip was used to remove as much liquid as possible, and the pellets were dried in an incubator at 30°C for approximately 30 min. DNA was resuspended in TE buffer (10 mM Tris-Cl [pH 8.0], 1 mM EDTA [pH 8.0]). DNA concentrations and purity were determined by separating the DNA sample by electrophoresis on 1% agarose gel and comparing DNA band intensities with a DNA standard of known DNA concentration under UV light.

Preselective amplification

The AFLP analysis was performed based on the protocol of Vos *et al.* (1995). Genomic DNA (30µl of sample) was digested to completion with the restriction enzymes *Eco*R1 and *Mse*1, following the manufacturer's directions (New England Biolabs, Marylands, USA). Preamplification of restriction fragments was carried out using the primers Eco and Mse, in 60 µl reactions. The concentration of the amplified DNA was checked in 1% agarose gel.

Selective amplification

Selective amplification was performed using 10 primer combinations (Table 1**Error! Reference source not found.**). Reactions contained 0.5 µM fluorescently labelled Mse+3 primer, 0.5 µM Eco+3 primer, 0.2 µM dNTP and 1.5 mM $MgCl_2$. Amplifications were performed using a touchdown PCR cycle from 60°C to 50°C. All amplifications were performed on Hybaid PCR express.

(insert Table 1**Error! Reference source not found.**)

Fragment analysis

The AFLP fragments were separated and detected using a CEQ GeXP Genetic Analysis System, following the manufacturer's procedures (Beckman Coulter, USA). The amplification product (0.8 µl) was added to 39 µl of sample loading solution and 0.5 µl internal size standard (600 base pairs; Beckman Coulter).

Data analysis

Leaf width was analysed using the generalised ANOVA in GenStat V13 (Payne *et al.* 2010).

The AFLP fragments were analysed using the GeXP software, with confidence limits set to 98%, a 10% slope threshold and two nucleotide separation differences. A binary matrix was obtained from the fragment size data using the CEQ GeXP (Beckman Coulter, USA) software with the Fragment Algorithms version 2.2.1 to give a genotype for each sample. The number of polymorphic fragments were calculated using the program GenAIEX v6.04 (Peakall and Smouse 2006). Analysis of molecular variance (AMOVA) was undertaken using the program GenAIEX (Peakall and

Smouse 2006) to examine diversity at the Chiltern site. Principal coordinate analysis (PCO) was undertaken using the program PAST (Hammer *et al.* 2001). Descriptive statistics indicating within-population genetic diversity of the populations was undertaken using the program PAST (Hammer *et al.* 2001) using the Lynch and Milligan (1994) method. Results were reported in terms of n: number of individuals scored; #loc: number of loci scored; #loc_P: number of polymorphic loci at the 5% level, i.e., loci with allelic frequencies lying within the range 0.05 to 0.95; PLP: proportion of polymorphic loci at the 5% level, expressed as a percentage; Hj: expected heterozygosity under Hardy-Weinberg genotypic proportions, also called Nei's gene diversity (analogous to H or He in most publications); S.E. (Hj): standard error of Hj; Var(Hj): variance of Hj; VarI(Hj): variance component of Hj due to sampling of individuals (finite sample size); VarI%: proportion of Var(Hj) due to sampling of individuals; VarL(Hj): variance component of Hj due to sampling of loci, VarL%: proportion of Var(Hj) due to sampling of loci. These calculations were performed in PAST (Hammer *et al.* 2001).

The number of polymorphic fragments was calculated using the program AFLP Surv 1.0 (Vekemans 2002), and allelic frequencies were calculated using a Bayesian method with non-uniform prior distribution with 1,000 permutations. Results were reported in terms of Ht: the total gene diversity; Hw: the mean gene diversity within populations (analogous to Nei's Hs); Hb: the average gene diversity among populations in excess of that observed within populations (analogous to Nei's Dst), or genetic differentiation among populations; Fst: Wright's fixation index, measuring the genetic correlation between pairs of genes sampled within a population relative to pairs of genes sampled within the overall set of populations (also interpreted as the proportion of the total gene diversity that occurs among, as opposed to within, populations).

Results

There were morphological differences among the microlaena populations sampled, with differences in the leaf widths and colours. Limited variation in terms of leaf width was found within each site, however significant variation between sites. The mean leaf widths from Site 1 (4.5 ± 0.1 mm) were greater ($P < 0.001$) than those from Site 2 (3.9 ± 0.1). The mean leaf colour from Site 1 was 7.5GY5/6 whereas Site 2 was 7.5GY4/6. These morphological differences may be the result of growing conditions.

An AFLP analysis was performed to determine the extent of genetic diversity that existed within the 85 individuals of the microlaena population. Ten polymorphic primer combinations amplified 1,612 fragments within the 85 samples (Table 1). Fragments within the range of 60 to 674 bp formed the basis of the analysis (the average fragment size analysed was 218.99; Sd = 123.92). There was no bias in the use of these markers because they detected diversity between each of the populations.

(insert Table 1)

The AMOVA indicated that only 4% of the genetic variation at Chiltern resided between, and 96% within the populations at the two sites ($P < 0.01$, Table 2).

(insert Table 2)

The PCO indicated that there was some overlap between the populations from Site 1 and Site 2. The first two axes explained a total of 26.7% (14.56% and 12.2% respectively) of the molecular variance among the populations (Figure 2).

The expected heterozygosity (Hj) is a measure of the genetic variation within a population. There was similar genetic variation within each of the sites sampled. When the two populations of microlaena that were sampled are considered, the gene diversity is in a range between 0.22975±0.00415 for Site 1 to 0.23888±0.00434 for Site 2 (Table 3).

The Wright's fixation index (F_{ST}) is a measure of population differentiation, genetic distance, based on genetic polymorphism data. An estimation of Wright's F_{ST} for the two sites at Chiltern was 2%

($P < 0.0001$, based on 1,000 random permutations of individuals among species) (Table 4). This F_{ST} value indicates that the samples were genetically more differentiated than random assemblages of the individuals. There was limited differentiation of the populations sampled.

(insert Table 3 and Table 4)

Discussion

Before European settlement, open eucalyptus woodlands (in which the main species were *Eucalyptus macrorhyncha* F. Muell. ex. Benth., *E. blakelyi* Maiden, *E. macrocarpa* [Maiden] Maiden and *E. sideroxylon* A.Cunn. ex Woolls) formed a relatively continuous vegetation throughout the sheep-wheat belt of south-eastern Australia (Beadle 1981). The understorey in these grassy woodlands was normally dominated by tall cool-season (e.g. *Poa sieberiana* Spreng.) or warm-season (e.g. *Themeda triandra* Forsk.) tussock-forming perennial grasses (Lindsay and Cunningham 2011). The woodlands in the area around Chiltern would have been cleared after the discovery of gold in the region in the 1850s (Ashley 1974). This vegetation clearing and the introduction of grazing by introduced livestock may have led to the development of the microlaena-dominated native pasture that exists in the area today.

Microlaena populations have moderate levels of polymorphism with the primer pairs used. This research found polymorphism for the markers ranging from 47 to 65% (Table 1). Microlaena has been recorded as a highly variable species in terms of its morphology, with leaves that ranges from fine to broad, coarse (Whalley and Huxtable 1993; Whalley and Jones 1998). The variation in leaf width and colour in our study was much less than was expected.

Given the potential of microlaena to spread vegetatively, via rhizome and stolons (Mitchell *et al.* 2013), it is possible that clonality would exist within the pasture. To detect this, a finer scale sampling would be required. The smallest between-plant distance within this study was 0.1 m.

Three breeding systems operate in microlaena: chasmogamy (fertilisation occurs in open flowers), cleistogamy (fertilisation occurs within closed flowers) and cleistogenes (produced from the axillary buds at the lower nodes of the flowering culm) (Clifford 1962; Edgar and Connor 1998; Groves and Whalley 2002). Previous literature has recorded chasmogamous flowering as infrequent in microlaena (Clifford 1962; Edgar and Connor 1998; Groves and Whalley 2002; Davies *et al.* 2005). The occurrence of chasmogamous (open) flowering and hence outcrossing in populations of microlaena may be higher than previously recorded (Mitchell *et al.* 2014). The F_{ST} is a measure of genetic differentiation, which can range from 0.0 (no differentiation) to 1.0 (complete differentiation). Heywood (1991) concluded that obligate outcrossing species have F_{ST} ranging from 0.004 to 0.08, and self-pollinating species have values between 0.026 to 0.78. The F_{ST} results (0.0219) from this study indicate that the microlaena populations at Chiltern are likely to be undergoing a degree of outcrossing. Even though the rate of seedling recruitment would appear to be low within the microlaena population at Chiltern (Mitchell *et al.* 2014), it would appear to be enough over time to produce a diverse population within the pasture.

This study found a significant amount of genetic variation within populations (96%; Table 2), indicating that the 85 microlaena individuals from the population sampled are genetically distinct individuals. This may indicate that sexual reproduction has played a significant role in the development of the microlaena population at Chiltern. The expected Hj is a measure of the genetic variation within a population. In this study, the mean value of the Hj was 0.2343 (Table 3), which could be considered a low degree of similarity, or that the populations are diverse. The patterns of AFLP variation observed in this study are comparable with other studies that have examined the genetic diversity within populations of grasses using AFLP markers. For example, 81% AFLP

variation was found within three populations of mountain rough fescue (*Festuca campestris* Rydb.) (Fu *et al.* 2005), 93% within six populations of little bluestem (*Schizachyrium scoparium* [Michx.] Nash) (Fu *et al.* 2004) and 64% within 12 populations of switchgrass (*Panicum virgatum* L.) (Cortese *et al.* 2010). In contrast, Subudhi *et al.* (2005) examined 19 populations of sea oats (*Uniola paniculata* L.), a species that is known to have low seed set, low rates of germination and seedling emergence, and to extensively spread via clonal reproduction, and found 34% AFLP variation within an accession. The authors attributed this result to the clonal nature of the species.

Future work is needed to understand the genetic diversity within microlaena more fully. One approach that could be used is to undertake AFLP analysis on microlaena seed. The collected seed from individual microlaena plants could be used to examine the genetic diversity that exists within each of the breeding systems that operate within microlaena.

This study has revealed that genetic variation exists within microlaena populations. Microlaena outcrosses more than previously thought; populations are composed of plants that have developed from both asexual and sexual reproduction. Since there are a number of microlaena cultivars that are commercially available, this result may have implications for the maintenance of these cultivars over time. The previous assumption has been that there is limited sexual reproduction within this species.

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Table 1. Selected primer combinations and polymorphism levels for AFLP analysis of *Microlaena stipoides*

Primer combination		Fragment range bp (min-max)	No. of loci	Mean % of polymorphic loci (se)	H_e mean (se)
Eco primer	Mse primer				
ACC	AGA	60-402	93	47.0 (9.7)	0.144 (0.007)
ACC	CTT	61-662	130	50.9 (10.5)	0.750 (0.006)
AAC	AGA	63-521	110	48.8 (11.3)	0.141 (0.006)
AAC	CTT	75-593	165	59.1 (8.4)	0.712 (0.005)
AGA	AGA	62-643	192	64.4 (7.1)	0.214 (0.005)
AGA	CTT	63-646	233	60.8 (7.6)	0.217 (0.005)
AGG	AGA	62-674	172	55.8 (8.2)	0.169 (0.005)
AGG	CTT	63-636	221	59.1 (8.4)	0.203 (0.005)
AGC	AGA	60-423	152	55.0 (9.1)	0.190 (0.006)
AGC	CTT	62-509	153	65.0 (7.0)	0.209 (0.006)

Table 2: Analysis of molecular variance (AMOVA) for 85 individuals from two sites within the Microlaena pasture at Chiltern as implemented by GenAlEx software (Peakall and Smouse 2006)

Source	df	Sum of squares	Means square	Estimated variance	% variation	*P* value
Between pops	1	672.09	672.09	10.50	4%	0.01
Within pops	83	18,986.71	228.76	228.76	96%	0.01
Total	84	19,658.80		239.26	100%	0.01

Table 3. Descriptive statistics indicating within-population genetic diversity of populations of *Microlaena stipoides* at a site at Chiltern, Victoria

Population	n	#loc	#loc_P	PLP	Hj	S.E.(Hj)	Var(Hj)	VarI(Hj)	VarI%	VarL(Hj)	VarL%
Site-1	39	1621	1104	68.1	0.22975	0.00415	0.000017	0.000001	6.6	0.000016	93.4
Site-2	46	1621	1061	65.5	0.23888	0.00434	0.000019	0.000001	4.9	0.000018	95.1

Where n: number of individuals scored; #loc: number of loci scored; #loc_P: number of polymorphic loci at the 5% level, i.e., loci with allelic frequencies lying within the range 0.05 to 0.95; PLP: proportion of polymorphic loci at the 5% level, expressed as a percentage; Hj: expected heterozygosity under Hardy-Weinberg genotypic proportions, also called Nei's gene diversity (analogous to H or He in most publications); S.E. (Hj): standard error of Hj; Var(Hj): variance of Hj; VarI(Hj): variance component of Hj due to sampling of individuals (finite sample size); VarI%: proportion of Var(Hj) due to sampling of individuals; VarL(Hj) : variance component of Hj due to sampling of loci, VarL% : proportion of Var(Hj) due to sampling of loci. Calculated in PAST (Hammer *et al.* 2001) using the Lynch and Milligan (1994) method.

Table 4. Descriptive statistics indicating between-population genetic diversity of populations of *Microlaena stipoides* at a site at Chiltern, Victoria

Number of populations	Ht	Hw	Hb	F_{ST}
2	0.2396	0.2343	0.0052	0.0219
S.E.		0.004568	0	0.019064
Var		0.000021	0	0.000363

Where Ht: the total gene diversity; Hw: the mean gene diversity within populations (analogous to Nei's Hs); Hb: the average gene diversity among populations in excess of that observed within populations (analogous to Nei's Dst), or genetic differentiation among populations; Fst: Wright's fixation index, measuring the genetic correlation between pairs of

genes sampled within a population relative to pairs of genes sampled within the overall set of populations (also interpreted as the proportion of the total gene diversity that occurs among as opposed to within populations).

Figure 1. Location of the two sample collection sites and transects within the Microlaena-dominated pasture at Chiltern. At each site there were four transects, north (orange), south (pink), east (red) and west (blue). Each transect was 102.4 m in length.

Figure 2. Principal coordinates analysis (PCO) of Microlaena AFLP data using Dice distances. Site 1 (   ) and Site 2 (   ) can be separated on the first two axes of the PCO and these cumulatively account for 26.73% (14.56% and 12.17% respectively) of the variance.

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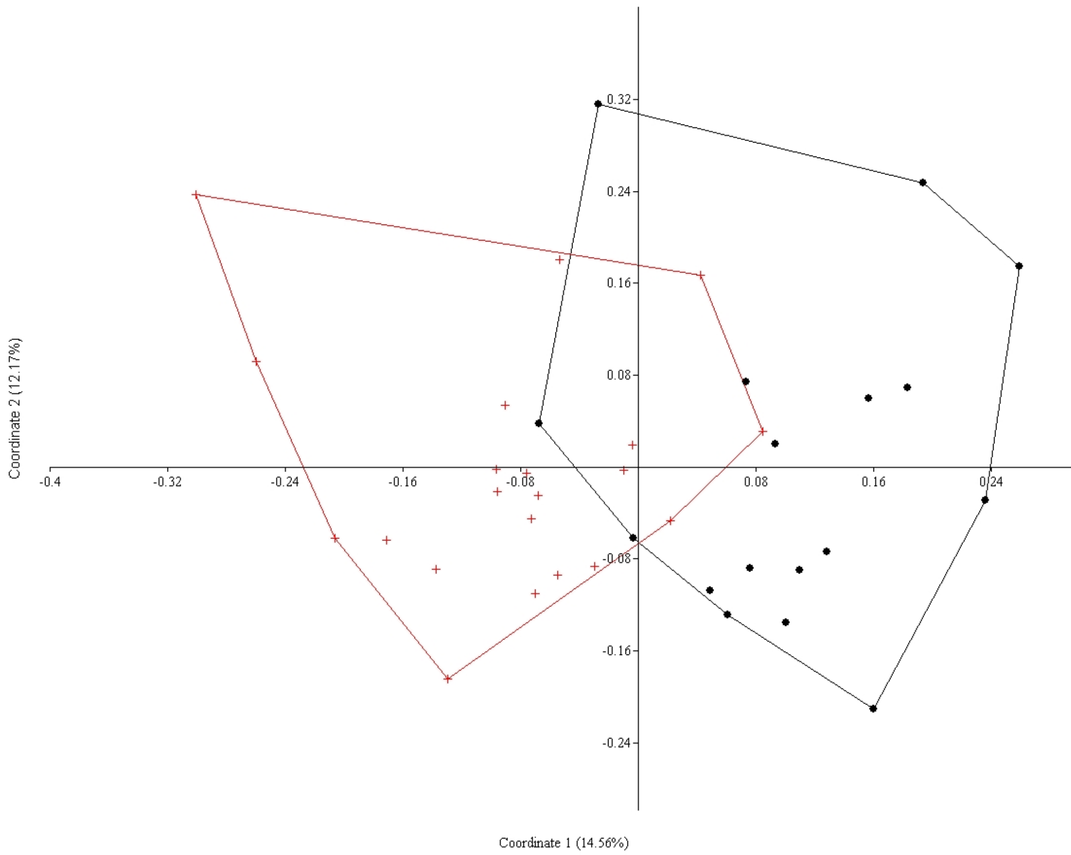

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