FINGERPRINTING *PENNISETUM PURPUREUM* SCHUMACH. VARIETIES AND CULTIVARS USING AFLP ANALYSES

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Dissertation submitted in partial fulfillment of the requirements for the degree Masters of Environmental Science at the North West University (Potchefstroom campus)

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2007

Grass

I have written of dawn, of the moon, and the trees;
Of people, and flowers, and the song of the bees.
But over these things my mind would pass,
And come to rest among the grass.

Grass so humble, that all things tread
Its tender blades. Grass - the bread,
The staff of life; a constant need
Of man and beast – a power indeed.

Grass, so vagrant – does anything stray
With such gallant courage? The hardest way
Is coaxed and beguiled by the wayward grace
Of the constant friend of every space.

God in His wisdom gave many friends
To grace our way, as long it wends.
But the grandeur of many, my mind would pass,
And come to rest among the grass.....

Mabel Duggan

ACKNOWLEDGEMENTS

First and foremost I would like to thank my Heavenly Father for the opportunity and for the courage and strength to complete this study to the best of the abilities that He gave me.

Prof J. van den Berg, and Dr. M.H. Buys for their endless patients and help.

Very special thanks to Dr. C.M.S. Mienie, without whom I could not have completed this study.

ARC-Grain Crops Institute for use of their laboratories and facilities, as well as the staff for all their help and friendliness.

The following persons and institutes who kindly provided me with plant material and information:

Sigrun Ammun, ARC Cedara

Karen Dearlove, Marike Trytsman, Magda Kleyn and Dr. At Kruger, ARC Roodeplaat

John Cunningham, KZN Department of Agriculture Estcourt

Peter Wandera, Department of Agricultural Research, Gaborone, Botswana

Jean Hanson, International Livestock Research Institute, Ethiopia

Dr. Domingos Cugala, Eduardo Mondlana University, Maputo, Mozambique

Anchèn van der Walt for all her encouragement and help with fieldwork.

My friends and family for their support and encouragement.

DECLARATION

I hereby declare that the work contained in this dissertation is my own work and has not previously in its entirety or in part been submitted at any university for a degree.

Signature: _____

Date: _____

ABSTRACT

Pennisetum Rich. is one of the most important genera in the family Poaceae because it includes forage and crop species such as *Pennisetum purpureum* Schumach. and *Pennisetum glaucum* (L.) R. Br. Both *P. purpureum* and *P. glaucum* have a number of cultivars and varieties arising due to natural crossing which are very difficult to distinguish morphologically. *P. purpureum* and *P. glaucum* also hybridize naturally because they are protogynous and cross pollinated. The resulting hybrids are highly sterile and resemble *P. purpureum*. Lepidopteran stem borers cause great yield loss in maize produced by resource-poor farmers in Africa and are managed by habitat management or push-pull strategies, in which *P. purpureum* cultivars and hybrids are used as a trap crop. The aims of this project were to genotype different *P. purpureum* cultivars and hybrids using Amplified Fragment Length Polymorphism (AFLP) as well as Random Amplified Polymorphic DNA (RAPD) in order to identify cultivars and hybrids and possible misidentifications, assess the congruency of results between AFLPs and RAPDs and to attempt to relate these results to the oviposition preference of *Chilo partellus* for different *P. purpureum* cultivars. The individuals to be fingerprinted were collected from several countries in sub-Saharan Africa, a few from the USA and one from China. The AFLP analysis of these individuals were done with primer combinations *Eco*RI/*Mse*I and *Mlu*I/*Mse*I on polyacrylamide gels and an ABI 3130 *x*l Genetic Analyzer respectively. The automated sequencer visualized more bands than the polyacrylamide gels. The RAPD technology was not developed any further after 17 primers were tested and no polymorphic bands detected. Overall results indicated that cultivars did not cluster according to geographical origin, and cultivars known by popular names did not always cluster together, indicating diversity within the cultivar or misidentifications. An example of a misidentification is the cultivar Green Gold being no other than cultivar Harare, or cultivar Swaziland 3 being cultivar Sanitas. Proper management by nursery managers cannot be stressed enough, as this will prevent plants getting mixed up, causing confusion. There was no relationship between the relatedness of cultivars and moth oviposition preference. The AFLP technology could be a powerful tool for the DNA fingerprinting and molecular characterization of this grass species, but poor germ plasm management negates its application.

Keywords: Napier grass, *Pennisetum* species, AFLP, RAPD, germ plasm management.

OPSOMMING

Pennisetum Rich. is een van die belangrikste genera in die familie Poaceae aangesien dit beide voer- en graangewasse soos *Pennisetum purpureum* Shumach. en *Pennisetum glaucum* (L) R. Br. insluit. Beide *P. purpureum* en *P. gluacum* word oop-bestuif met die gevolg dat die groot aantal kultivars wat deur natuurlike kruisings ontstaan morfologies moeilik onderskeibaar is. *P. purpureum* en *P. glaucum* hibridiseer natuurlik aangesien hul protoginies en kruisbestuiwend is. Die gevolglike hibried is steriel en lyk soos *P. purpureum.* Stam boorders (Lepidoptera) veroorsaak skade aan mielies wat deur hulpbron-arm boere in Afrika verbou word. Hierdie insekplae word beheer deur habitat beheerstelsels of stoot-trek strategië, waar *P. purpureum* kultivars en hibriede as vanggewasse gebruik word. Die doelwitte van die projek was om die verskillende *P. purpureum* kultivars met behulp van Amplified Fragment Length Polymorphism (AFLP) en Random Amplified Polymorphic DNA (RAPD) te analiseer sodat verskillende kultivars en hibriede, asook moontlike verkeerde identifikasies geidentifiseer kan word, die ooreenstemmigheid van die AFLP en RAPD resultate te bepaal en om die resultate met die eierlegging voorkeur van *Chilo partellus* op die verskillende *P. purpureum* kultivars in verband te bring. Die individue wat ondersoek is, is van verskeie lande in sub-Sahara Afrika, asook die VSA en China afkomstig. Die AFLP analysis van hierdie individue is gedoen met primer kombinasie *Eco*RI/*Mse*I en *Mlu*I/*Mse*I op poli-akrielamiedgelle en 'n ABI 3130*x*l Genetic Analyzer onderskeidelik. Die outomatiese DNA analiseerder het meer bande waargeneem as die poli-akrielamiedgelle. Die RAPD tegniek is nie verder ontwikkel nadat 17 primer getoets en geen polimorfiese bande gevind is nie. Oorhoofse resultate dui daarop dat kultivars nie volgens geografiese oorsprong groepeer nie en kultivars met 'n gegewe naam groepeer ook nie saam nie, wat 'n aanduiding kan wees van intra-kultivar diversiteit of verkeerde identifikasies. 'n Voorbeeld van 'n verkeerde identifikasie is kultivar Green Gold wat ook as kultivar Harare identifiseer of kultivar Swaziland 3 wat ook bekend is as kultivar Sanitas. Deeglike kwekerybestuur kan nie genoeg beklemtoon word nie aangesien dit sal verhoed dat plante deurmekkaar raak en verwarring veroorsaak. Daar was geen verband tussen die verwantskappe tussen die kultivars en die motte se eierlegging voorkeur nie. Die AFLP

tegniek kan 'n kragtige metode wees vir die DNS-vingerafdrukanalises en molekulêre karakterisering van die grasspesie, maar swak kiemplasmabestuur beperk die tegniek se toepassing.

Sleutelwoorde: Napier gras, *Pennisetum* spesies, AFLP, RAPD, kiemplasmabestuur

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CHAPTER 1: INTRODUCTION

1.1 *Pennisetum* Rich.

Pennisetum Rich. is one of the most important genera in the Poaceae (Dahlgren *et al.*, 1985) and consists of 140 species distributed throughout the tropics of the old and the new world (Brunken, 1977). *Pennisetum* can be divided into five sections based on morphological characters: section *Pennisetum* (previously section *Penicillaria* (Willd.) Steud. (Brunken, 1977)), *Brevivalvula* Stapf & C.E. Hubbard, *Gymnothrix* Stapf & C.E. Hubbard, *Heterostachya* Stapf & C.E. Hubbard and section *Eu-Pennisetum* Stapf & C.E. Hubbard (Table 1.1) (Stapf & Hubbard, 1934).

Pennisetum section *Pennisetum* includes all the cultivated *Pennisetums* (Harlan *et al.*, 1976) and consists of two closely related species *viz. P. purpureum* Schumacher and *P. glaucum* (L.) R. Br. (Brunken, 1977). It is thought that *P. glaucum* is the progenitor of *P. purpureum* (Ingham *et al.*, 1993). Internal transcribed spacer (ITS) studies done by Martel *et al.* (2004) showed that these two taxa form a monophyletic group and share a basic chromosome number of x = 7. *Pennisetum purpureum* has a chromosome number of 2n = 28 (Barbaso *et al.*, 2003; Techio *et al.*, 2002) and *P. glaucum* has a chromosome number of 2n = 14 (Gupta & Mhere, 1997). Their chromosome sizes differ (Martel *et al.*, 2004) however, with *P. purpureum* being smaller than *P. glaucum* (Jahuar & Hanna, 1998).

1.2 *Pennisetum* in Southern Africa

There are 22 *Pennisetum* species in Southern Africa. These include species of both the primary gene pool (*P. glaucum*) and the secondary gene pool (*P. purpureum)* of pearl millet, as well as the hybrid (*P. purpureum* X *P. glaucum*). *P. foemeranum* Leeke and *P. stapfianum* L. Bolus are endemic to the region (Karivu & Mithen, 1987). Gibbs Russell *et al.* (1991) describe 13 species of *Pennisetum* occurring in southern Africa of which four have been naturalized *i.e. P. clandestinum* Choiv., *P. glaucum, P. purpureum* and *P. villosum* R. Br. ex. Fresen. Henderson (2002) declared *P. setaceum* (Forssk.) Choiv. and *P. villosum* as category 1 invasive species (these plants are prohibited and must be controlled) and proposed that *P. clandestinum* and *P. purpureum* should be placed in category 3 (new plants may no longer be planted,

but existing plants may remain, as long as all reasonable steps are taken to prevent their spreading, except within the flood line of watercourses and wetlands).

1.3 *Pennisetum purpureum* Schumacher

Pennisetum purpureum, commonly known as Napier grass, is native to tropical Africa. Its natural distribution ranges from Guinea in the west, through the forest belt of West Africa, south through Angola and Zimbabwe and in the east from Mozambique to southern Kenya (Brunken, 1977) (Fig. 1.1). Its habitat includes riversides, valley bottoms and forest margins. It prefers rich soil (Gibbs Russell *et al.,* 1991) and grows best in high rainfall areas. *Pennisetum purpureum* has been introduced to most of the tropics throughout the world where it has frequently become naturalized (Brunken, 1977).

Pennisetum purpureum is valued for its high biomass, perennial nature and pest resistance. It is therefore an important forage crop for dairy cattle in smallholder farming systems in the tropics and subtropics (Lowe *et al.,* 2003; Bhandari *et al.,* 2004). It also has potential for industrial use in alcohol and methane production (Muldoon & Pearson, 1979).

Pennisetum purpureum is a tall, perennial grass with a long vegetative growth phase (Bhandari *et al.,* 2004) and propagates clonally as it does not produce much seed (Lowe *et al.,* 2003). Because it is open pollinated, the number of cultivars and genetic diversity arising due to natural crossings is very high (Augustin & Tcacenco, 1993). Morphological characters pertaining to reproductive parts, traditionally the mainstay of taxonomy, cannot be used to distinguish these cultivars from one another due to their long vegetative phase and perennial nature (Bhandari *et al.,* 2004) and partly due to a lack of variation in these characters (Fig. 1.2).

Different morphological forms of these cultivars are collected and maintained clonally as germplasm and these germplasm are exchanged without proper pedigree records (Bhandari *et al.,* 2004). Various techniques have been used to distinguish between the various *P. purpureum* accessions, e.g. Bhandari *et al.* (2004) used isozymes and total proteins to develop accession specific fingerprints for 64 Napier grass accessions maintained at the Indian Grassland and Fodder Research Institute as

well as the Regional Research Station. This information is used to complement morphological evaluations and to maintain identity and purity of germplasm for proper conservation and management.

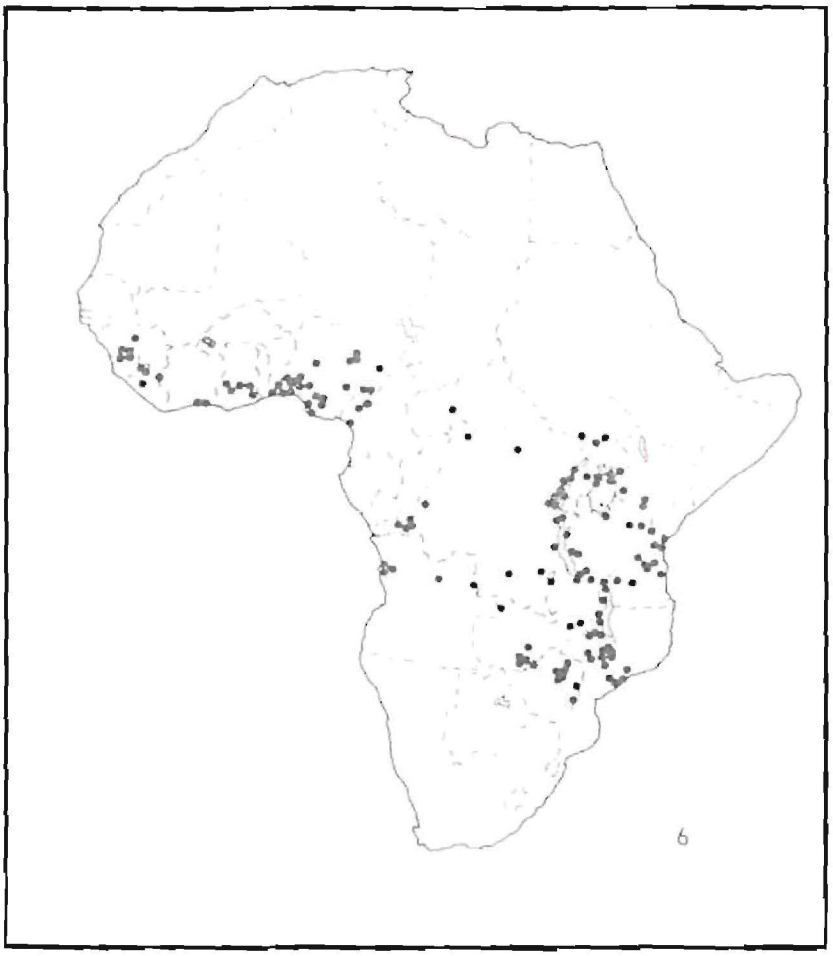

Figure 1.1 The natural distribution of *Pennisetum purpureum* Schumacher in Africa (Brunken, 1977).

The International Livestock Research Institute (IRLI) used Random Amplified Polymorphic DNA (RAPD) to fingerprint 56 accessions maintained at Zwai and Debre Zeit field stations in Ethiopia. The results led to the identification of two accessions that were accidentally switched during transfer from the germplasm collection at Zwai to Debre Zeit. It also confirmed a morphological analysis which indicated that two of the accessions, originally imported as hybrids, were not of hybrid origin. The overall conclusion was that RAPD were sufficient to identify

clonal propagation, duplication and misplantings in germplasm collections (Lowe *et al.*, 2003).

Daher *et al.* (2002) made similar conclusions when he fingerprinted the Napier group from the active germplasm bank at Embrapa in Brazil, also using RAPD. Originally only two cultivars, Napier and Merker, were introduced to Brazil in 1920 from Cuba for forage purposes, but over time new genotypes developed and new cultivars were introduced. These genotypes and cultivars are maintained without their original identification. Nine accessions were fingerprinted and results show that so-called Merker accession was not from the original Merker group.

Figure 1.2 *Pennisetum purpureum* cultivar.

1.4 *Pennisetum glaucum* (L.) R. Br.

Pennisetum glaucum (Pearl millet) is grown in Asia and Africa for grain and in the Americas for feed and forage (Gupta & Mhere, 1997). It was domesticated in the Sahel zone of West Africa. The crop spread along the southern border of the Sahara to the Sudan and later to east and central Africa and India (Harlan *et al.*, 1976) (Fig. 1.3). *Pennisetum glaucum* is a coarse, annual bunch grass (Gupta & Mhere, 1997) (Fig. 1.4) with a nutrient value that is higher than that of rice or wheat (Uprety & Austin, 1972). It is a highly cross-pollinated species (Gupta & Mhere, 1997) and genetic exchange between wild and cultivated genotypes frequently takes place (Brunken, 1977). It possesses high levels of phenotypic and genotypic polymorphism (Liu *et al.*, 1994), especially in cultivated material that is reflected in RFLP and AFLP studies (Pilat-Andre *et al.*, 1992; Busso *et al.*, 2000).

Busso *et al.* (2000) used AFLP analysis to study three genotypes of pearl millet collected in two villages 150 km apart in the north-eastern region of Nigeria in order to determine whether landraces with the same name, but grown in the two different regions, had a similar genetic identity and to test whether individual farmers play an active role in the development of the landraces that they grow. Overall results showed that there were greater levels of similarity between different landraces grown on the same farm than between identically named landraces grown by different farmers in the same village. These results stressed the need for documenting the details of the collection site, details on the individual farmers and the manner in which landrace material is maintained and selected by those famers. It also calls into question the use of landrace names and highlights the need of both germ plasm curators and breeders to have a method of coping with diversity that will likely be associated with a single name and potential duplication (Busso *et al.*, 2000).

Figure 1.3 The natural distribution of *Pennisetum glaucum* in Africa (Brunken, 1977).

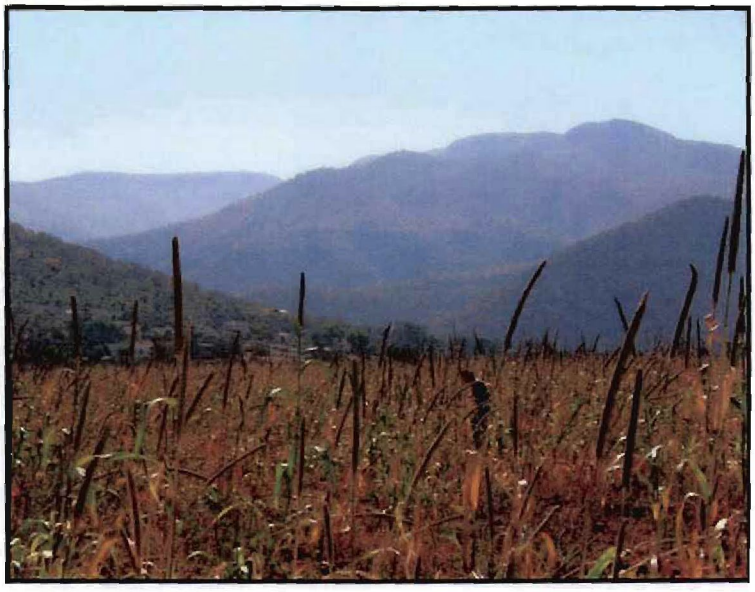

Figure 1.4 *Pennisetum glaucum* (L.) R. Br. cultivated in the Limpopo Province, South Africa.

1.5 *Pennisetum glaucum* X *Pennisetum purpureum*

Pennisetum purpureum and *Pennisetum glaucum* hybridize naturally because they are protogynous and cross-pollinated. The resulting hybrid (Fig. 1.5) does not shed pollen and is highly sterile (Burton, 1944). The hybrid can be propagated as a forage plant without the danger of it becoming a weed as in the case of *P. purpureum* (Gupta & Mhere, 1997). The hybrid resembles *P. purpureum* because of its larger genomic contribution and dominance (Gonzales & Hanna, 1984), but it has some of the fineness and leafiness of pearl millet (Muldoon & Pearson, 1979). The hybrid has a chromosome number of 2n = 21 (Barbaso *et al.*, 2003).

The first man-made hybrids were made by Burton (1944) in Georgia, USA, using a Napier and two late maturing Pearl millets. The resulting hybrids resembled Napier grass and showed considerable hybrid vigor along with a much leafier habit due to pronounced branching at the nodes. In comparison with Napier Grass, the heads that developed were longer, the spikelets were arranged more densely and the bristles were shorter. The stigmas appeared normal but the anthers were shriveled, failed to dehisce and were empty but for a few irregular pollen grains. The anthers were therefore, highly sterile. Some of the hybrids appeared yellow due to a chlorophyll deficiency and did not perform as well as the greener colored counterparts.

Similar crossings were later done by Gildenhuys (1950) in South Africa who used three types of Napier grass and a wide variety of pearl millet types. The hybrids showed great variation in habit, but in general it resembled Napier grass. They were perennial with the tufty appearance of Napier grass, but grew more vigorous, sprouted more abundantly and were taller. When mature, they were less course and more palatable than Napier grass. The hybrids were also completely sterile. The hybrid was named "Bana", derived from the two popular names of the parent species used in the breeding process. The first two letters of the word Babala (*P. glaucum*) and Napier grass (*P. purpureum*) were used to come up with the name Bana.

One of the primary reasons for these crossings was to transfer the above mentioned desirable forage characteristics of *P. glaucum* (succulence and palatability) to the perennial *P. purpureum* (Whyte, 1964; Jodhpur, 1965).

Figure 1.5 Contour planting of a *Pennisetum glaucum* X *Pennisetum purpureum* crop in the Limpopo province.

1.6 The cultivation of *Pennisetum purpureum*

Although *P. purpureum* was first described in 1827 (Schumacher, 1827), the documented utilization thereof has a relatively brief history with the first published note dating back to 1905 when Mynhardt, a Hungarian missionary in Barume, north western Zimbabwe, sent material to the Zurich Botanical Garden in Germany. It was however, named after a certain Colonel Napier of Bulawayo who brought this grass to the attention of the Agricultural Department. It was this grass' usefulness as a soil regenerator and mulch in especially coffee plantation crops that first attracted the attention to it (Boonman, 1993).

Dr. Pole Evans collected seeds of *P. purpureum* in West Africa which were used in the 1940's and 1950's by Dr. Codd at Prinshof, South Africa to develop a number of West African varieties, of which Gold Coast is better known. These varieties subsequently became popular in southern and eastern Africa (Boonman, 1993). Today

however, great confusion exists with the identity and names of varieties, because a single variety is often in circulation with more than one name. Sometimes old, almost forgotten varieties are reintroduced and become popular under a new name, or well known varieties may be present under old names, as well as different names (Boonman, 1993; Bhandari *et al.*, 2004). The confusion surrounding the variety Gold Coast or Ghana and Bana illustrate this well. Gold Coast was renamed to Ghana following Ghana's independence in 1956, but somewhere along the line the name Ghana got confused with the name "Bana" (given for the hybrid developed by Gildenhuys (1950) in South Africa) and was referred to and distributed for a long time under the incorrect name of Bana (Boonman, 1993).

No keys have ever been published to distinguish the better known varieties, but Boonman (1993) distinguish Gold Coast, French Cameroon, Clone 13, Uganda Hairless and Babala-Napier (hybrid) by qualitative and quantitative characters. These characters differ in the density and diameter of stems, the degree of hairiness, aerial tillering, head production and commonness of "black comb" on the upper edge of the leaf sheath in mature stems (Table 1.2). These characters are especially useful when comparing different varieties that are grown side by side. Hairiness has proven to be the most useful character to distinguish individual plants and varieties that grow in isolated stands either in the wild or in cultivation.

The history of Napier Grass in southern Africa is poorly documented resulting in general confusion surrounding the use of cultivar names. Most cultivars are in circulation with more than one name or no definite name and their origins are most often mere speculation. The name "Bana", is often synonymous with Napier and is also often used for cultivars that are not of hybrid origin, leading to further confusion.

1.7 The use of *Pennisetum purpureum* in crop pest management

Maize is the staple food of the majority of resource-poor farmers in Africa. These cereals are pestered by lepidopterous insects (moths) such as the indigenous maize stalk borer, *Busseola fusca* (Fuller) (Noctuidae) and the exotic sorghum stem borer, *Chilo partellus* (Swinhoe) (Pyralidae) (Khan *et al.*, 2000; Van den Berg *et al.*, 2001). The adult moths locate a suitable host plant (maize) and lay their eggs on the leaves

of these plants. Larvae that hatch from these eggs feed on the leaves before they enter into the stems. This damage to the leaves and stems result in yield loss (Fig 1.6) (Khan *et al.*, 2000).

In eastern and southern Africa, stem borers are managed by means of habitat management or 'push-pull' strategies (Fig.1.7) (Khan *et al.*, 2000). In these strategies a repellent plant species is planted between the main crop. *Desmodium uncinatum* (Jacq.) DC. (Fabaceae) is used as such as crop since it produces volatile compounds that repel the female stem borer moths away from the crop. The moths are then attracted to a trap crop, such as Napier grass, which is planted around the maize field (Khan *et al.*, 2000). The stem borer moths deposit their eggs on this crop where the resulting larvae are not able to develop and complete their life cycle. Trap crops that do not allow the survival of the offspring are known as dead-end trap crops (Shelton & Nault, 2004).

Apart from its pest management role and consequent yield increase, the Napier grass trap crop has many other benefits in the farming system and contributes significantly towards sustainable farming. It reduces soil erosion, conserves soil moisture and prevents maize plants from lodging in strong wind. Napier grass also provides good-quality fodder, which can be fed to cattle, often resulting in increased milk production. The availability of Napier grass also reduces the time spent searching for fodder when cattle are stall-fed. Excess fodder can be sold for much needed cash (Gatsby Charitable Foundation, 2005).

As mentioned, there are many natural occurring hybrids, cultivars and varieties. Many of these could possibly be used with better effect in this push-pull system. Van den Berg. (2006) has researched the oviposition preference of *C. partellus* moths and their larval survival rates on maize and various Napier grass varieties and cultivars found in South Africa. Results indicate that *C. partellus* prefers to oviposit on the majority of Napier grass cultivars and varieties rather than maize. However, although larval survival was very poor on the majority of Napier grass varieties, some did, however, allow larval survival, making them unsuitable as a dead-end trap crop. It would appear therefore, that although the majority of these grasses can potentially be used as trap crops around maize fields, the adaptability of each specific Napier genotype to

Figure 1.6 Damage done by stem borers to a stem and ear of maize.

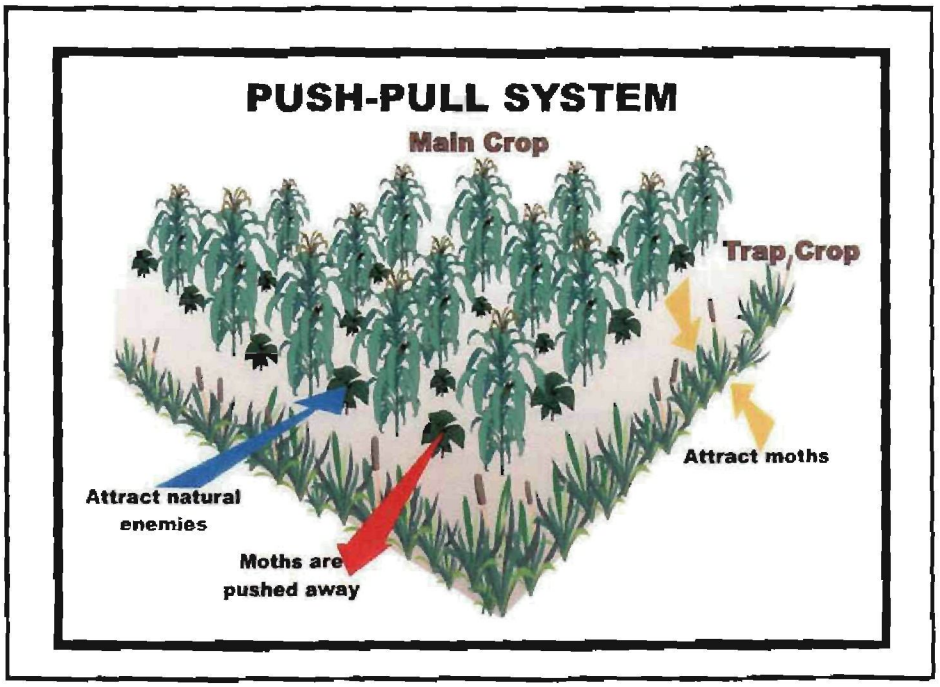

Figure 1.7 Diagrammatic representation of the "push-pull" strategy.

farming conditions, rainfall, temperature requirements and suitability as animal fodder should be taken into account when selecting a genotype for use in the habitat management systems (Van den Berg, 2006).

Due to the rapid expansion and use of Napier grass in the habitat management of stem borers, a need has arisen to determine the relationship between the different cultivars and to determine the identity of cultivars that could be used as trap cops.

1.8 Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and *Pennisetum*

Numerous studies have been done on Napier grass using RAPD as method of choice. All of the studies found RAPD a useful tool to quantify genetic distances and to distinguish between accessions (Smith *et al.*, 1993; Lowe *et al.*, 2003; Daher *et al.*, 2002; Passo *et al.*, 2005). RAPDs, however, have the disadvantage of inconsistent fragment amplification resulting in polymorphisms unsuitable for unbiased and objective scoring and thereby, necessitating duplicating analyses (Smith *et al.*, 1993). AFLPs (Vos *et al.*, 1995). however, has recently become the method of choice for genotyping plants (Koopman *et al.*, 2001), animals (Van Haeringen *et al.* 2002), fungi (Vandemark, 1999) and bacteria (Huys *et al.*, 1996), because it is a relatively cheap, easy, fast and a reliable method to generate unique, reproducible fingerprints for each individual being analysed (Meudt *et al.*, 2006; Mueller & Wolfenbarger, 1999). To date, no published work exists on Napier grass utilizing AFLP and in order to confirm results, both RAPD and AFLP will be done.

1.8 Aims

The aims of this project were to genotype different *P. purpureum* cultivars and hybrids using Amplified Fragment Length Polymorphism (AFLP) as well as Random Amplified Polymorphic DNA (RAPD) in order to:

1. identify cultivars and hybrids and possible misidentifications
2. assess the congruency of results between AFLPs and RAPDs
3. attempt to relate these results to the oviposition preference of *C. partellus* for different *P. purpureum* cultivars.

Table 1.1. Morphological differences between the various *Pennisetum* sections (Stapf & Hubbard, 1934; Brunken, 1977).

Sections	*Pennisetum (Penicillaria)*	*Brevivalvula*	*Heterostachya*	*Gymnothrix*	*Eu-Pennisetum*
Anther tips	Penicillate	Glabrous	Glabrous	Glabrous	Glabrous
Valves of upper floret	± Hardened and chartaceous, or coriaceous at maturity, smooth and shining in the lower two thirds		Upper glume and lower valve much larger than external spikelets	Valves scarcely change at maturity	Valves scarcely change at maturity, membranous or thinly chartaceous, not shining
Lodicules	Absent		Present	Often present	Often present or very minute
Styles				Free or connate	Free, rarely connate
Bristles				Glabrous or rarely ciliate	All bristles or the inner plumose rarely glabrous
Involucre				Sessile or subsessile	Sessile or shortly stalked
Spikelets				Usually solitary, rarely 2-3 in each involucre	1-4 (more) in each involucre
			Spikelets heteromorphous, external male, laterally compressed and keeled, central female, subterete or dorsally compressed		If clustered, all alike in shape and sex, or the outer sometimes male, not keeled
			Floral bracts within the spikelet are heteromorphic. Glumes and lower lemma membranous. Bracts of upper floret indurated in fruit. Lower lemma has a tridentate apex. Rachis, below each involucre in the inflorescence, has decurrent wings		

Table 1.2. Characters used by Boonman (1993) to distinguish between different *Pennisetum purpureum* varieties and the Babala-Napier hybrid.

Variety/ Cultivar	Number of stems at maturity (m^2)	Stem diameter	Hairs on leaf sheath	Black "comb" of hairs on upper fringe of leaf sheath	Long hairs on leaf blade near ligule	Hairiness of upper surface of leaf blade	Aerial tillering from nodes	Special characters
Gold Coast	1-15	Thick	2-3 mm dense	Very common	Common	Hairy to the touch, very dense short hairs	Uncommon	Palish colour; old leaves remain attach to the stems; top leaves give stem top fan-shape appearance; leaf sheaths diverging which flattens the stem
French Cameroon	15-20	Thin	2-3 mm not dense	Uncommon	Uncommon	Not hairy to the touch; scattered short hairs	Common	Very fast establishment from cane cuttings
Clone13	30-40	Very thin	3-4 mm not dense	Uncommon	Very common	Not hairy to the touch; scattered short hairs	Very common	Erect shoots; very poor establishment from cane cuttings
Unganda Hairless	20-25	Thin	2-3 mm not dense	Uncommon	Uncommon short hairs	Not hairy to the touch; scattered short hairs	Uncommon	Old leaves purplish; open clumps
Babala-Napier (hybrid)	15-20	Thin	4-5 mm	Very common	Very common	Hairy to the touch' long hairs not dense	Uncommon	Not cultivated; very hairy

CHAPTER 2: MATERIALS AND METHODS

2.1 AFLP introduction

Amplified Fragment Length Polymorphism (AFLP) is a technique that can detect levels of genetic variation within and between natural populations in many plant species (Tremetsberger *et al.,* 2003). This technique is based on the selective Polymerase Chain Reaction (PCR) amplification of restriction fragments from a total digest of genomic DNA of any origin and complexity. The technique involves three steps (Fig. 2.1):

(1) restriction of the DNA and the ligation of oligonucleotide adapters.

(2) the amplification of restriction fragments. This step involves two amplifications: pre-amplification and selective amplification.

(3) gel analysis of the amplified fragments.

DNA is cut with restriction enzymes and the amplification of these fragments is achieved by using the adapters and restriction site sequence target sites for the annealing of the primers. The primers used in the pre-amplification step have a single selective nucleotide while those used in the selective amplification have a longer selective extension (Vos *et al.,* 1995).

2.1.1 Plant material

The history and origins of the samples used in this study are not well documented and are based on personal communication with nursery managers.

Plant material of the different *Pennisetum* cultivars was obtained from South Africa, Botswana, Mozambique, Ghana and Ethiopia (Table 2.1). These samples included landraces (a distinct crop variety or cultivar developed and maintained agriculturally (Allaby, 2004)), cultivars (a variety of plant which has been produced by horticultural techniques and is not normally found in wild populations (Allaby, 2004)), hybrids (an

individual plant which is the result of a cross between parents of different genotypes (Allaby, 2004)) as well as pure *Pennisetum glaucum* and *Pennisetum purpureum*. *Pennisetum setaceum* (Forssk.) Choiv. and *Pennisetum macrourum* Trin. were also included in the study to potentially root trees.

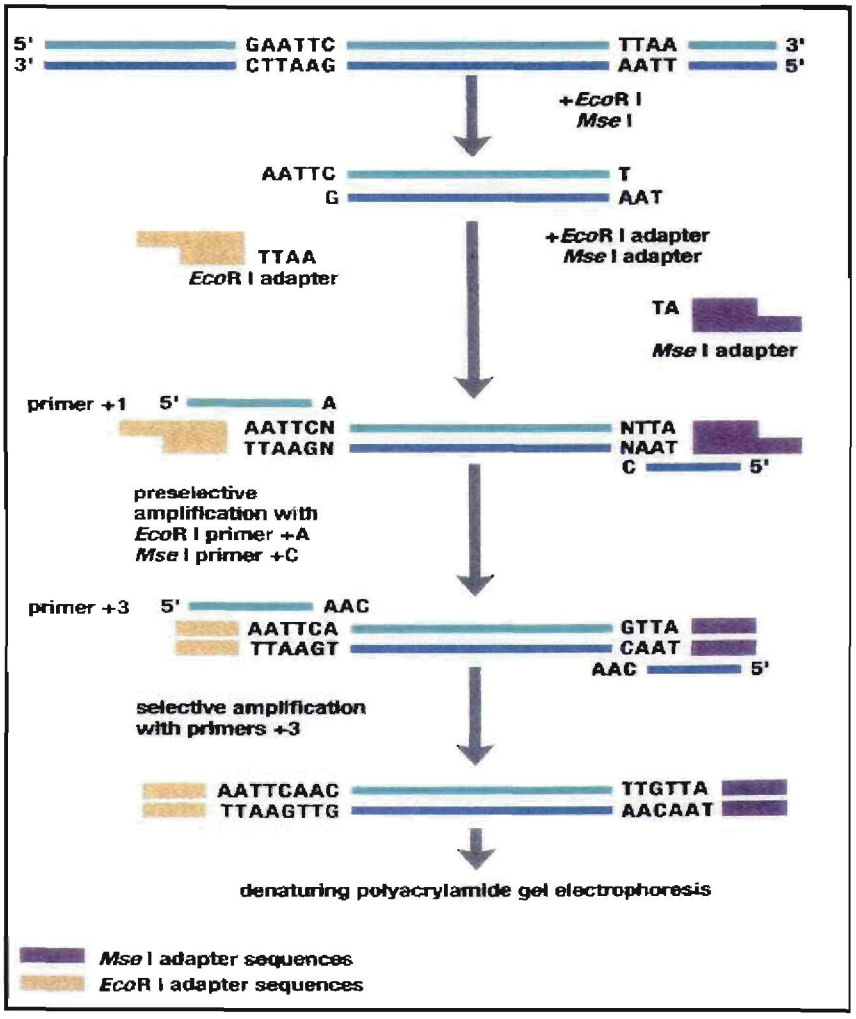

Figure 2.1: Diagram showing the various steps followed during the application of the AFLP-technique (From: AFLP™ Analysis System I, AFLP Starter Primer Kit, Life Technologies).

Table 2.1: *Pennisetum* plant material and the localities/institutes from which they were obtained*

Numbers used in PCA diagrams	Accession	Locality/Institute	Cultivar/Hybrid/Landrace	Country of origin
1 & 2	Estcourt 1 & 1.3	Agricultural Department (KZN) Estcourt, South Africa	Cultivar	Unkown
3, 4 & 5	Estcourt 2, 2.2 & 2.3	Agricultural Department (KZN) Estcourt, South Africa	Cultivar	Unkown
6, 7 & 8	Estcourt 3, 3.2 & 3.3	Agricultural Department (KZN) Estcourt, South Africa	Cultivar	Unkown
9, 10 & 11	Estcourt 4, 4.2 & 4.3	Agricultural Department (KZN) Estcourt, South Africa	Cultivar	Unkown
12, 13 & 14	Estcourt 5, 5.2 & 5.3	Agricultural Department (KZN) Estcourt, South Africa	Cultivar	Unkown
15, 16 & 17	Estcourt 6, 6.2 & 6.3	Agricultural Department (KZN) Estcourt, South Africa	Cultivar	Unkown
18 & 19	Estcourt 8 & 8.2	Agricultural Department (KZN) Estcourt, South Africa	Cultivar	Unkown
20, 21 & 22	Estcourt 9, 9.2 & 9.3	Agricultural Department (KZN) Estcourt, South Africa	Cultivar	Unkown
23, 24 & 25	Estcourt 10, 10.2 & 10.3	Agricultural Department (KZN) Estcourt, South Africa	Cultivar	Unkown
26, 27 & 28	Estcourt 11, 11.2 & 11.3	Agricultural Department (KZN) Estcourt, South Africa	Cultivar	Unkown

Numbers used in PCA diagrams	Accession	Locality/Institute	Cultivar/Hybrid/Landrace	Country of origin
29, 30 & 31	Estcourt 12, 12.2 & 12.3	Agricultural Department (KZN) Estcourt, South Africa	Cultivar	Unkown
32, 33 & 34	Estcourt 13, 13.2 & 13.3.	Agricultural Department (KZN) Estcourt, South Africa	Cultivar	Unkown
35, 36 & 37	Estcourt 14, 14.2 & 14.3	Agricultural Department (KZN) Estcourt, South Africa	Cultivar	Unkown
38, 39 & 40	Estcourt 15, 15.1 & 15.2	Agricultural Department (KZN) Estcourt, South Africa	Cultivar	Unkown
41 & 42	Estcourt 16.2 & 16.3	Agricultural Department (KZN) Estcourt, South Africa	Cultivars	Unkown
43	Estcourt 18	Agricultural Department (KZN) Estcourt, South Africa	Cultivar	Unkown
44: 45 & 46	Estcourt 19, 19.2 & 19. 3	Agricultural Department (KZN) Estcourt, South Africa	Cultivar	Unkown
47, 48 & 49	Estcourt 20, 20.2 & 20. 3	Agricultural Department (KZN) Estcourt, South Africa	Cultivar	Unkown
50	Estcourt Bana	Agricultural Department (KZN) Estcourt, South Africa	Hybrid	Unkown
51	Nanzindlela	Nanzindlela Farm Pietermaritzburg, South Africa	Unkown	South Africa
52, 53 & 54	Potchefstroom Bana 1, 2 & 3	Agricultural Research Council (ARC) Potchefstroom South Africa	Hybrid	South Africa

Numbers used in PCA diagrams	Accession	Locality/Institute	Cultivar/Hybrid/Landrace	Country of origin
55	China	Agricultural Department (KZN) Estcourt, South Africa	Cultivar	China
56, 57 & 58	Roodeplaat Harare 1, 2 & 3	Agricultural Research Council (ARC) Roodeplaat, South Africa	Landrace	Zimbabwe
59, 60 & 61	Roodeplaat Nyle Source 1, 2 & 3	Agricultural Research Council (ARC) Roodeplaat, South Africa	Landrace	Africa (possibly origin of Nyle river?)
62, 63 & 64	Roodeplaat Mfufu 1, 2 & 3	Agricultural Research Council (ARC) Roodeplaat, South Africa	Landrace	Unkown
65, 66 & 67	Roodeplaat Gold Coast Napier 1, 2 & 3	Agricultural Research Council (ARC) Roodeplaat, South Africa	Cultivar	Unkown, east or west coast of Africa
68, 69, 70 & 71	Roodeplaat Green Gold 1, 2 & 3	Agricultural Research Council (ARC) Roodeplaat, South Africa	Cultivar	Zimbabwe
72, 73 & 74	Roodeplaat Bana 1, 2 & 3	Agricultural Research Council (ARC) Roodeplaat, South Africa	Hybrid	South Africa
75, 76 & 77	*P. purpureum* cv Swaziland 1, 2 & 3	Department of Agricultural Research, Gaborone, Botswana	Cultivar	South Africa
78, 79 & 80	*P. purpureum* ex Sanitas 1, 2 & 3	Department of Agricultural Research, Gaborone, Botswana	Cultivar	Nigeria
81, 82 & 83	*P. purpureum* Umbeluzi 1, 2 & 3	Mozambique	Cultivar	Unknown

Numbers used in PCA diagrams	Accession	Locality/Institute	Cultivar/Hybrid/Landrace	Country of origin
84, 85 & 86	*P. purpureum* Agro Farm 1, 2 & 3	Mozambique	Cultivar	Unknown
87	Ethiopia 1026	International Livestock Research Institute, Ethiopia	Cultivar	Burundi
88	Ethiopia 14355	International Livestock Research Institute, Ethiopia	Cultivar	Ethiopia
89	Ethiopia 14389	International Livestock Research Institute, Ethiopia	Cultivar	Nigeria
90	Ethiopia 14984	International Livestock Research Institute, Ethiopia	Cultivar	USA
91	Ethiopia 15357H	International Livestock Research Institute, Ethiopia	Hybrid	Unkown
92	Ethiopia 15743	International Livestock Research Institute, Ethiopia	Cultivar	USA
93	Ethiopia 16621	International Livestock Research Institute, Ethiopia	Cultivar	Namibia
94	Ethiopia 16783	International Livestock Research Institute, Ethiopia	Cultivar	Tanzania
95	Ethiopia 16784	International Livestock Research Institute, Ethiopia	Cultivar	Tanzania
96	Ethiopia 16785	International Livestock Research Institute, Ethiopia	Cultivar	Tanzania
97	Ethiopia 16786	International Livestock Research Institute, Ethiopia	Cultivar	Swaziland
98	Ethiopia 16787	International Livestock Research Institute, Ethiopia	Cultivar	Swaziland

Numbers used in PCA diagrams	Accession	Locality/Institute	Cultivar/Hybrid/Landrace	Country of origin
99	Ethiopia 16789	International Livestock Research Institute, Ethiopia	Cultivar	Swaziland
100	Ethiopia 16790	International Livestock Research Institute, Ethiopia	Cultivar	Swaziland
101	Ethiopia 16791	International Livestock Research Institute, Ethiopia	Cultivar	Swaziland
102	Ethiopia 16792	International Livestock Research Institute, Ethiopia	Cultivar	Mozambique
103	Ethiopia 16793	International Livestock Research Institute, Ethiopia	Cultivar	USA
104	Ethiopia 16794	International Livestock Research Institute, Ethiopia	Cultivar	Mosambique
105	Ethiopia 16797	International Livestock Research Institute, Ethiopia	Cultivar	Zimbabwe
106	Ethiopia 16798	International Livestock Research Institute, Ethiopia	Cultivar	Zimbabwe
107	Ethiopia 16799	International Livestock Research Institute, Ethiopia	Cultivar	Zimbabwe
108	Ethiopia 16800	International Livestock Research Institute, Ethiopia	Cultivar	Zimbabwe
109	Ethiopia 16801	International Livestock Research Institute, Ethiopia	Cultivar	Zimbabwe
110	Ethiopia 16802	International Livestock Research Institute, Ethiopia	Cultivar	Zimbabwe
111	Ethiopia 16803	International Livestock Research Institute, Ethiopia	Cultivar	Zimbabwe
112	Ethiopia 16804	International Livestock Research Institute, Ethiopia	Cultivar	USA

Numbers used in PCA diagrams	Accession	Locality/Institute	Cultivar/Hybrid/Landrace	Country of origin
113	Ethiopia 16806	International Livestock Research Institute, Ethiopia	Cultivar	USA
114	Ethiopia 16807	International Livestock Research Institute, Ethiopia	Cultivar	USA
115	Ethiopia 16808	International Livestock Research Institute, Ethiopia	Cultivar	USA
116	Ethiopia 16809	International Livestock Research Institute, Ethiopia	Cultivar	USA
117	Ethiopia 16810	International Livestock Research Institute, Ethiopia	Cultivar	USA
118	Ethiopia 16812	International Livestock Research Institute, Ethiopia	Cultivar	USA
119	Ethiopia 16813	International Livestock Research Institute, Ethiopia	Cultivar	USA
120	Ethiopia 16814	International Livestock Research Institute, Ethiopia	Cultivar	USA
121	Ethiopia 16815	International Livestock Research Institute, Ethiopia	Cultivar	USA
122	Ethiopia 16816	International Livestock Research Institute, Ethiopia	Cultivar	USA
123	Ethiopia 16817	International Livestock Research Institute, Ethiopia	Cultivar	USA
124	Ethiopia 16818	International Livestock Research Institute, Ethiopia	Cultivar	USA
125	Ethiopia 16821	International Livestock Research Institute, Ethiopia	Cultivar	Zimbabwe
126	Ethiopia 16834H	International Livestock Research Institute, Ethiopia	Hybrid	Zimbabwe

Numbers used in PCA diagrams	Accession	Locality/Institute	Cultivar/Hybrid/Landrace	Country of origin
127	Ethiopia 16835H	International Livestock Research Institute, Ethiopia	Hybrid	Zimbabwe
128	Ethiopia 16836	International Livestock Research Institute, Ethiopia	Cultivar	Zimbabwe
129	Ethiopia 16837H	International Livestock Research Institute, Ethiopia	Hybrid	Zimbabwe
130	Ethiopia 16838H	International Livestock Research Institute, Ethiopia	Hybrid	Zimbabwe
131	Ethiopia 16839	International Livestock Research Institute, Ethiopia	Cultivar	Zimbabwe
132	Ethiopia 16840H	International Livestock Research Institute, Ethiopia	Hybrid	Zimbabwe
133	Ethiopia 16902	International Livestock Research Institute, Ethiopia	Cultivar	Zimbabwe
134	Ethiopia 18438	International Livestock Research Institute, Ethiopia	Cultivar	Tanzania
135	Ethiopia 18448	International Livestock Research Institute, Ethiopia	Cultivar	Tanzania
136	Cedara *P. glaucum*	Agricultural Research Council (ARC) Cedara Hilton South Africa	-	South Arica
137	*P. purpureum* ex Ghana	Pampram, Ghana	-	Ghana
138, 139 & 140	*P. setaceum* 1, 2 & 3	Vredefort Dome, South Africa	-	South Africa
141, 142 & 143	*P. macrourum* 1, 2 & 3	Jonkershoek Nature Reserve, Stellenbosch, South Africa	-	South Africa
144	Venda Bana	Venda, South Africa	Hybrid	South Africa
145	Venda Flower	Venda, South Africa	Hybrid	South Africa

Note that there are no Estcourt 7 or Estcourt 17 individuals as there were no cultivars in the Estcourt germ plasm with that specific numbers. Roodeplaat Green Gold * refers to the *P. purpureum* cultivar that is used as a control throughout the different analysis.

* Voucher specimens were deposited in the A.P. Goossens Herbarium, North West University, South Africa.

The Estcourt individuals were collected over two seasons (2005 and 2006). The second season is indicated by .2 and .3, which also indicate that it is the second and third individual of the given number e.g. Estcourt 2.2 shows that it is the second individual of Estcourt 2, collected in the second season, 2006.

2.1.2 DNA extraction

Leaf tissue was freeze-dried and grounded to a fine powder. DNA was extracted from 250-300 µl of lyophilized leaf tissue using a CTAB (Cetieltrimethylammoniumbromide) method of Saghai-Maroof *et al.* (1984) with slight modifications. The powdered leaf tissue was incubated with 1X CTAB buffer (100 mM Tris pH 8, 20 mM EDTA, 1.4 M NaCl, 1% CTAB, 0.2% ß-merk) at 65 °C for 1 hour after which the suspension was extracted with chloroform:iso-amylalcohol (24:1). The phases were separated by centrifuging at 10 000 rpm (4 °C) for 10 minutes. DNA was precipitated from the top aqueous layer with 600 µl isopropanol for 20 minutes at room temperature and DNA was pelleted at 12 000 rpm (4 °C) for 30 minutes. The pellets were washed with 70% ETOH and air dried and then resuspended in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8) overnight. The quality of the DNA was estimated with 0.8% agarose gel electrophoresis and the concentration was spectophotometrically determined at 260 and 280 nm.

2.1.3 AFLP analysis

The protocol used was based on the AFLP technology developed by Marc Zabeau and colleagues (Vos *et al.*, 1995).

2.1.4 Restriction digest

Approximately 1 µg of genomic DNA was digested at 37 °C with two restriction enzymes, *Mse*I (for 5 hours) followed by *Eco*RI or *Mlu*I overnight, after which *Mse*I-adapters and *Eco*RI- or *Mlu*I-adapters (Table 2.2) were ligated to the fragments as described by Vos *et al.* (1995) in a total volume of 60 µl.

2.1.5 Pre-amplification reaction

Five µl digested DNA was pre-amplified in a reaction volume of 50 µl containing 30 ng of each primer with one selective nucleotide (Table 2.2), 2 mM $MgCl_2$, 200 µM dNTP's, Taq polymerase buffer (10 mM Tris-HCl pH 9, 50 mM KCl, 0.1% Triton ®X-100) and 0.75 U Taq DNA polymerase (Promega, Madison WI). Each sample was overlaid with mineral oil and pre-amplified with a ThermoHybaid MBS Thermal Cycler (ThermoElectron Corporation, UK) of 30 cycles for 30 seconds at 94 °C, one minute at 56 °C and one minute at 72 °C. The quality of the pre-amplification was determined with 1.5% agarose gel electrophoresis.

2.1.6. Protocol used for primer combination *Eco*RI/*Mse*I

2.1.6.1 Selective amplification reaction

The pre-amplification products were diluted 1:10 with 0.1 x TE (1 mM Tris-HCl, 0.1 mM EDTA, pH 8) after which 5 µl diluted DNA was amplified in a 20 µl reaction volume containing *Mse*I and *Eco*RI primers with 3 selective nucleotides, 0.2 mM dNTP's, 2 mM $MgCl_2$, 100 µg/ml BSA, Taq polymerase buffer (10 mM Tris-HCl pH 9, 50 mM KCl, 0.1% Triton ®X-100) and 0.75 U Taq DNA polymerase (Promega, Madison WI). Each sample was overlaid with mineral oil and amplified with an ThermoHybaid MBS Thermal Cycler utilizing a touchdown protocol (one cycle at 94 °C for 2 minutes, then 94 °C for 30 seconds, 65 °C for 30 seconds and 72 °C for one minute, after which the annealing temperature was lowered 1 °C for each of 9 cycles down to 57 °C, followed by 30 cycles

for 30 seconds at 94 °C, 30 seconds at 56 °C and one minute at 72 °C). Samples were stored at 4 °C overnight. The following primer combinations were used: *Eco*RI-AAC, *Mse*I-ACA, *Mse*I-ACC, *Mse*I-CGT, *Mse*I-CCG and *Mse*I-TAC (Table 2.2).

2.1.6.2 Denaturing polyacrylamide gel electrophoresis

Two glass plates were used for casting the gel. The plates were treated before casting: one plate was previously prepared with Acrylease (Stratagene, Whitehead Scientific Suppliers) and washed with cold isopropanol and ethanol in order to repel the gel, and to prevent it from binding to the plate. The other plate was treated with bind silane (950 µl absolute ethanol, 5 µl acetic acid and 3 µl bind silane, Promega) in order to bind the gel to this plate and to keep it stable through the staining process.

Three µl of the selective amplification products were separated on a 5% (m/v) denaturing polyacrylamide (19 acrylamide : 1 N,N'-methylene-bis-acrylamide ratio) gel containing 7 M urea and 1 x TBE buffer (89 mM Tris-borate, 2.5 mM EDTA, pH 8.3) was used as running buffer. Electrophoresis was carried out at 80 W constant power for approximately 2 hours using a standard DNA sequencing unit (C.B.S. Scientific Company, California, USA).

2.1.6.3 Silver staining

The separated amplified DNA fragments were visualized with a silver staining kit from Promega (Madison WI) according to the manufacturer's instructions. The plates were separated and the gel fixed with 10% acetic acid for 20 minutes after which it was rinsed three times (three minutes each) with Ultra Pure water (18.2 µOhm). The gel was stained with silver nitrate (1 g/l) containing formaldehyde (0.056%) for 30 minutes and washed for 10 seconds before developing with sodium carbonate (30 g/l) containing formaldehyde (0.056%) and sodium thiosulfate (2 mg/l). The developing process was stopped with 10% acetic acid and left to fix for 3 minutes, followed by two washes with water. The gel was air dried and photographed by exposing photographic paper (Kodak Polymax II RC) directly

Table 2.2: Sequences of adapters and primers used in the AFLP analysis of the *Pennisetum* plant material.

Name	Type	Sequence (5'-3')	Keygene Primer Code
*Eco*RI	Adapter	5'-CTCGTAGACTGCGTACC CATCTGACGCATGGTTAA-5'	
*Mse*I	Adapter	5'-GACGATGAGTCCTGAG TACTCAGGACTCAT-5'	
*Mlu*I	Adapter	5'-CTCGTAGACTGCGTAAC CGCGGTTACGCAGTC-5'	
*Eco*RI-A	Primer + 1	GACTGCGTACCAATTCA	E 01
*Mse*I-A	Primer + 1	GATGAGTCCTGAGTAAA	M 01
*Mse*I-C	Primer + 1	GATGAGTCCTGAGTAAC	M 02
*Mse*I-G	Primer + 1	GATGAGTCCTGAGTAAG	M 03
*Mse*I-T	Primer + 1	GATGAGTCCTGAGTAAT	M 04
*Mlu*I-*T*	Primer + 1	GACTGCGTAACCGCGT	
*Eco*RI-AAC	Primer + 3	GACTGCGTACCAATTCAAC	E 32
*Mlu*I-TCA	Primer + 3	VIC-GACTGCGTAACCGCGTCA	
*Mlu*I-TAA	Primer + 3	6-FAM-GACTGCGTAACCGCGTAA	
*Mse*I-ACA	Primer + 3	GATGAGTCCTGACTAAACA	M 35
*Mse*I-ACC	Primer + 3	GATGAGTCCTGACTAAACC	M 36
*Mse*I-CGT	Primer + 3	GATGAGTCCTGACTAACGT	M 58
*Mse*I-CCG	Primer + 3	GATGAGTCCTGACTAACCG	M 53
*Mse*I-TAC	Primer + 3	GATGAGTCCTGACTAATAC	M 80

under the gel to approximately 20 seconds of dim light in order to produce a negative image of the same size as the gel.

2.1.6.4 Data analysis

Bands were scored manually as present or absent. Each band was considered to represent a single locus and only reproducible bands were scored. Distance estimation was done using methods described by Nei & Li (1979) and cluster analysis was performed using UPGMA (unweighted pairgroup method using arithmetic averages). Bootstrap analysis was performed using 1000 replicates. Analyses were done with TREECON version 1.3b (Van den Peer & Watcher, 1994).

Principal Coordinate Analysis was done in NTSYSpc version 2.02j (Rohlf, 1998), on the basis of similarity measures computed with the SIMQUAL module using the Jaccard (1908) or Dice (1945) coefficient and the DCENTER and EIGEN procedures.

The polymorphic index, which can be used to compare the extent of DNA polymorphism in different populations, was calculated using the following formula: PI=PBx1000/(TBxTI) where PB is the total number of polymorphic DNA bands (sum of all polymorphic bands across all analysed individuals), TB is the total number of bands (sum of bands separated by electrophoresis) and TI is the total number of analysed plant individuals (Labra *et al.*, 2001).

2.1.7 Protocol used for primer combination *Mlu*I/*Mse*I

2.1.7.1 ABI 3130 *x*l Genetic Analyzer

The ABI 3130 *x*l Genetic Analyzer (Applied Biosystems) (Fig 2.2) is a high- performance, fluorescence based machine capable of analysing 2 x 96 samples simultaneously, using a 16 capillary system. It is fully automated and provides continuous, unattended operation, from

automated polymer loading and sample injection to separation, detection and data generation (Anonymous, 2005a).

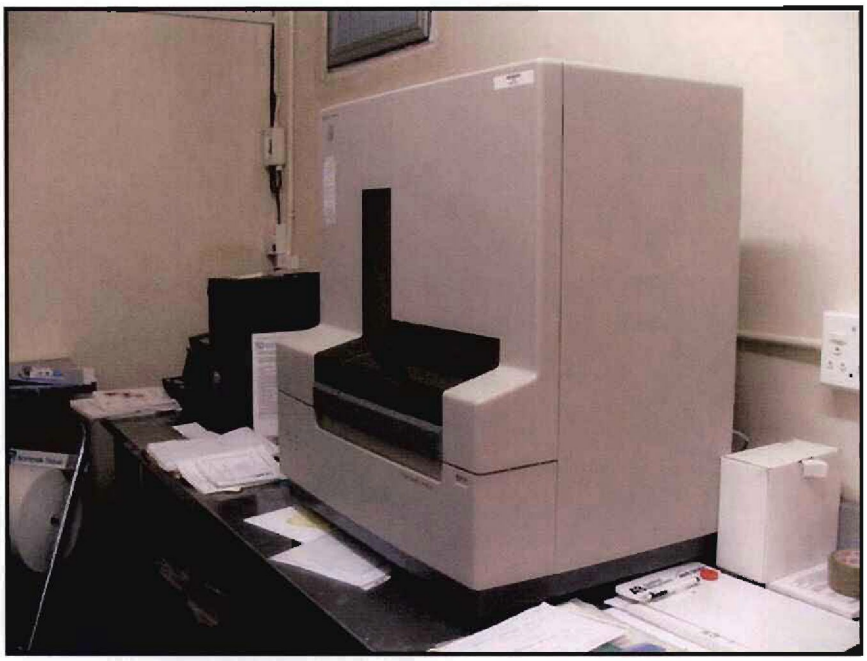

Figure 2.2 The ABI 3130 *xl* Genetic Analyzer used for the separation of fragments digested by *MluI*/*MseI* primer combination.

PCR products are dye-labelled during selective amplification with a fluorescent dye (fluorophore) so that all the strands that are synthesized from this primer are fluorescently labelled. Different primers are labelled with different fluorophores. The capillary instrument detects fragments present in the spectrum of each fluorophore and produces an electronic profile (Meudt & Clarke, 2006). An internal lane size standard of another colour is added to every lane to size all amplification fragments accurately (Anonymous, 2005b).

2.1.7.2. Spectral calibration of ABI 3130 *xl* Genetic Analyzer

Before analysis of samples can take place, a spectral calibration must be performed in order to create a matrix that is used during a run to reduce raw data from the instrument to the dye data stored in the sample files. The calibration is similar to performing a sample run

except that calibration standards are run in place of samples and a spectral calibration module is used in place of a run module (Anonymous, 2004).

Hundred and ninety µl of Hi-Di formamide were added to the 10 µl Matrix standard for fragment analysis after which it was denatured for 5 minutes at 95 °C and then cooled on ice for 2 minutes. Ten µl denatured standard was added to the first two rows of wells of the plate and the spectral calibration program was run.

2.1.7.3 Selective amplification reaction

The pre-amplification products were diluted 1:20 with 0.1 x TE (1 mM Tris-HCl, 1 mM EDTA, pH 8) after which 1.3 µl, 2.5 µl or 5 µl diluted DNA was amplified in a 20 µl reaction volume containing *Mse*I and fluorescent dye-labelled *Mlu*I primers with 3 selective nucleotides, 0.2 mM dNTP's, 2 mM $MgCl_2$, 100 µg/ml BSA, Taq polymerase buffer (10 mM Tris-HCl pH 9, 50 mM KCl, 0.1% Triton ®X-100) and 0.75 U Taq DNA polymerase (Promega, Madison WI). Samples were then amplified with an ThermoHybaid MBS Thermal Cycler utilizing a touchdown protocol (one cycle at 94 °C for 2 minutes, then 94 °C for 30 seconds, 65 °C for 30 seconds and 72 °C for one minute, after which the annealing temperature was lowered 1 °C for each of 9 cycle down to 57 °C, followed by 30 cycles for 30 seconds at 94 °C, 30 seconds at 56 °C and one minute at 72 °C). Samples were stored at 4 °C overnight. The following primer combinations were used *Mlu*I-TCA and *Mlu*I-TAA; *Mse*I-ACA, *Mse*I-ACC, *Mse*I-CCG and *Mse*I-TAC (Table 2.2).

2.1.7.4 Sample preparation for ABI 3130 *xl* Genetic Analyzer

One µl selective amplification product was added to 10 µl Hi-Di formamide containing GeneScan- 500 LIZ Size Standard (Applied Biosystems) in a 96 well plate. After denaturation at 94 °C for 5 minutes, with immediate cooling on ice, the plates were centrifuged and loaded into the Genetic Analyzer fitted with 16 capillaries of 36 centimetre length and the following setting: G 5 dye set, POP -7 Conformational Analysis Polymer (Applied Biosystems) as running matrix and 1 X Genetic Analyzer Buffer with EDTA as

running buffer (supplied by Applied Biosystems). Injection was done at 1.2 kVoH for 55 seconds while the run voltage was 15 kVH. Oven temperature was set to 60 °C.

2.1.7.5 Data analysis

The spectral data is displayed in Relative Fluorescent Units (RFU). Analysis was done with the GeneMapper Software Version 4.0 (Applied Biosystems) using auto panel generation and auto panel binning. Peaks were detected between 50 – 500 basepairs with the detection algorithm set to advanced and the peak amplitude threshold set to 500. Minimum peak half width was set to 2 points, polynomial degree to 3 points and peak window size to 15 points. Maximum peak width was 1 basepairs. The local southern method was used as the size calling method.

Results were exported as present/absent tables. Distance estimation, Principal Coordinate Analysis and Polymorphic Index were done as described in 2.1.6.4.

In order to obtain a better resolution, the data was divided and analysed in two sets. The first set consisted mainly of the Estcourt individuals which were collected over two seasons (2005 and 2006). *P. setaceum* and *P. macrourum* were included as the outgroup and *P. glaucum, P. purpureum* ex Ghana, Potchefstroom Bana and Roodeplaat Green Gold* as controls. The second subset consisted of all the remaining individuals after excluding the Estcourt individuals.

2.2. RAPD introduction

Random Amplified Polymorphic DNA (RAPD) (Fig. 2.3) is a technique that amplifies random genomic DNA segments of any species from which DNA can be prepared, with the use of primers with arbitrary nucleotide sequences. It is a fast and simple technique, requiring no prior sequencing knowledge of the genome (Welsh & McClelland, 1990; Williams *et al.*, 1990).

Genomic DNA is amplified with primers chosen without regard to the sequence of the genome to be fingerprinted in a PCR amplification reaction. Fragments are then detected with high resolution agarose gel electrophoresis and visualized with ethidium staining, resulting in the identification of polymorphic bands (Welsh & McClelland, 1990).

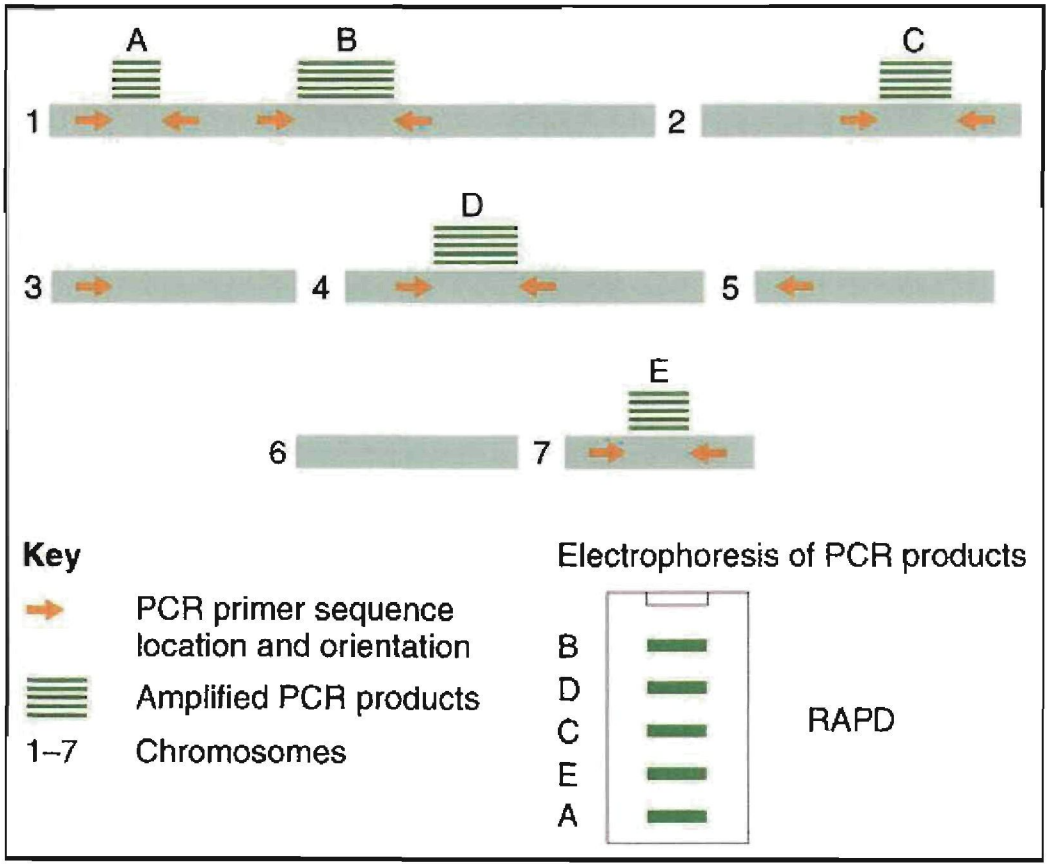

Figure 2.3 Flow diagram indicating the process of RAPD analysis using arbitrary chosen primers resulting in polymorphism detected as bands on agarose gels (From: Pawlik, D. Marker assisted breeding in the 21st century: www.usask.ca)

2.2.1 Plant material

The same plant material used for the AFLP analysis (Table 2.1) was also used for the RAPD analysis.

2.2.2. RAPD analysis

RAPD analysis was based on the protocol developed by Williams *et al.* (1990).

2.2.3 RAPD reaction

One µl (50 ng/µl) DNA was amplified in a 25 µl reaction volume containing 5 pmol primer (Table 2.3), 0.2 mM dNTP's, 2 mM $MgCl_2$, 10 x reaction polymerase buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl pH 8.8 0,1% Tween-20) and 0.5 U Biotaq DNA polymerase (Bioline). Amplification was performed using an ThermoHybaid MBS Thermal Cycler of one cycle at 94 °C for 2 minutes, followed by 30 cycles of 94 °C for 30 seconds, 36 °C for one minute and 72 °C for one minute and one cycle at 72 °C for 5 minutes. Fragments were separated with 3% agarose gel electrophoresis at constant power overnight and visualised with ethidium bromide.

2.2.4 Data analysis

Distance estimation, Principal Coordinate Analysis and Polymorphic Index were done as described in 2.1.6.4.

2.3 Correlation between the oviposition preference and larval survival of *Chilo partellus* Swinhoe on Napier grass and results of the AFLP and RAPD analysis.

Van den Berg (2006) studied the oviposition preference of *Chilo partellus* Swinhoe moths and their larval survival rates on various Napier grass varieties and cultivars found in South Africa. Results indicated that *C. partellus* prefered to oviposit on the majority of Napier grass cultivars and varieties and, although larval survival was very poor on the majority of these Napier grass varieties, some did allow larval survival.

Since the Napier grass varieties and cultivars used by Van den Berg (2006) were analysed in the present study, an attempt was made to bring the oviposition preference and larval survival observed into context with the results generated by AFLP and RAPD.

Table 2.3: Sequences of primers used in the RAPD reactions of the *Pennisetum* plant material.

Primer	**Sequence**
Operon A6	5'-GGTCCCTGAC-3'
Operon F5	5'-CCCAATTCCC-3'
Operon Z1	5'-TCTGTGCCAC-3'
Operon Z2	5' CCTACGGGGA-3'
Operon Z3	5'-CAGCACCGCA-3'
Operon Z4	5'-AGGCTGTGCT-3'
Operon Z5	5'-TCCCATGCTG-3'
Operon Z6	5'-GTGCCGTTCA-3'
Operon Z8	5'-GGGTGGGTAA-3'
Operon Z9	5'-CACCCCAGTC-3'
Operon Z10	5'-CCGACAAACC-3'
Operon Z12	5'-TCAACGGGAC-3'
Operon Z13	5'-GACTAAGCCC-3'
Operon Z14	5'-TCGGAGGTTC-3'
Operon Z15	5'-CAGGGCTTTC-3'
Operon Z18	5'-AGGGTCTGTF-3'
Operon Z20	5'-ACTTTGGCGG-3'

CHAPTER 3: RESULTS

3.1 *Eco*RI/*Mse*I analysis of 23 individuals

Twenty-three individuals were analysed with restriction enzymes *Mse*I and *Eco*RI and the separation of the amplified fragments was done with PAGE and silver staining. Of these, 19 were *Pennisetum purpureum* cultivars obtained from Estcourt and a single suspected hybrid (Estcourt Bana), obtained from Estcourt, as well as a single known hybrid specimen from Potchefstroom (Potchefstroom Bana), a single suspected *P. purpureum* cultivar (Roodeplaat Green Gold*) from Roodeplaat, a single *P. glaucum* from Cedara and a suspected *P. purpureum* individual from Ghana. The five primer combinations (*Eco*RI-AAC/*Mse*I-ACA; *Eco*RI-AAC/*Mse*I-ACC; *Eco*RI-AAC/*Mse*I-CGT; *Eco*RI-AAC/*Mse*I-CCG; *Eco*RI-AAC/*Mse*I-TAC) resulted in 276 bands (Figure 3.1 shows an example of a gel with two of the above mentioned primer combinations). Of these, three were unique to cluster A, 13 for cluster B, and one for the single *P. glaucum* analysed. The polymorphism index (PI value) for the entire analysis was 2.7.

The UPGMA tree grouped the 23 individuals into five main clusters, each with good bootstrap support (Fig. 3.2):

Cluster A: With the exception of Estcourt 1, 2, 12, 14 and 15, the Estcourt individuals, as well as the Bana hybrids obtained from Estcourt and Potchefstroom, cluster with a bootstrap value of 100%.

Cluster B: Estcourt 1, 14 and 15 forms a distinct cluster with a bootstrap value of 100%.

Cluster C: The Roodeplaat Green Gold* cultivar clusters with Estcourt individuals 4 and 12, supported by a bootstrap value of 92%.

Cluster D: *P. glaucum* clusters to the above with a bootstrap value of 100%.

Cluster E: *P. purpureum* ex Ghana clusters in turn with the former four groups.

In order to access the influence of bands with low frequencies, bands with an occurrence in samples of up to 5% (Fig. 3.3), 10 % (Fig. 3.4) and 20% (Fig 3.5) were discarded. The resultant trees are highly congruent with the tree utilising all the bands, with the position of Estcourt 19 in Figure 3.4 being the exception—bearing in mind the lack of bootstrap support.

The Principal Coordinate Analysis of the 23 individuals and all 276 bands obtained with the *Eco*RI/*Mse*I primer combination (Fig. 3.6) supports the above-mentioned clusters. The five clusters can clearly be distinguished from each other, especially so cluster B (*i.e.* Estcourt 1, 14 and 15). Estcourt 19 forms part of cluster A.

The reproducibility test, where the pre-amplification, selective amplification and gel electrophoresis were repeated using the same individuals and reaction conditions, resulted in near identical results. Figure 3.7 illustrates two gels with the same primer combination and individuals but run on different days (confirm for example, bands A, B and C repeated in both runs).

3.2 AFLP results of primer combination *Mlu*I/*Mse*I

3.2.1 *Mlu*I/*Mse*I analysis of 23 individuals

The twenty-three individuals that were analysed with primer combination *Eco*RI and *Mse*I (Section 3.1) were also analysed with primer combination *Mlu*I and *Mse*I on an automatic ABI 3130 *xl* capillary electrophoresic genetic analyser using four primer combinations (*Mlu*I-TCA/*Mse*I-ACA; *Mlu*I-TCA/*Mse*I-CCG; *Mlu*I-TAA/*Mse*I-ACC; *Mlu*I-TAA/*Mse*I-TAC), resulting in 1026 bands of which three bands were unique to cluster B and C respectively; 45 bands for cluster D; two bands for cluster E; 14 bands for cluster F and 55 bands for cluster G. The polymorphism index for the entire analysis was 5.2.

The UPGMA tree grouped the 23 individuals into seven main clusters, most with good bootstrap support (Fig. 3.8). Here Estcourt 1, 14 and 15 (Fig. 3.8: B) as well as Estcourt 4, 12 and Roodeplaat Green Gold* (Fig. 3.8: D) also form distinct clusters (as they did with the *Mse*I and *Eco*RI analyses) with bootstrap values of 99% and

100% respectively. The tree (Fig. 3.8) differs mainly from the one based on *Mse*I and *Eco*RI (Fig. 3.2) in that Estcourt 10 and Nanzindlela form a separate cluster (Fig. 3.8: C)—here with a higher bootstrap support than in the *Mse*I and *Eco*RI analyses—clustering to A and B. However, the clustering of A and B received relatively low bootstrap support. Furthermore, the position of *P. purpureum* ex Ghana and *P. glaucum* also changes in relation to each other, while Estcourt 19 occurs on its own—in this sense similar to Figure 3.4—and with a bootstrap value of 69%.

The influence of bands with low frequencies were also tested with the *Mlu*I/*Mse*I primer combination and bands with an occurence of up to 5% (Fig. 3.9), 10% (Fig. 3.10) and 20% (Fig. 3.11) were discarded. In all three cases, Estcourt 19 now clusters with *P. purpureum* ex Ghana, albeit with no good bootstrap support. In contrast, the marked differences in the position of cluster C in Fig. 3.9–3.10 in relation to Fig. 3.11 all receive moderate bootstrap support.

The Principal Coordinate Analysis of the 23 individuals and 1026 bands obtained with *Mlu*I/*Mse*I (Fig 3.12) did not fully support the existence of seven main clusters identified in the aforementioned UPGMA tree. Cluster A and cluster C in the UPGMA tree are not discernable in the PCA analysis. In addition, Estcourt 1 is more distant from Estcourt 14 and 15, than the UPGMA tree would suggest. The PCA analysis also suggests Estcourt 19 (cluster E) to be a separate entity. Estcourt 9 (number 20) a member of cluster A in the UPGMA tree, also appears to be a distinct entity in the PCA analysis.

3.2.2 *Mlu*I/*Mse*I analyses of 145 individuals

In an expanded taxon sample, 145 individuals (Table 2.1) were analysed with the restriction enzymes *Mlu*I and *Mse*I on an automatic ABI 3130 *x*l capillary electrophoresic genetic analyser.

Figure 3.13 provides an example of the profiles of six individuals generated by the ABI 3130 *x*l Genetic Analyser. The profiles show the bands generated from 0 to 800 basepairs. A high number of bands were generated in the lower size range (up to 150 bp) from where it decreased. The profiles also give an indication of the DNA

concentration. The DNA concentration of profiles Penn 179 (*P. purpureum* Umbeluzi3), Penn 50 (Estcourt 5.3) and Penn 97 (*P. setaceum*) were satisfactory but needed to be increased for profiles Penn 215 (Ethiopia 16804) and Penn 217 (Ethiopia 16806) and lowered for profile Penn 14 (Estcourt 14). Monomorphic and polymorphic bands can also be seen on the profiles. Arrow A indicates a band that was present in all the individuals except for Penn 97 (*P. setaceum*) and arrow C indicates a band that was only present in Penn 179 (*P. purpureum* Umbeluzi3) and Penn 14 (Estcourt 14). Arrow B indicates a monomorphic band that was present in all the individuals.

Figure 3.14 shows overlayed profiles of 9 individuals, which were produced with *Mlu*I-TCA/*Mse*I-CCG. Each individual's profile is represented by a different color, therefore, bands unique to an individual and bands shared among individuals can clearly be seen (arrows A and B). Estcourt 12 (Penn 12 (black)) has 5 unique bands, as well as bands that it shares with other individuals and bands that are absent while it is present in other individuals. Nanzindlela (Penn 21 (orange)), Estcourt Bana (Penn 22 (brown)) and Potchefstroom Bana (Penn 23 (yellow)) have two bands that they share and Nanzindlela has three bands unique to its profile. Nanzindlela and Estcourt 12 share one band with one another but not with any of the other individuals. Ethiopia 16813 (purple) has three bands unique to its profile and shares two bands with Estcourt 12 and one band with Nanzindlela. Estcourt 8 (Penn 8 (green)) has two unique bands although one of the bands is off scale (indicated by the pink line running from top to bottom of profile). The rest of its bands is shared with the other individuals, including one that it shares with Nanzindlela. Estcourt 13 (Penn 13 (light blue)) does not have any unique bands and shares all its bands with the other individuals. Estcourt 11 (Penn 11 (dark blue)) has two unique bands and shares the rest of the bands with the other individuals, including one band with Nanzindlela.

The 145 individuals were analysed with four *Mlu*I/*Mse*I primer combinations (*Mlu*I-TCA/*Mse*I-ACA; *Mlu*I-TCA/*Mse*I-CCG; *Mlu*I-TAA/*Mse*I-ACC; *Mlu*I-TAA/*Mse*I-TAC), resulting in 1026 bands of which three were unique to cluster G, and 24 for cluster H. The PI value for the entire analysis was 0.2.

The UPGMA tree grouped the 145 individuals into nine main clusters, of which only one is supported by a bootstrap value (Fig. 3.15):

Cluster A: The majority of the Ethiopian individuals clustered together, with a few individuals from Estcourt, Roodeplaat, and all the individuals obtained from Botswana (*P. purpureum* cv. Swaziland and *P. purpureum* ex. Sanitas). A number of hybrids, except for five (Potchefstroom Bana 2 and 3; Roodeplaat Bana 1 and 2 and Ethiopia 16834H) also clustered in this group.

Cluster B: This group contains all the remaining individuals obtained from Ethiopia (except Ethiopia 16621), a few individuals from Estcourt, all of the individuals obtained from Mozambique (*P. purpureum* Umbeluzi and *P. purpureum* Agro Farm), the majority of the Roodeplaat individuals, and the five hybrids (Potchefstroom Bana 2 and 3; Roodeplaat Bana 1 and 2 and Ethiopia 16834H) that did not group with the other hybrids in the first cluster.

Cluster C: This cluster also consists exclusively of Estcourt individuals.

Cluster D: This cluster also consists exclusively of Estcourt individuals.

Cluster E: As in the majority of previous analyses above, Estcourt 19 also clusters separately.

Cluster F: All the Roodeplaat Green Gold and Harare individuals obtained from Roodeplaat clustered together with Ethiopia 16621 and a number of Estcourt individuals (3.2, 3.3, 4, 9.2, 9.3, 12, 19.2 and 19.3). This is the only cluster that receives bootstrap support.

Cluster G: *P. purpureum* ex Ghana clusters on its own.

Cluster H: *P. glaucum* clusters on its own.

Cluster I: This cluster contains *P. setaceum* and *P. macrourum* individuals.

The Estcourt indivuals of the same number sequence (*e.g.* Estcourt x, x.2 and x.3) collected over two seasons (2005 and 2006) did not always cluster together.

The PCA of the 145 individuals analysed (Fig. 3.16) is difficult to interpret due to the large number of samples present and the graphical limitations of the NTSYS software employed. The PCA does not reflect the main clusters obtained in the dendrogram, although cluster F clearly clusters separate in the diagram.

3.2.3. *Mlu*I/*Mse*I analysis of the Estcourt individuals

The UPGMA tree of the *Mlu*I/*Mse*I anlaysis confined to individuals obtained from Estcourt resulted in eight main clusters (Fig. 3.17), which were not supported by good bootstrap values. One band was unique for cluster C and E respectively, four for cluster G and 44 for cluster H. The Polymorphism index (PI) for the analysis was 0.7.

The composition of the clusters were similar to that of Figure 3.15:

Cluster A: The biggest cluster contains most of the individuals obtained from Estcourt, as well as the hybrids obtained from Potchefstroom.

Cluster B: This cluster is identical to cluster C in figure 3.15.

Cluster D: This cluster mirrors to a large extent cluster D in figure 3.15, but now also includes Estcourt 20.2 and 20.3

Cluster E: Estcourt 19 is again a separate entity clustering to A and B above.

Cluster F: The Estcourt individuals that cluster with the Roodeplaat Green Gold* individual (Fig. 3.2 and 3.8) were the same Estcourt individuals as in Figure 3.15.

Cluster G: *P. purpureum* ex Ghana again clusters on its own.

Cluster H: *P. glaucum* still clusters on its own.

Cluster I: *P. setaceum* and *P. macrourum* cluster distinctly from the rest of the individuals as a separate group.

The PCA of the Estcourt individuals (Fig. 3.18) only distinguished cluster F and I as clear separate entities.

The indivuals of the same number sequence (*e.g.* Estcourt x, x.2 and x.3) collected over two seasons (2005 and 2006) did not always cluster together, *e.g.* Estcourt 11. Figure 3.19 a-e shows the profiles of Estcourt 11 collected during 2005 (Penn 11) and 2006 (Penn 64 and 66). The profiles of the two individuals collected during 2006 (green and red profiles) were the same except for slight differences, but the profiles of both individuals collected during 2006 differ from the individual collected during 2005. The differences are indicated by arrows A and similarities by arrows B on figure 3.19 a-e.

3.2.4 *Mlu*I/*Mse*I analysis of all the individuals except for the Estcourt individuals.

The UPGMA tree based on a second subset consisting of all the remaining individuals after excluding the Estcourt individuals (Fig. 3.20) results in similar clusters suggested by the UPGMA tree based on an analsyis of all individuals (Fig. 3.15), although the sequence of the individuals in the main clusters differ slightly. Noteworthy is that whereas cluster A and B in Fig 3.15 had no bootstrap support, in essentially the same split, they now have a bootstrap support of 89%. Similarly, cluster F possesses a higher bootstrap support here (84%) than the corresponding cluster (Cluster F) in Fig. 3.15. The split between *P. glaucum* and the rest of the clusters now receives a bootstrap suport of 55%. Roodeplaat Mfufu and Ethiopia 16790 which clustered in cluster B (Fig. 3.15), now cluster in cluster A (Fig. 3.20). Cluster H has 29 unique bands and cluster G six. The Polymorphism Index (PI) is 0.4.

In the Principal Coordinate Analysis of all the individuals except for the Estcourt individuals (Fig 3.21), the three individuals of *P. setaceum* and *P. macrourum* as well as cluster F cluster separately from the rest of the clusters which are scattered thoughout the diagram.

The reproducibility of the bands was tested by repeating the analysis and results were found to be highly reproducible. Reproducibility was tested both with a repeat of the selective amplification and a full repeat from the restriction digestion step. Figure

3.22 a-c shows the profiles of Estcourt 11 repeated three times on three different days from 50 – 420 basepairs. The three repeats are each coloured with a different colour, and except for slight differences (arrow A), the amplification on the three days were the same and generated the same bands, although with slight differences in intensities (arrow B).

3.3 Results obtained from the RAPD method

The same individuals that were analysed with the AFLP method were also analysed with the RAPD method. Seventeen primers were tested but only four primers (Operon Z6, Z10, Z18, Z20) revealed bands, but not enough polymorphism (Fig. 3.23) to distinguish the individuals sufficiently from one another. The technique was therefore not developed any further. Since there were no unique bands, the polymorphism index (PI) had a value of 0.

3.4 Correlation between the oviposition preference and larval survival of *Chilo partellus* on Napier grass and results of the AFLP and RAPD analysis

Since the RAPD technology was not developed any further, no evaluation of the relationships between the oviposition preference and larval survival with plant relatedness could be made.

As a contingency, those individuals used by Van den Berg (2006) (Table 3.1) were indicated in the UPGMA tree based on an analysis of all individuals and all bands (Fig 3.24).

No clear pattern in oviposition preference as indicated by the numbers of eggs per plant on the different cultivars was observed. Similarly, no distinct pattern was observed when the larval survival rate was plotted on the dendrogram. In the oviposition experiments, cultivars Estcourt 12 and 18 both were non-preferred for oviposition. These cultivars were very dissimilar to one another, occuring in cluster F and A respectively. Estcourt 8 had the highest oviposition preference and occured in cluster A that also housed the most individuals analsyed in this case.

Table 3.1: The mean number of eggs laid on the leaves and the percentage survival of larvae on different Napier grass individuals (Van den Berg, 2006)

Individual	Mean number or persentage eggs laid	Persentage survival
Estcourt 2	142.7	0
Estcourt 3	283.8	0
Estcourt 6	125.0	0
Estcourt 8	397.8	0
Estcourt 9	178.0	0
Estcourt 10	237.5	0
Estcourt 11	194.3	0
Estcourt 12	45.7	2
Estcourt 13	144.5	0
Estcourt 18	70.3	0
Estcourt 19	243.7	0
Potchefstroom Bana	330.0	0
Nanzindlela	127.7	1
Roodeplaat Gold Coast Napier	230.0	0

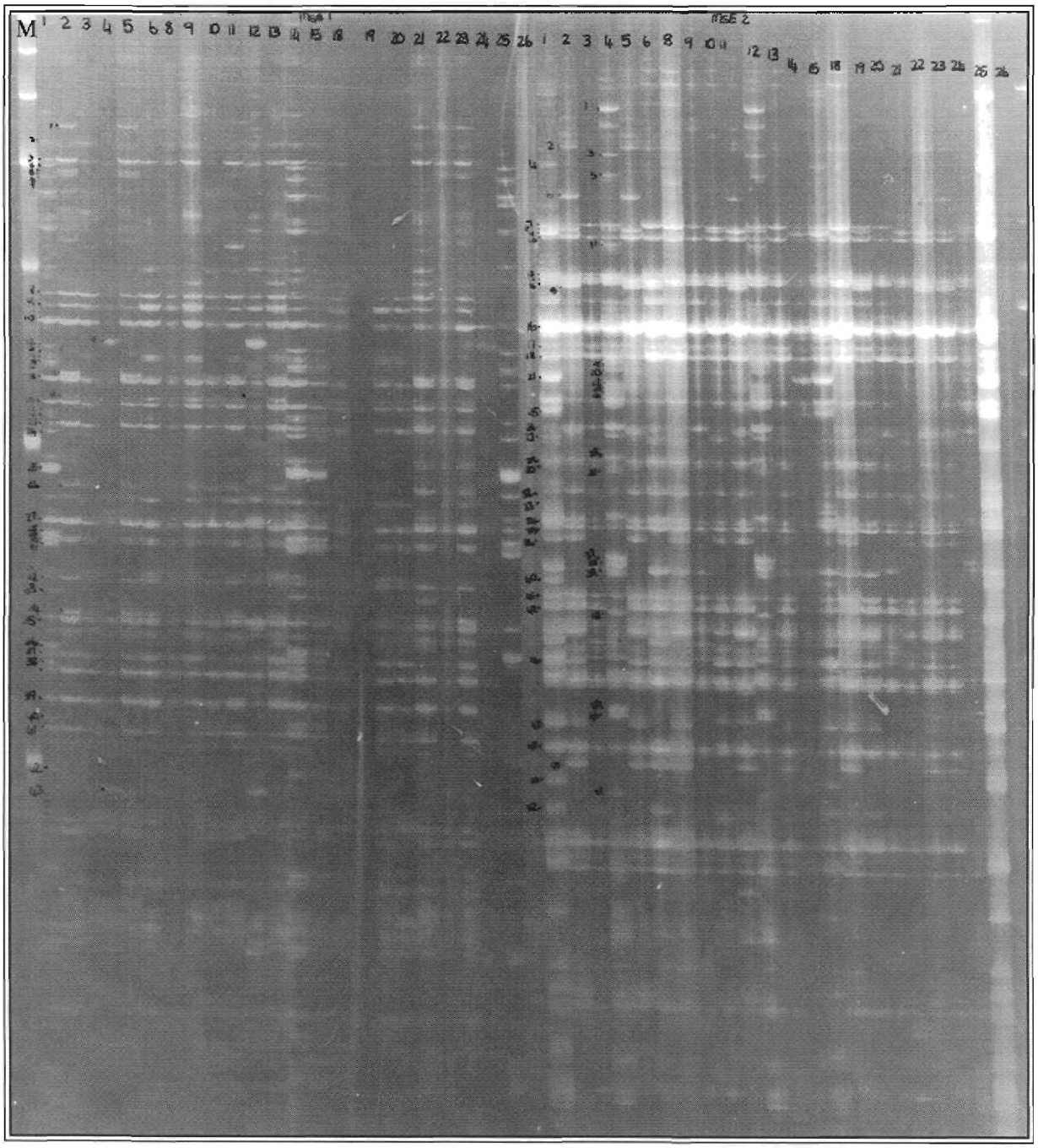

Figure 3.1 Example of a gel showing bands obtained with primer combinations *Eco*RI-AAC/*Mse*I-ACA and *Eco*RI-AAC/*Mse*I-ACC. Column M contains a molecular marker (100 bp ladder). Lanes 1 – 23 are the different individuals analysed. Sample numbers are as follows: 1 - 20 are Estcourt individuals. Sample 21 = Nanzindlela; 22 = Estcourt Bana; 23 = Potchefstroom Bana; 24 = Roodeplaat Green Gold*; 25 = Cedara *P. glaucum*; 26 = *P. purpureum* ex Ghana.

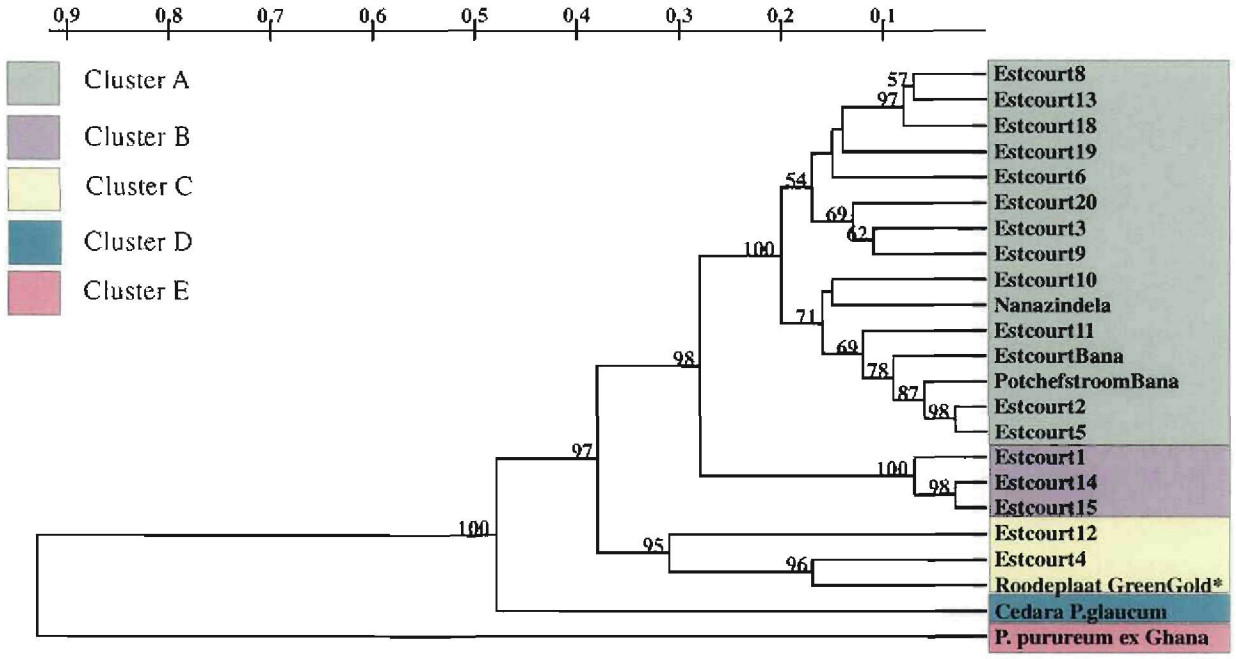

Figure 3.2 UPGMA tree based on AFLP data using *Eco*RI/*Mse*I and PAGE. Numbers above branches are bootstrap values.

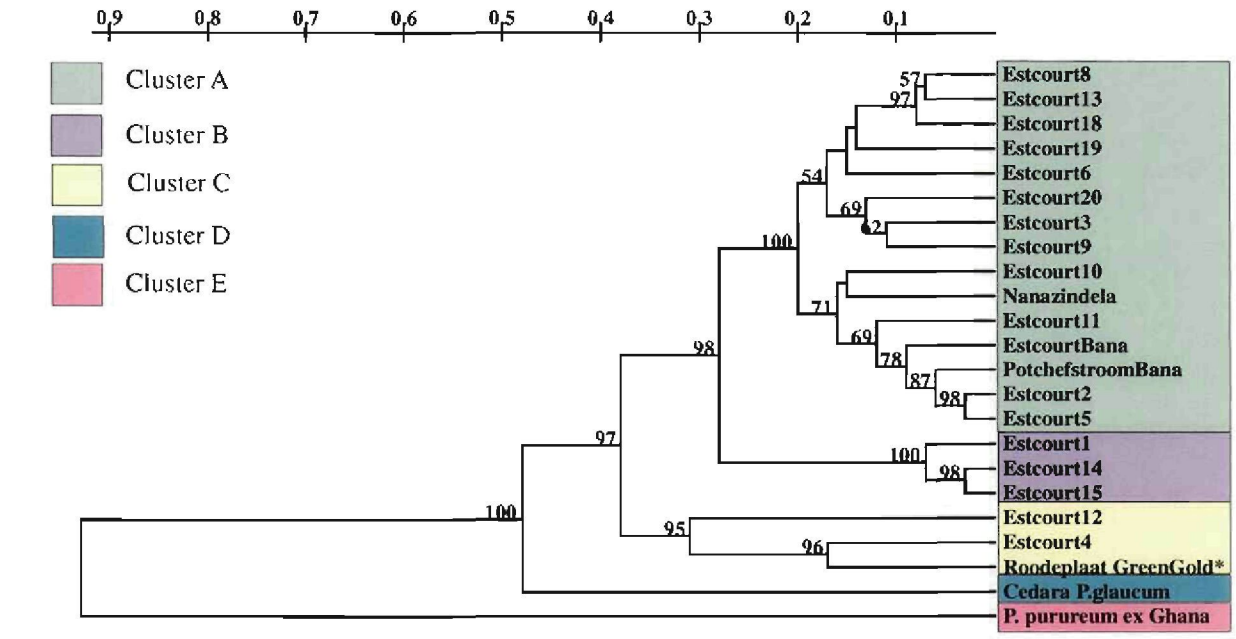

Figure 3.3 UPGMA tree of the AFLP analysis using *Eco*RI/*Mse*I and PAGE, excluding bands with a 5% frequency. Numbers above branches are bootstrap values.

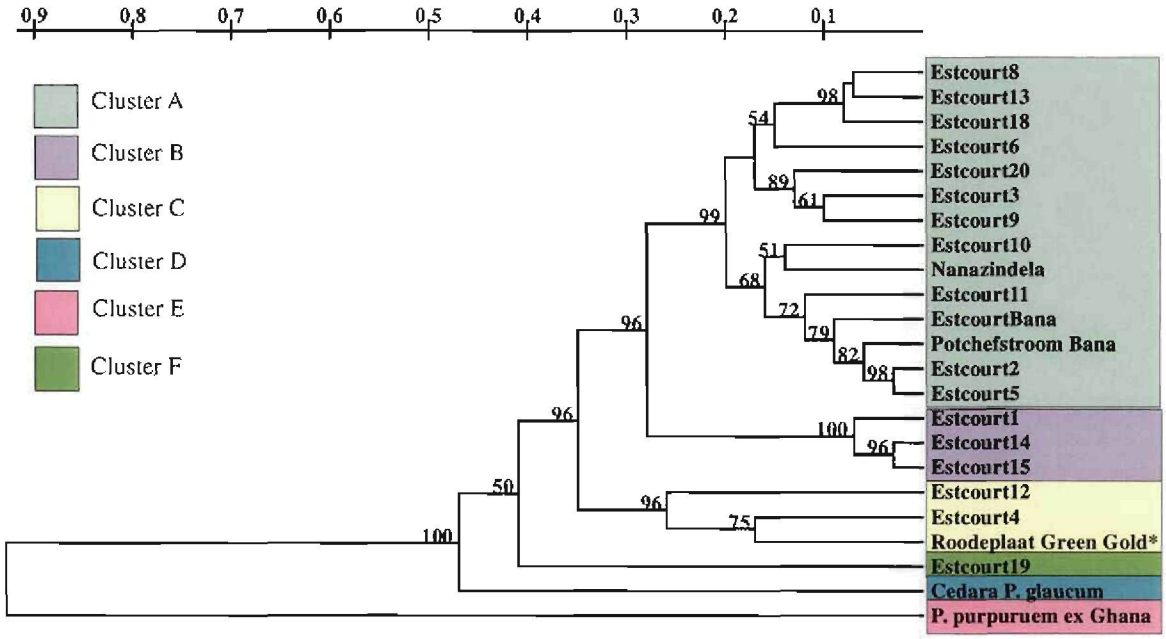

Figure 3.4 UPGMA tree of the AFLP analysis using *Eco*RI/*Mse*I and PAGE, excluding bands with a 10% frequency. Numbers above branches are bootstrap values.

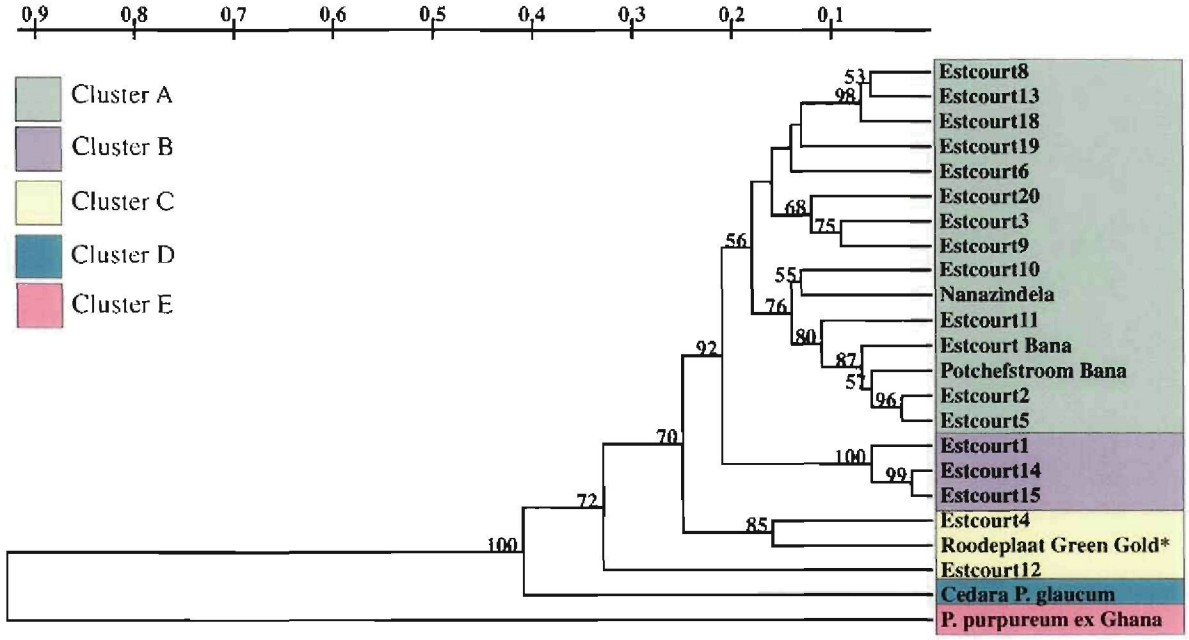

Figure 3.5 UPGMA tree of the AFLP analysis using *Eco*RI/*Mse*I and PAGE, excluding bands with a 20% frequency. Numbers above branches are bootstrap values.

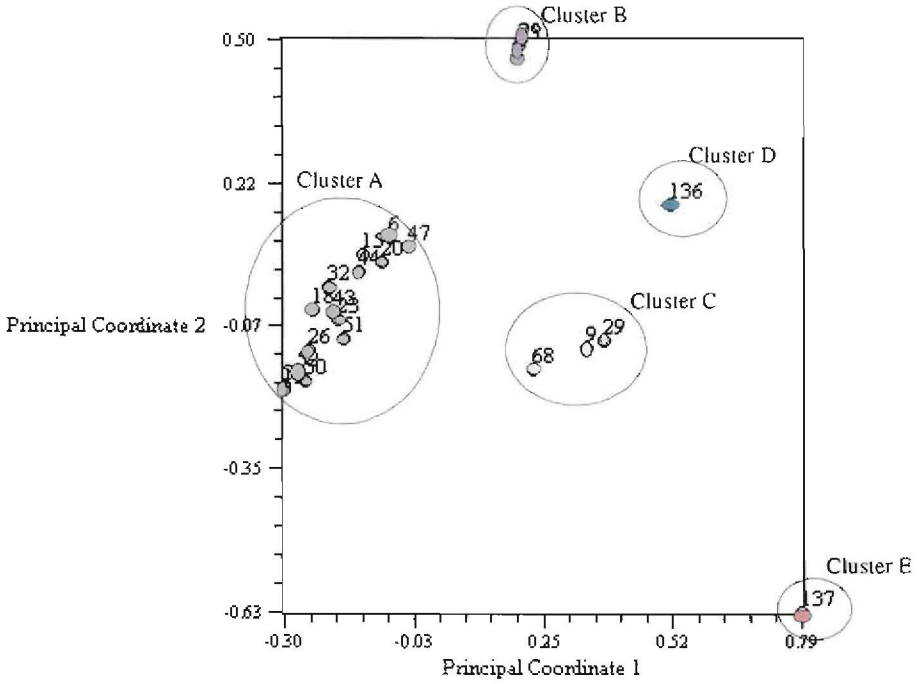

Figure 3.6 Principal Coordinate Analysis of all the bands obtained with AFLP analysis using *Eco*RI/*Mse*I and PAGE indicating five main clusters. Refer to Table 2.1 for sample numbers.

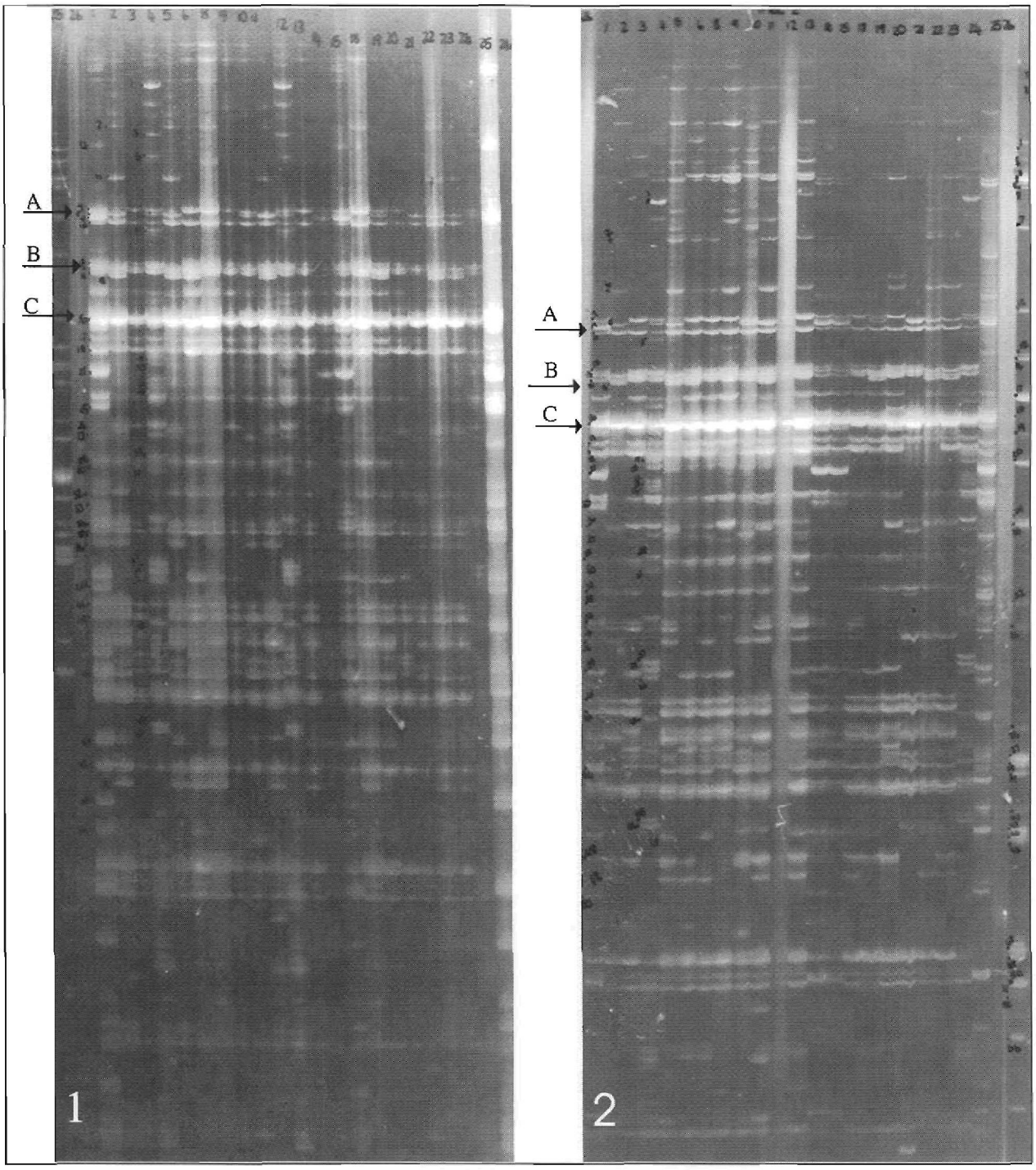

Figure 3.7 The two gels with the primer combination *Eco*RI/*Mse*I and individuals run on different days. Arrow A, B and C indicate some of the bands identical in both runs. Sample numbers: 1 - 20 = Estcourt individuals. Sample 21 = Nanzindlela; 22 = Estcourt Bana; 23 = Potchefstroom Bana; 24 = Roodeplaat Green Gold*; 25 = Cedara *P. glaucum*; 26 = *P. purpureum* ex Ghana.

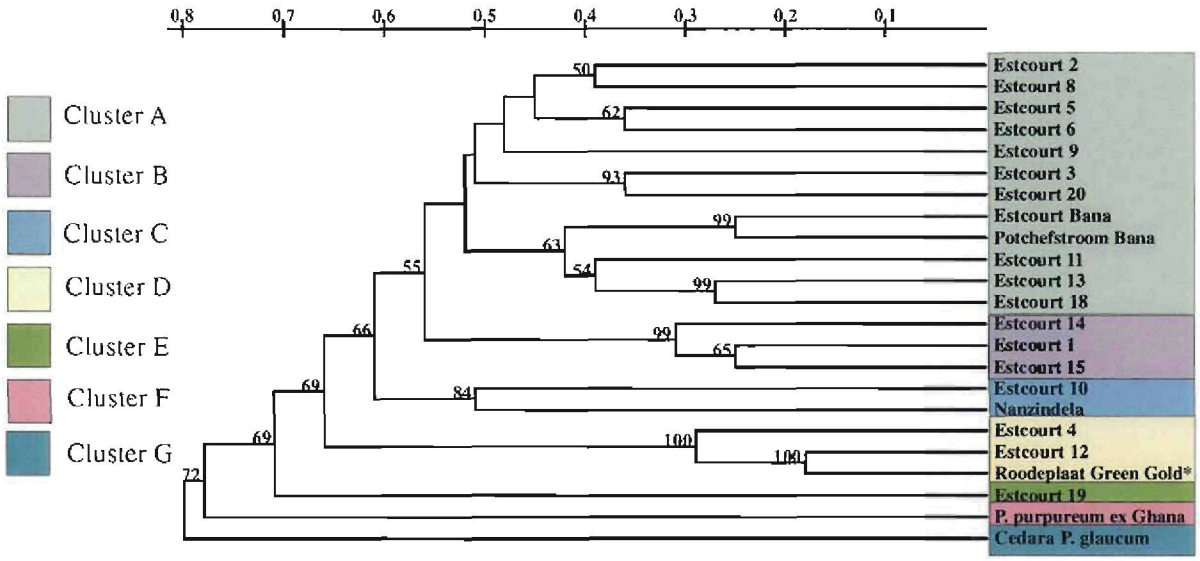

Figure 3.8 UPGMA tree based on AFLP data using *MluI*/*Mse*I an ABI 3130 *xl* Genetic Analyser. Numbers above branches are bootstrap values.

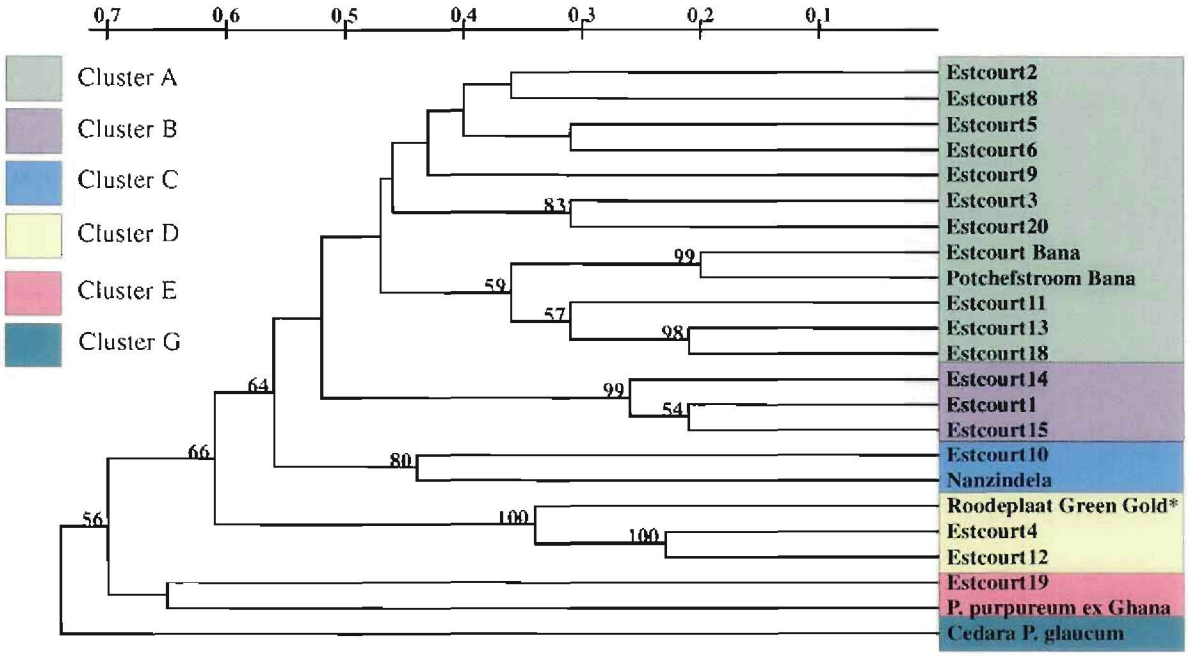

Figure 3.9 UPGMA tree of AFLP analysis using *MluI*/*Mse*I and an automatic sequencer excluding bands with a 5% frequency. Numbers above branches are bootstrap values.

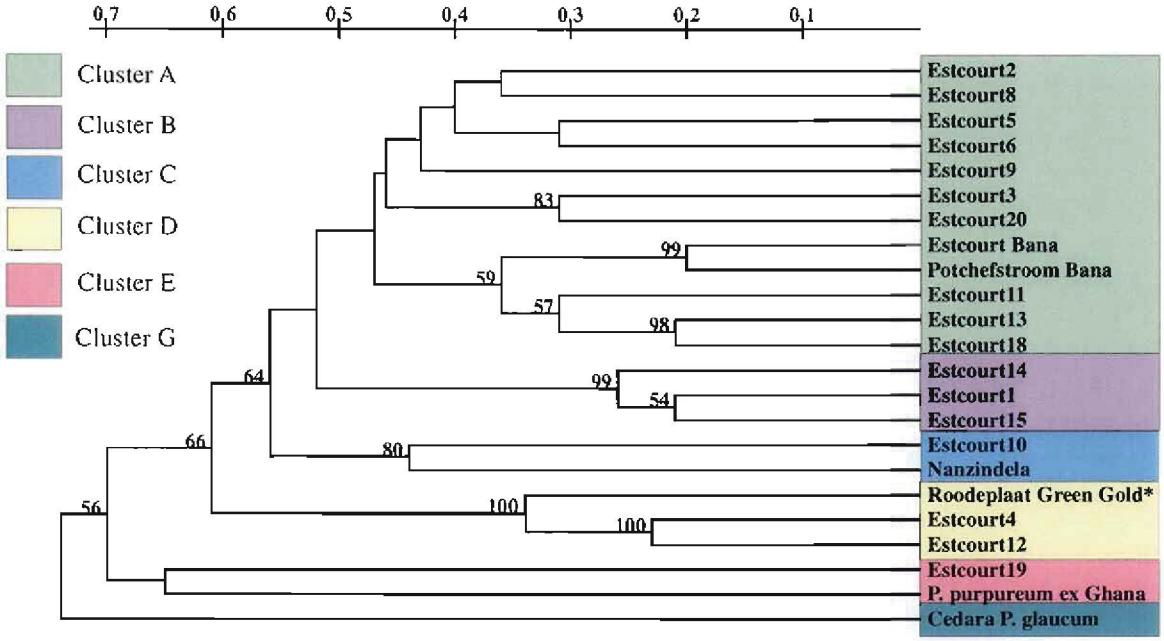

Figure 3.10 UPGMA tree of AFLP analysis using *MluI/Mse*I and an automatic sequencer excluding bands with a 10% frequency. Numbers above branches are bootstrap values.

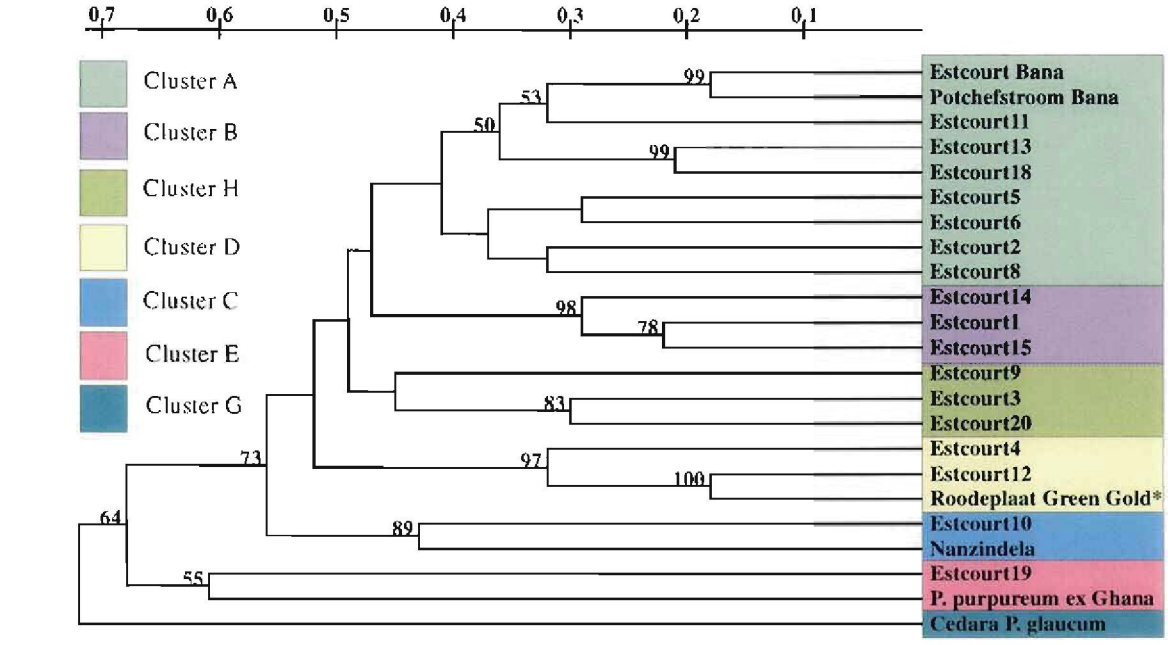

Figure 3.11 UPGMA tree of AFLP analysis using *MluI/Mse*I and an automatic sequencer excluding bands with a 20% frequency. Numbers above branches are bootstrap values

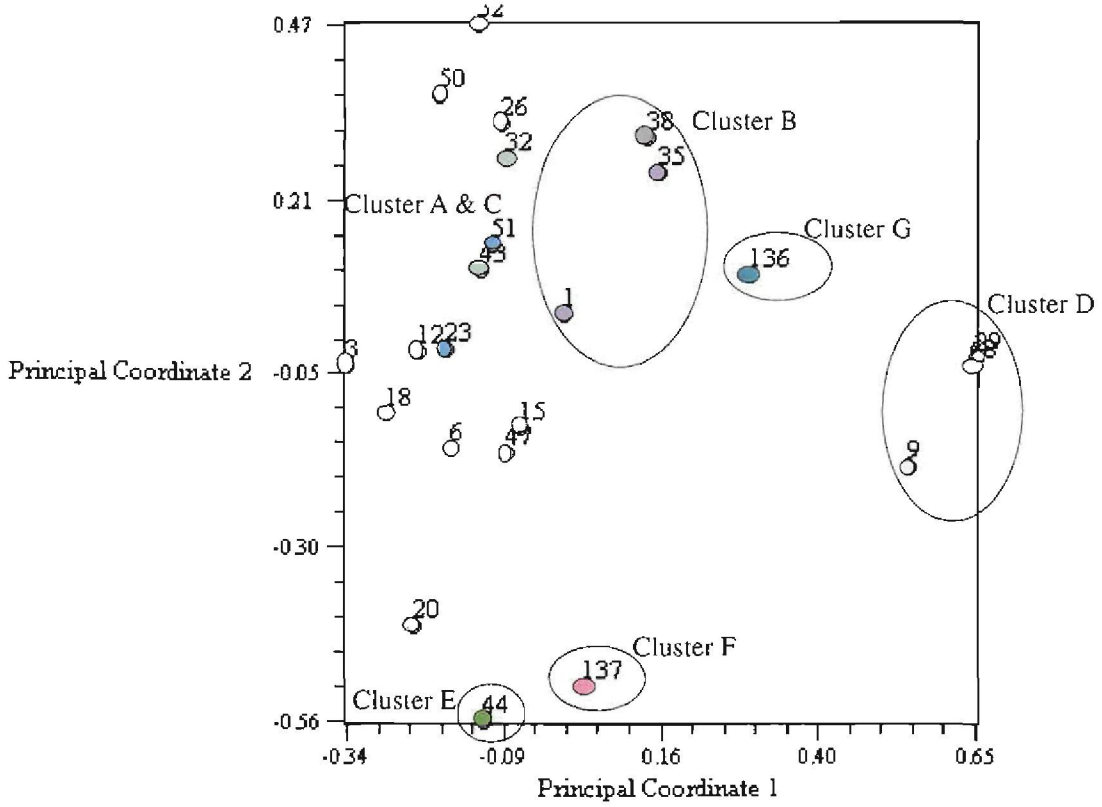

Figure 3.12 Principal Coordinate Analysis showing the seven clusters in which the 23 individuals analysed with *MluI/MseI* and the ABI 3130 *xl* Genetic Analyser cluster. Refer to Table 2.1 for sample numbers.

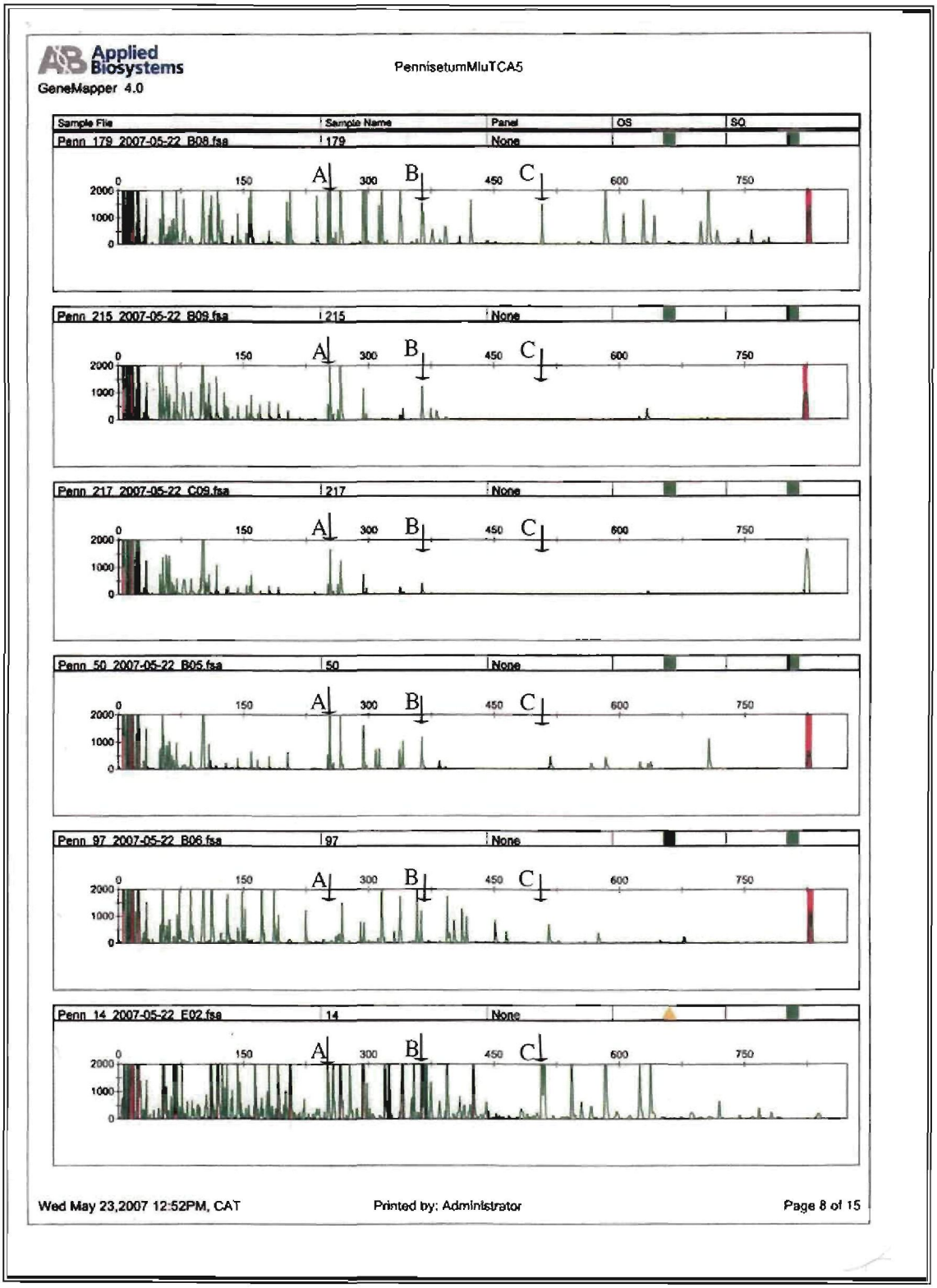

Figure 3.13 Examples of the profiles of six *Pennisetum* individuals visualized by the ABI 3130 *x*l Genetic Analyser. Penn 179 is *P. purpureum* Umbeluzi3; Penn 215 = Ethiopia 16804; Penn 217 = Ethiopia 16806; Penn 50 = Estcourt 5.3; Penn 97 = *P. setaceum* and Penn 14 = Estcourt 14. Arrows A and C indicate polymorphic bands and arrow B indicates a monomorphic band.

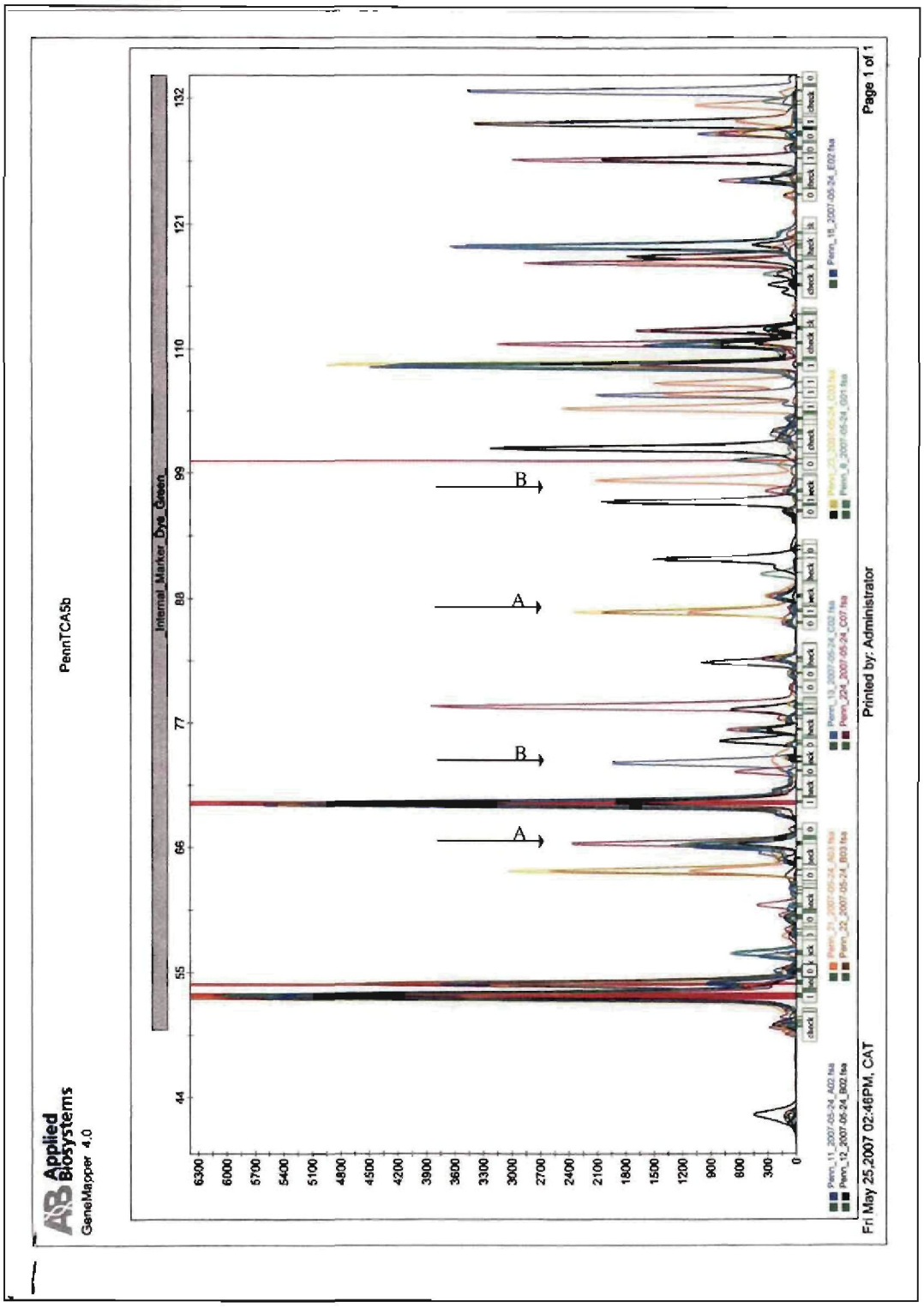

Figure 3.14 Profiles of nine individuals, overlayed, indicating shared bands (A) and unique bands (B) for the individuals analysed. Each color represents one individual. Penn 8 – 18 = Estcourt 8 – 18; Penn 21 = Nanazindela; Penn 22 = Estcourt Bana; Penn 23 = Potchefstroom Bana; Penn 224 = Ethiopia 16813.

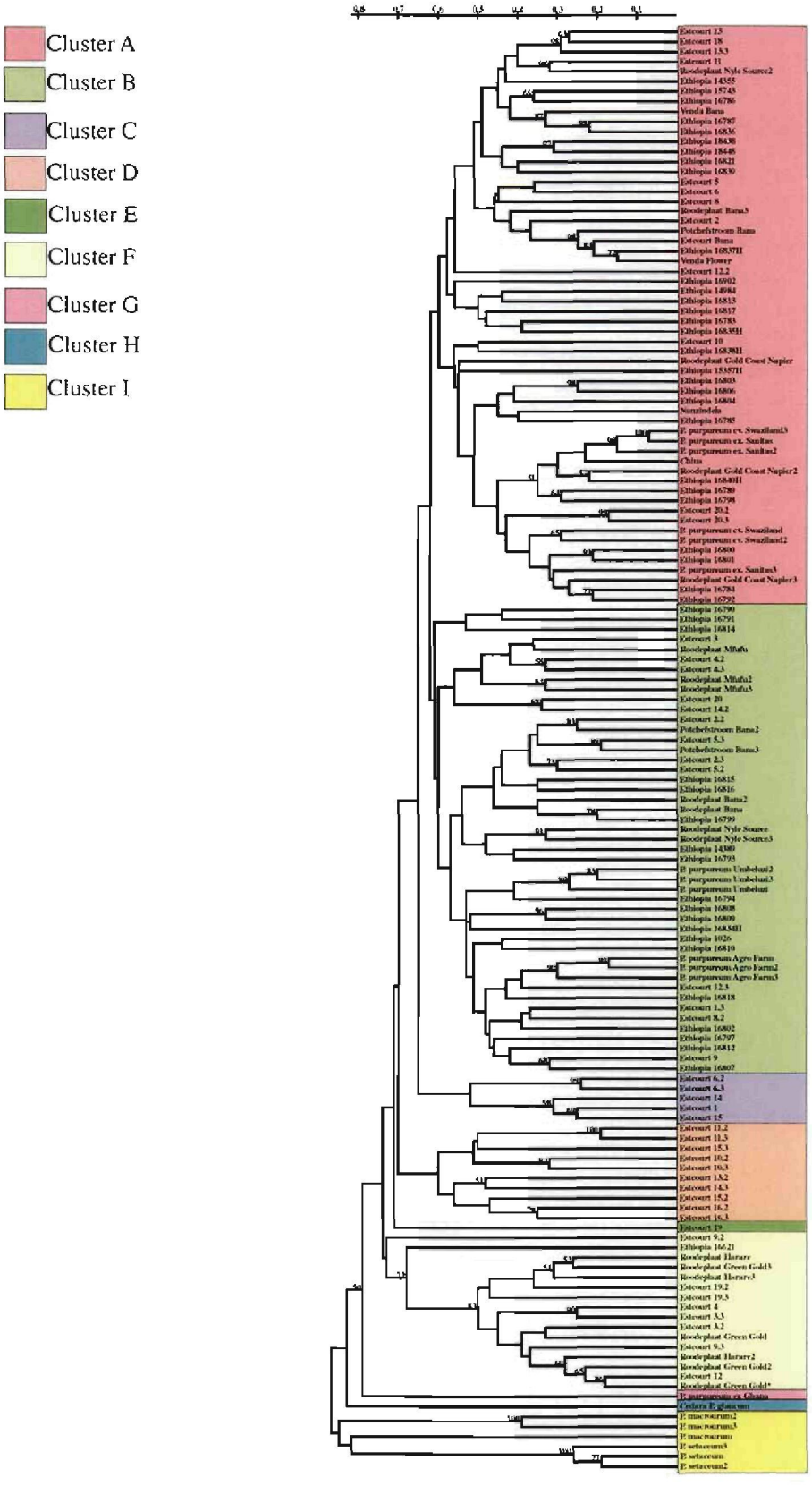

Figure 3.15 Dendrogram constructed with an UPGMA clustering algorithm indicating the nine clusters obtained from the AFLP analysis using four *MluI*/*Mse*I primer combinations and the ABI 3130 *x*l Genetic Analyser. Numbers above the branches are bootstrap values.

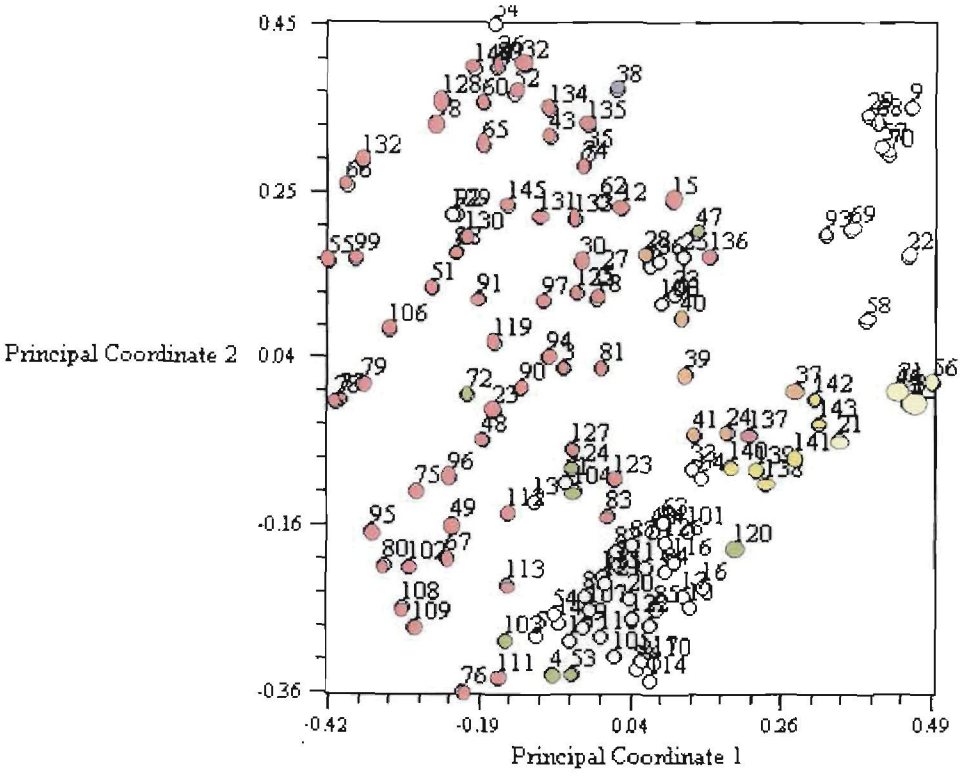

Figure 3.16 Principal Coordinate Analysis of the 145 individuals and all the bands obtained with the AFLP analysis using four *MluI/MseI* primer combinations and the ABI 3130 *xl* Genetic Analyser. Refer to Table 2.1 for sample numbers.

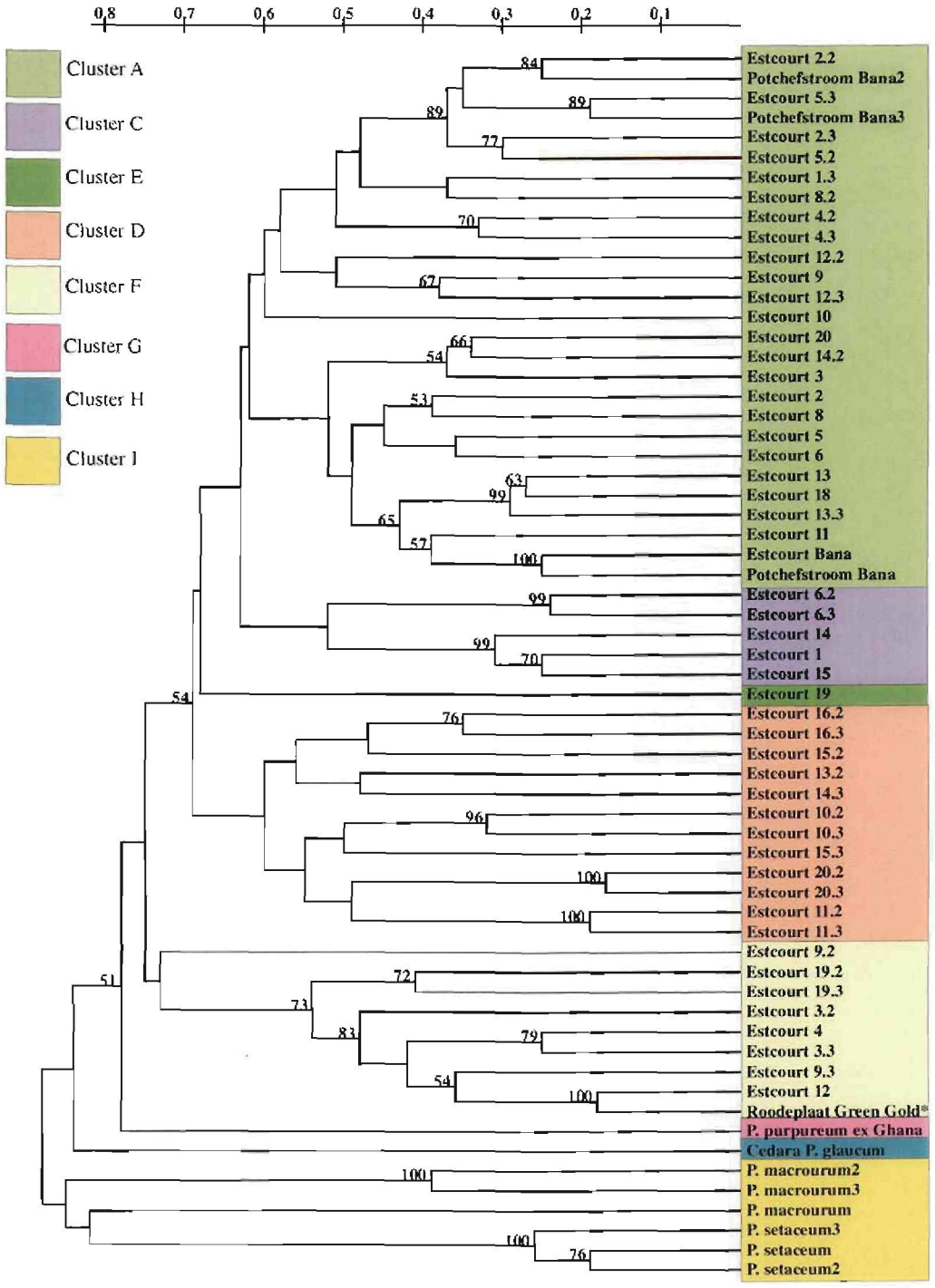

Figure 3.17 UPGMA tree of a subset of AFLP data using four *MluI/MseI* primer combinations and including only the Estcourt individuals as well as *P. macrourum, P. setaceum, P. purpureum* ex Ghana, *P. glaucum,* Potchefstroom Bana and Roodeplaat Green Gold* as controls. Numbers above the branches are bootstrap values.

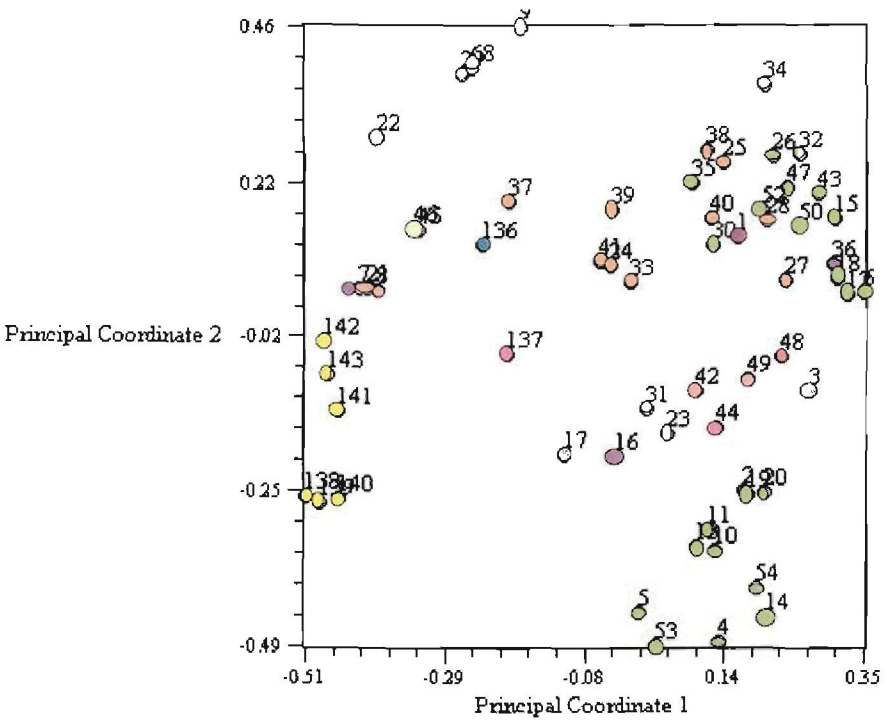

Figure 3.18 Figure 3.18 Principal Coordinate Analysis of a subset of AFLP data using four *MluI/Mse*I primer combinations and only including Estcourt individuals as well as *P. macrourum, P. setaceum, P. purpureum* ex Ghana, *P. glaucum,* Potchefstroom Bana and Roodeplaat Green Gold* as controls. Refer to Table 2.1 for sample numbers.

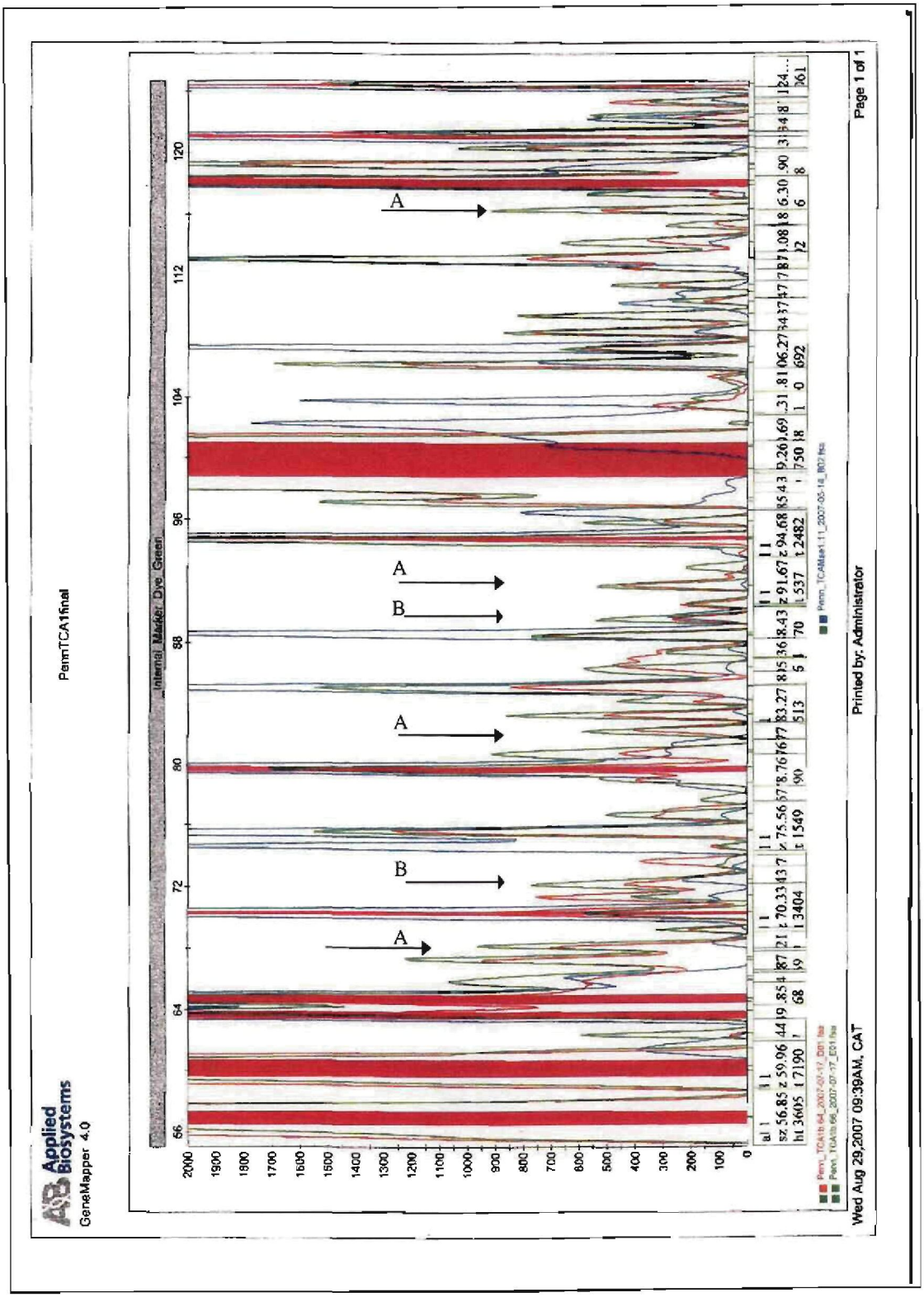

Figure 3.19a Profiles of the three Estcourt 11 individuals, Estcourt 11 (Penn 11, 2005); Estcourt 11.2 (Penn 64, 2006) Estcourt 11.3 (Penn 66, 2006), collected over two years from 50 – 125 basepairs. Arrows A indicate differences and arrows B similarities.

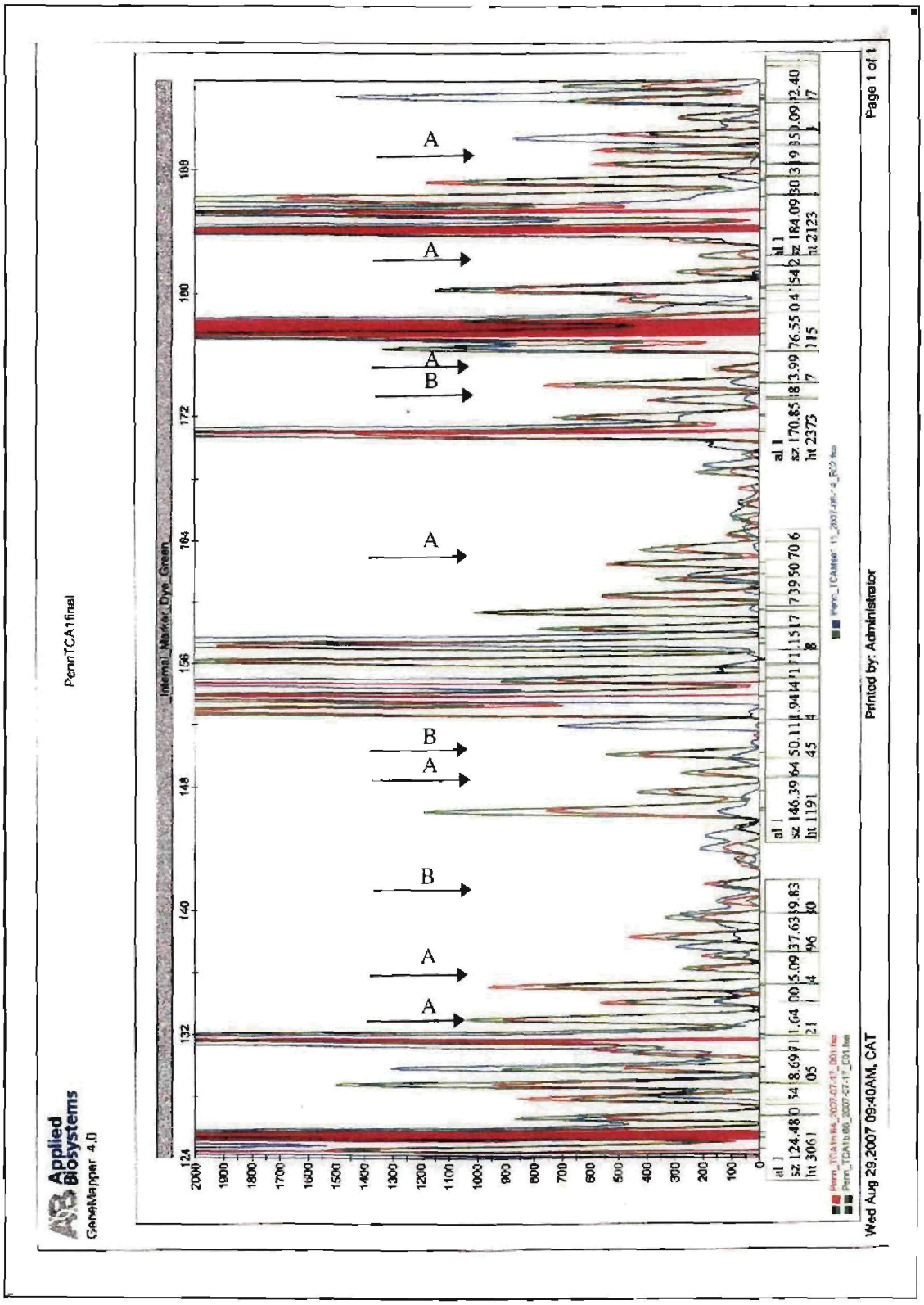

Figure 3.19b Profiles of the three Estcourt 11 individuals from 124– 188 basepairs.

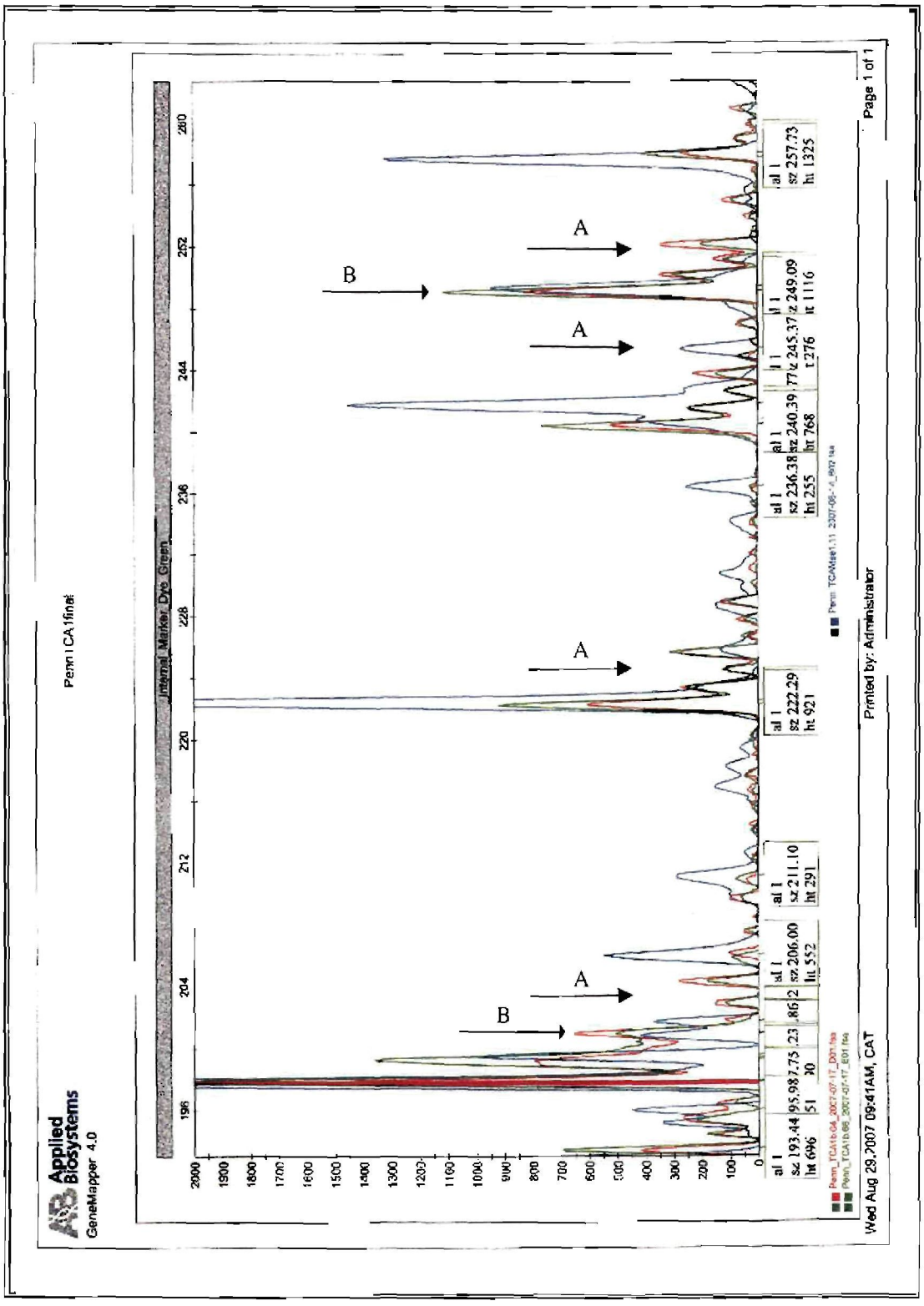

Figure 3.19c Profiles of the three Estcourt 11 individuals from 196 - 260 basepairs.

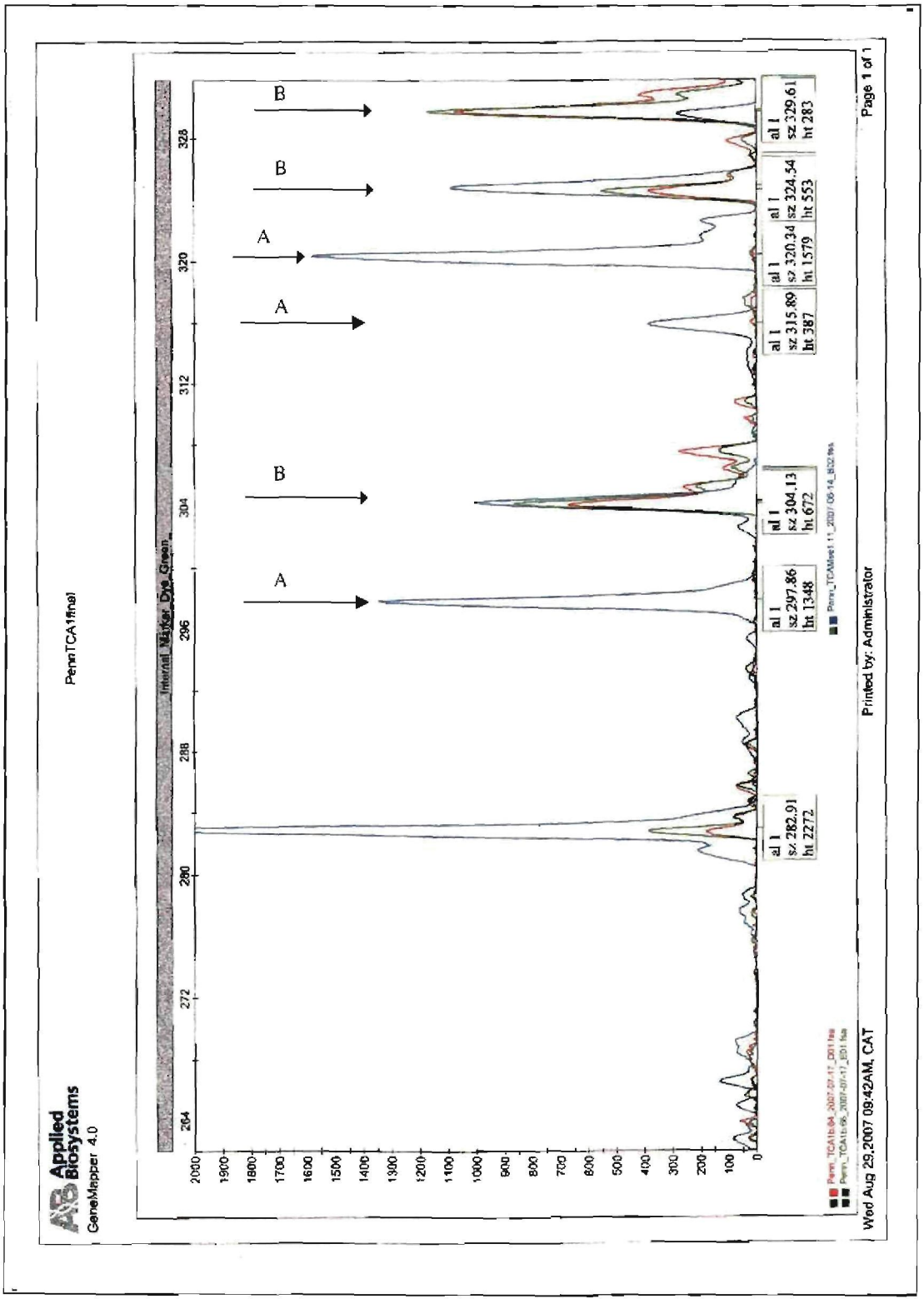

Figure 3.19d Profiles of the three Estcourt 11 individuals from 264 – 328 basepairs.

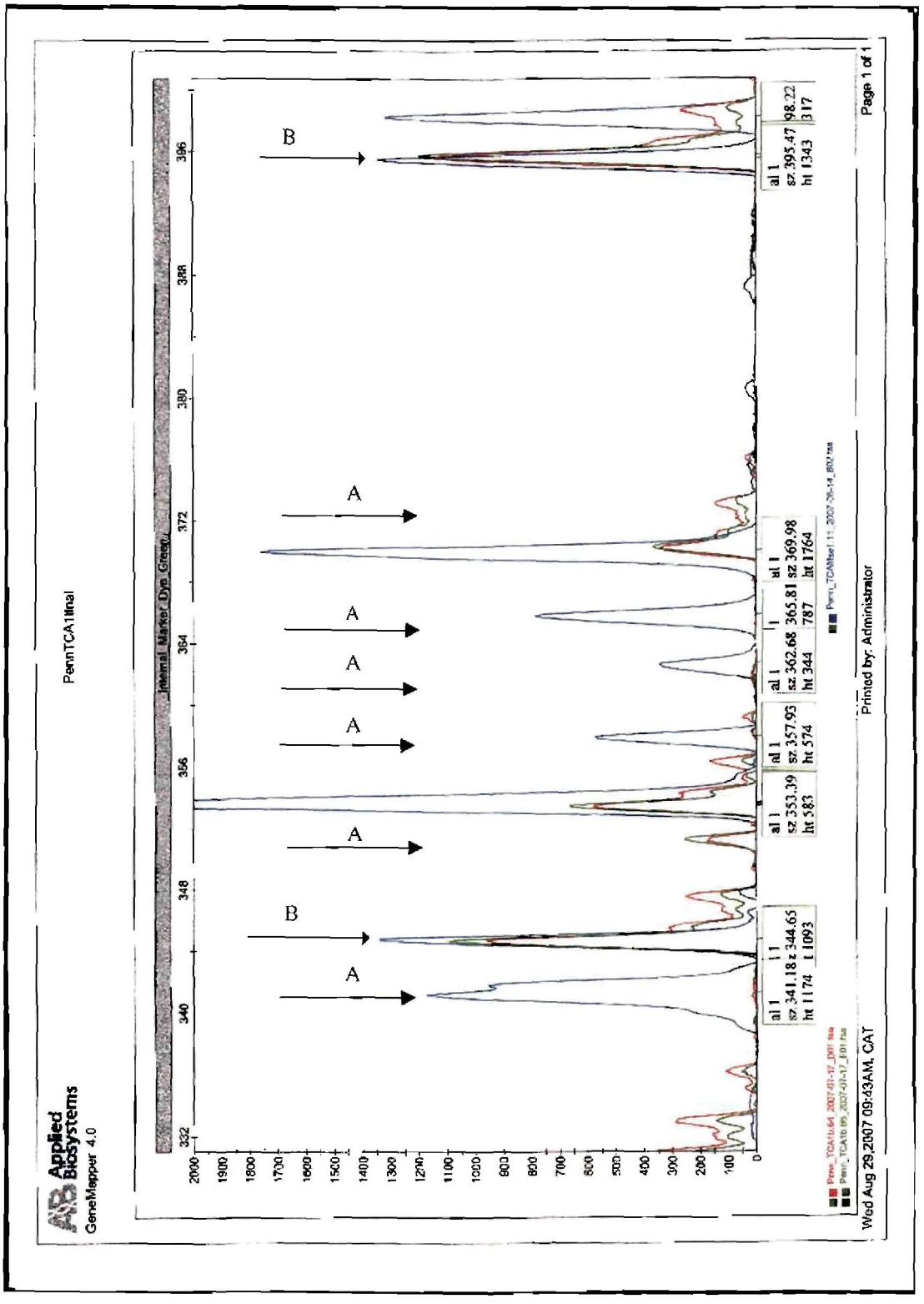

Figure 3.19e Profiles of the three Estcourt 11 individuals from 332 – 396 basepairs.

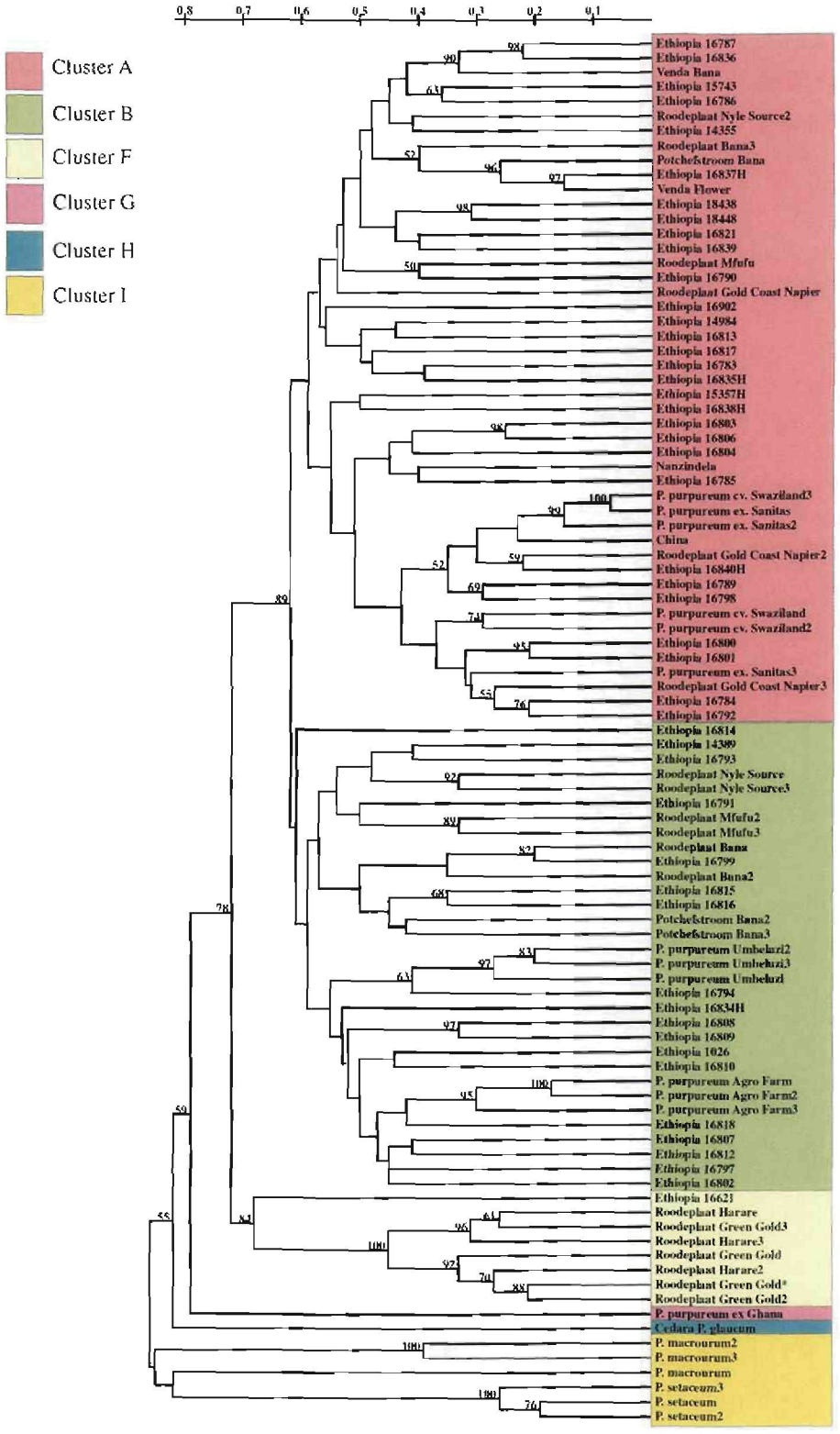

Figure 3.20 UPGMA tree of a subset of AFLP data using four *MluI/MseI* primer combinations and excluding all Estcourt individuals. Numbers above the branches are bootstrap values.

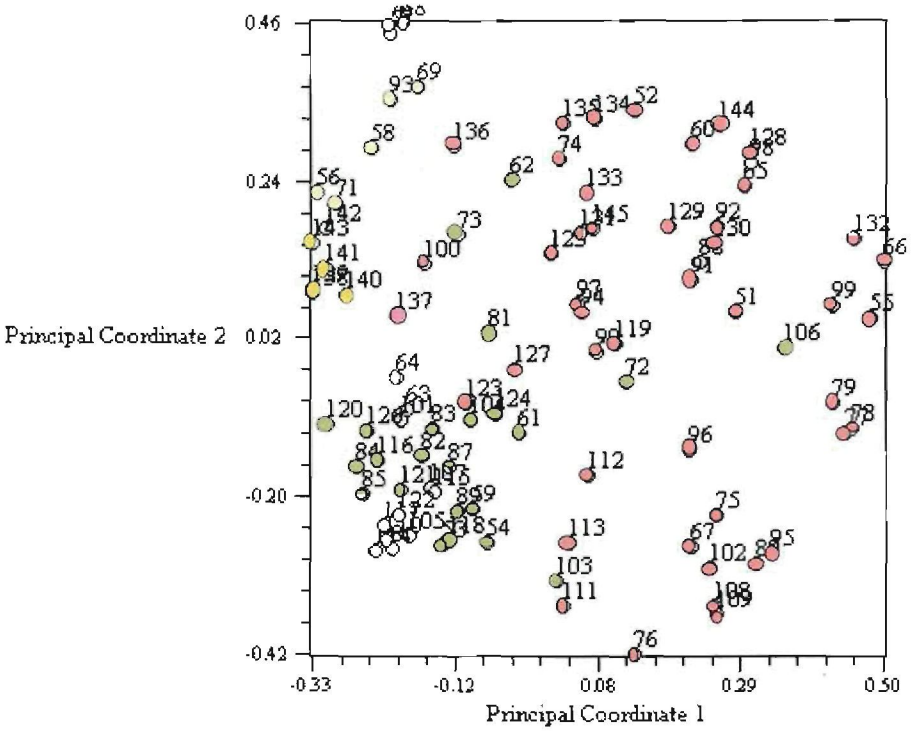

Figure 3.21 Principal Coordinate Analysis of a subset of AFLP data using four *MluI*/*Mse*I primer combinations and excluding all Estcourt individuals. Refer to Table 2.1 for sample numbers.

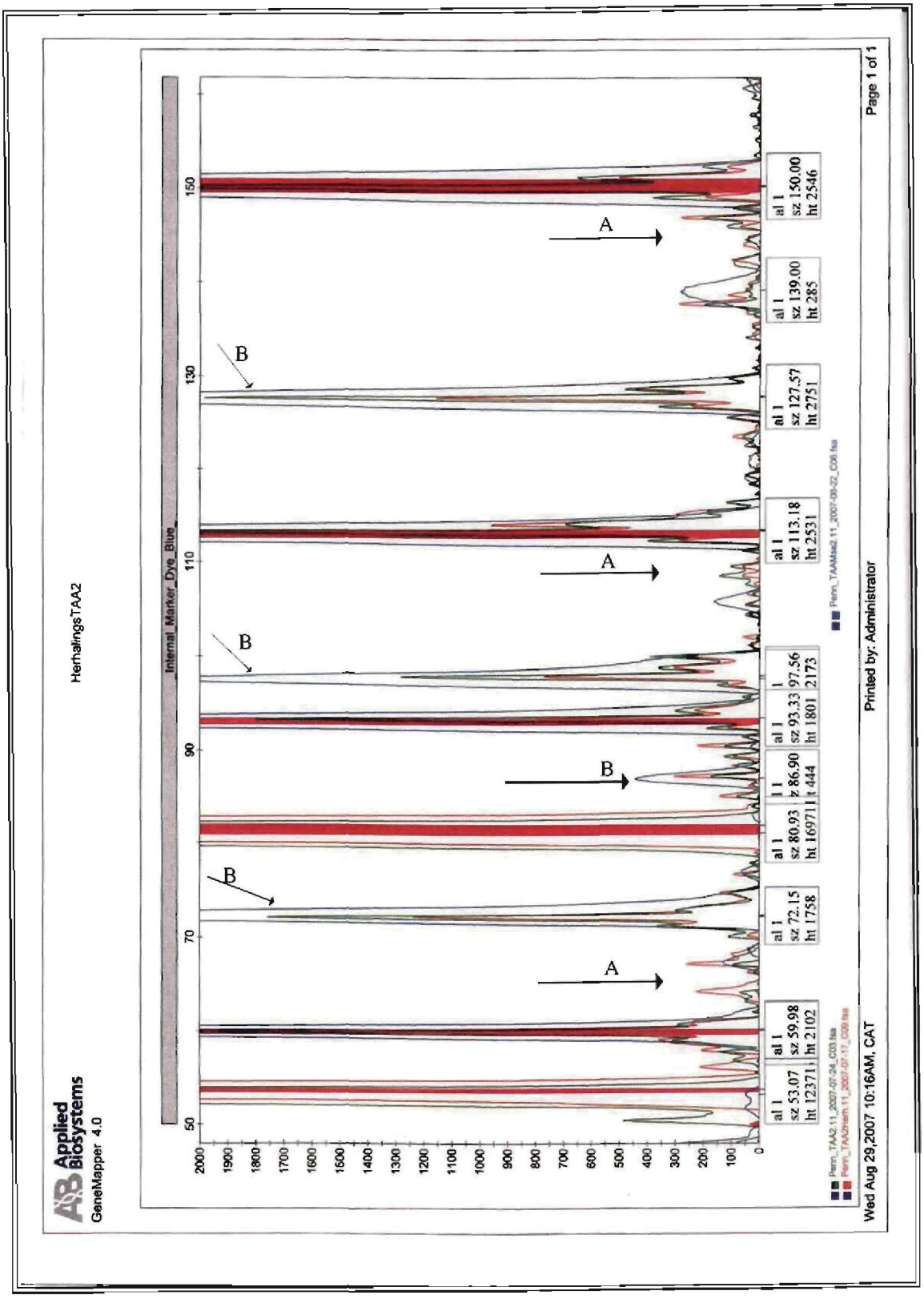

Figure 3.22a Profiles of the three repeats of Estcourt 11 (Estcourt 11 (Penn 11); Estcourt 11.2 (Penn 64) Estcourt 11.3 (Penn 66)) run on the ABI 3130 *xl* Genetic Analyser on three different days, from 50 - 150 basepairs. Arrow A indicates differences and arrow B similarities.

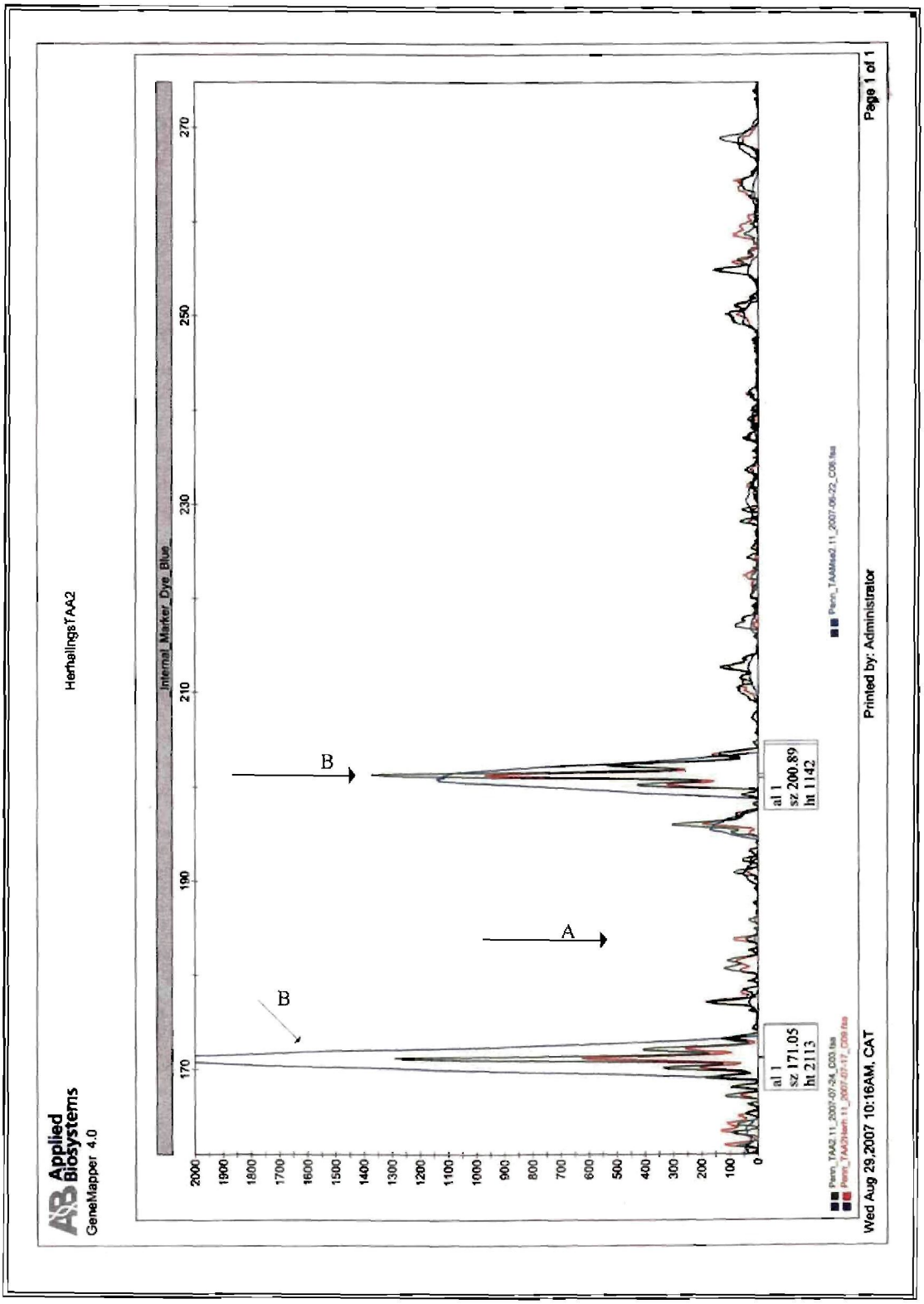

Figure 3.22b Profiles of the three repeats of Estcourt 11 from 170 – 270 basepairs.

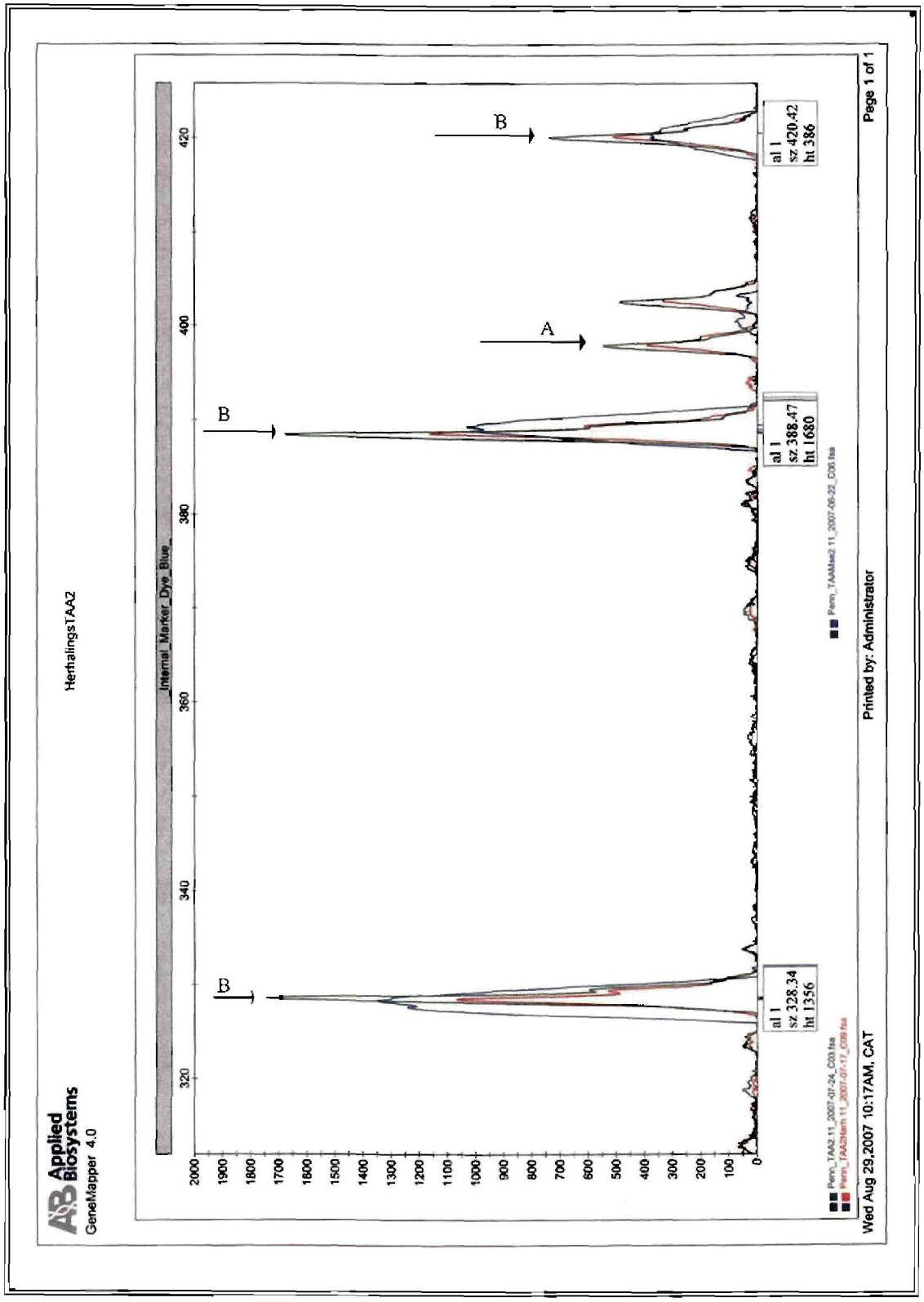

Figure 3.22c Profiles of the three repeats of Estcourt 11 from 320 – 420 basepairs.

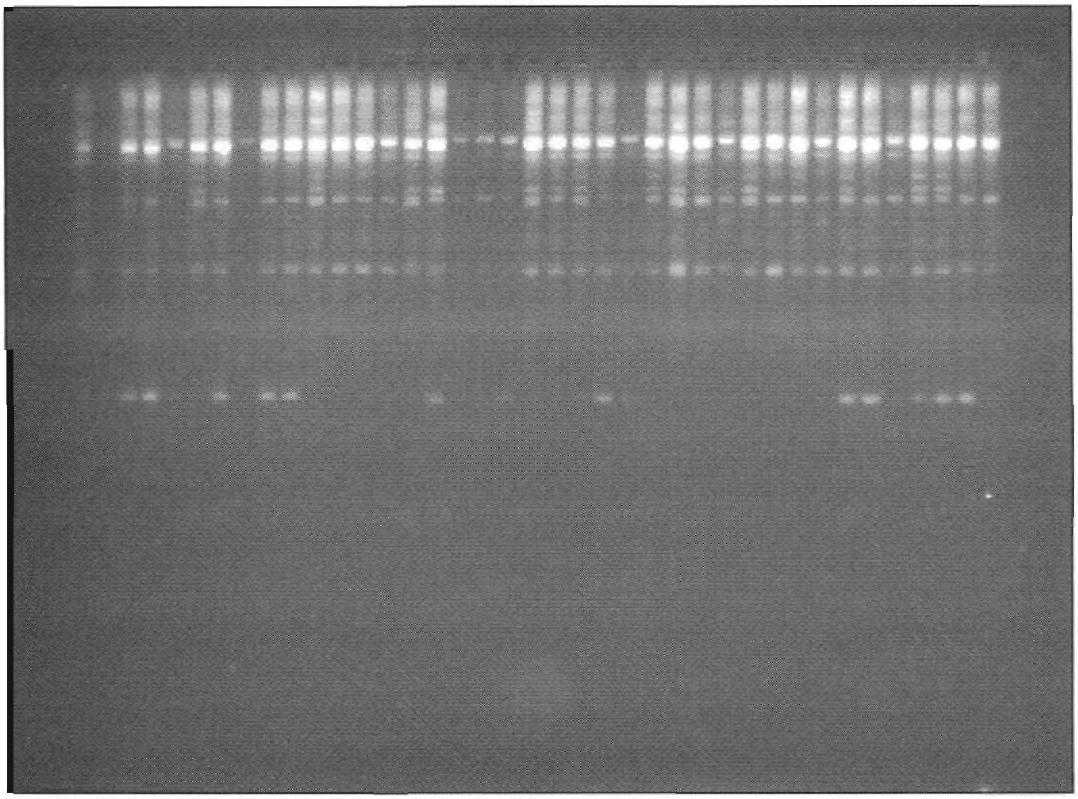

Figure 3.23 RAPD gel showing little polymorphism between individuals after amplification with primer, Operon Z 6. Individuals analysed from left to right. Estcourt 1, Ethiopia 16791, Ethiopia 16792, Ethiopia 16793, Ethiopia 16794, Ethiopia 16797, Ethiopia 16798, Ethiopia16799, Ethiopia 16800, Ethiopia 16801, Ethiopia 16802, Ethiopia16803, Ethiopia16804, Ethiopia 16806, Ethiopia 16807, Ethiopia 16808, Ethiopia 16809, Ethiopia 16810, Ethiopia16811, Ethiopia 16812, Ethiopia 16813, Ethiopia 16814, Ethiopia 16815, Ethiopia 16816, Ethiopia 16817, Ethiopia 16818, Ethiopia 16821, Ethiopia 16834, Ethiopia 16835, Ethiopia 16836, Ethiopia 16837, Ethiopia 16838, Ethiopia 16839, Ethiopia 16840, Ethiopia 16902, Ethiopia 18438, Ethiopia 18448, Venda Bana and Venda Flower.

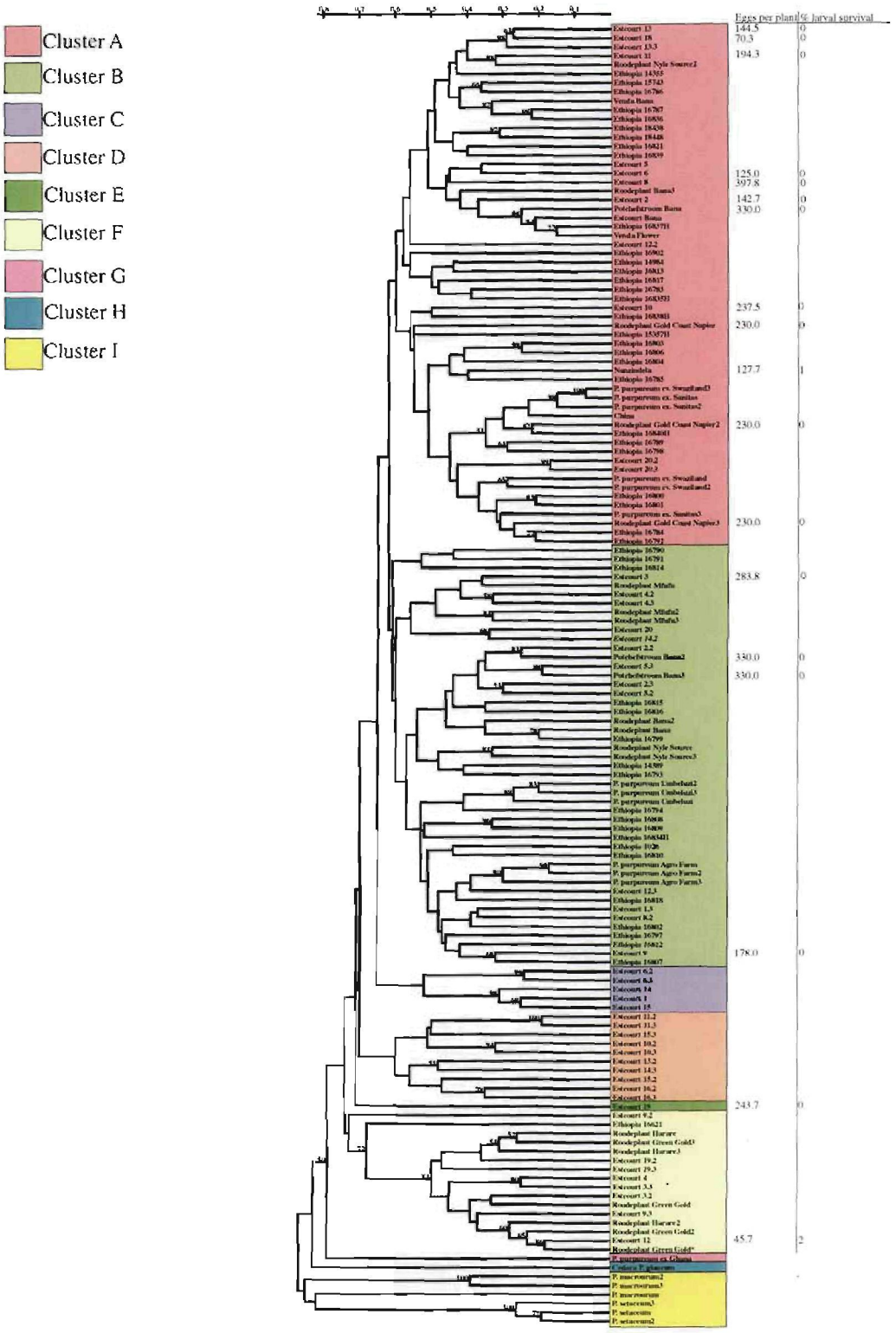

Figure 3.24 Dendrogram of the AFLP analysis together with the results of the *Chilo partellus* oviposition preference and larval survival studies. The first column indicates the mean number of eggs laid per plant and the second column the percentage survival of larvae on the plants. Numbers above the branches indicate bootstrap values.

CHAPTER 4: DISCUSSION

4.1 Interpretations of the UPGMA trees

4.1.1 Analyses of the 23 individuals

The UPGMA trees based on twenty-three individuals analysed with restriction enzymes *Eco*RI/*Mse*I (Fig.3.2) as well as *Mlu*I/*Mse*I (Fig.3.8) are to a large extent congruent, strongly suggesting that the AFLP data convey a noteworthy signal. The significance of the main clusters is further emphasized with their respective bootstrap support. In addition, the main clusters also result in the absence of bands with low frequencies. What these clusters represent is a different matter however due to the uncertainty regarding the origin of the individuals. If Potchefstroom Bana is a true Bana, then Estcourt 2, 5, 10, 11 Nanzindlela and Estcourt Bana could possibly also be hybrids and if Roodeplaat Green Gold* is a true *P. purpureum* then Estcourt 4 and 12 could possibly also be true *P. purpureums.*

In general, the *Eco*RI/*Mse*I analysis resulted in clusters with higher bootstrap values than the *Mlu*I/*Mse*I analysis, possibly suggesting that the former should rather be considered for future AFLP work on *Pennisetum.* While acknowledging the limitations of representing clusters in a phenogram, the value of doing a PCA analysis (Fig. 3.6 and 3.12) is evidenced especially when looking at Estcourt 1 which appeared more distant from Estcourt 14 and 15, than the UPGMA trees would suggest.

4.1.2 Analysis of the 145 individuals

Whereas the UPGMA trees based on an AFLP analysis of 23 individuals above resulted in moderate bootstrap support for major clusters (especially so with *Mse*I and *Eco*RI), such support is absent in an analysis of AFLP data from the extended sample of 145 individuals using *Mlu*I/*Mse*I (Fig. 3.15). By and large, meaningful bootstrap values are

present only in the clustering of terminal entities, although clade F, excluding Estcourt 9.2 is the exception with a bootstrap support of 72 %.

The individuals did not group according to geographical origin (Fig 3.15) indicating that they are either genetically similar due to extensive gene flow or that the primers employed could not sufficiently distinguish between major groupings.

Due to the uncertainty regarding the true original origin of most individuals, the results cannot be interpreted with any amount of confidence and what follows is by necessity speculative.

The samples collected from Potchefstroom are said to be of hybrid origin obtained from a farmer near Hoopstad who claims it originated from the hybrid Bana developed by Gildenhuys (1950). The Venda samples are supposedly clones taken from the Potchefstroom Banas. The Potchefstroom Bana (cluster A) cluster in a group together with Roodeplaat Bana 3, Estcourt Bana, a hybrid from Ethiopia (Ethiopia 16837H), Venda Flower and Estcourt 2, 5, 6, and 8. They are all therefore most likely hybrids. Potchefstroom Bana 2 and 3 (cluster B) cluster with Estcourt 2.2, 2.3, 5.2 and 5.3. These Estcourt individuals could therefore also be hybrids.

Roodeplaat Nyle Source, Gold Coast and Harare were apparently part of Napier grasses collected in Africa south of the Sahara by Mr. C. J. J. van Rensburg until the Second World War. Roodeplaat Nyle Source is speculated to come from the source of the Nyle River, its exact location unknown. The Nyle Source individuals cluster in cluster A, together with Estcourt 11 and in cluster B together with Ethiopia 14389 and 16793. Estcourt 11 and the two Ethiopia individuals could therefore also be Nyle Source individuals. Whether these Nyle source individuals really originated from the Nyle cannot be determined by the present analysis.

The origin of Roodeplaat Gold Coast is also unknown, possibly somewhere along the east or west coast of Africa. This cannot be determined by the present analysis, because most

of the individuals were sampled along eastern Africa and almost none from West Africa. Whether this Gold Coast originated from the Gold Coast developed by Dr. Codd is also unknown and cannot be determined because control individuals of Gold Coast developed by Dr. Codd, was not included in the analysis. The three Gold Coast individuals analysed in the present study all clustered in cluster A.

Roodeplaat Harare was collected outside Salisbury (Zimbabwe) from a locality known as Harare. Roodeplaat Green Gold, Mfufu and Bana were brought into the Roodeplaat collection in later years and were collected from farmers in the Limpopo Province. Roodeplaat Green Gold was collected from a farmer (Mr. van Zyl) in the Limpopo who collected it in the highlands of Zimbabwe (Gouws, 1987). It is morphologically very similar to Harare and Kleyn (1995) speculates that it was selected from Harare. Cluster F mainly consists of Roodeplaat Harare and Roodeplaat Green Gold individuals. The Roodeplaat Green Gold individuals are genetically similar to Roodeplaat Harare individuals confirming the observation of Kleyn (1995). Since both Roodeplaat Harare and Roodeplaat Green Gold originated from Zimbabwe, it is quite possible that Mr. van Zyl (Gouws, 1987) obtained nothing other than Harare, but failed to identify it as such. The AFLP data suggests that Estcourt 3.2, 3.3, 4, 9.2, 9.3, 12 and Ethiopia 16621 are possibly also Harare and that this is an example of an old cultivar that has been introduced under a new name.

Roodeplaat Mfufu appears to be morphologically intermediate between Gold Coast and Nyle Source, and cluster between the Gold Coast individuals in cluster A and two Roodeplaat Nyle Source individuals in cluster B. Estcourt 3, 4.2, 4.3, 14.2 and 20 cluster together with Roodeplaat Mfufu and could possibly also be individuals of cultivar Mfufu, although this cluster is not supported by a meaningful bootstrap value.

Roodeplaat Bana is claimed to be of hybrid origin, possibly from the progeny of the Bana hybrid developed by Gildenhuys (1950). This claim could not be addressed in the present analysis.

P. purpureum cv. Swaziland and *P. purpureum* cv. Sanitas are used as animal feed in the dairy industry in Botswana. The former entered Botswana from Swaziland and the latter was collected by Dr Gus Nilsson in Nigeria. *P. purpureum* cv. Swaziland 3 (cluster A) is genetically very similar to *P. purpureum* ex Sanitas, suggesting that they have a common ancestry.

P. purpureum Umbeluzi and *P. purpureum* Agro Farm are cultivars collected from plantings on farms near Maputo in Mozambique. Whether it is a variety that occurs naturally in Mozambique or whether it is an introduced cultivar is not known and could not be determined.

Seven of the Estcourt samples originated from the Roodeplaat cultivars, but no record exists of the names of these cultivars. The remaining Estcourt samples were collected in Estcourt and surrounds. In the absence of pedigree information, as well as bootstrap support, it is very difficult to explain what the clusters C, D and E represent exactly. That Estcourt 14, 1 and 15 forms a distinct cluster, is exemplified by the fact that the three mentioned individuals also cluster in all the analyses of the reduced sample set. The differences observed between the Estcourt individuals collected in 2005 and 2006 (Fig 3.17 and 3.19) is either due to incorrect identification or possibly increased genetic diversity after the plants were first harvested. The cultivars are open pollinated and different cultivars could have cross-pollinated one another. "China" is a cultivar that originated in China without known origins and forms part of the collection at Estcourt, clustering in cluster A.

The Ethiopian samples have a diverse origin, but mostly from Zimbabwe and the USA. The USA is geographically isolated from Africa, but these individuals still cluster between the individuals collected from Africa (cluster A and B). This indicates that they have not yet diversified from their African relatives.

The *P. purpureum* ex Ghana clusters at the base of the UPGMA tree. Being collected in West Africa, with the remaining individuals mainly collected in East Africa, it may

merely reflect geographical isolation. This possibility can only be proven/disproven with more samples from West Africa. *P. glaucum* (one of the parents of the *P. glaucum* x *P. purpureum* hybrid) clustered separately throughout, as did *P. setaceum* and *P. macrourum*.

The PCA of the 145 individuals analysed (Fig. 3.16) does not reflect the main clusters obtained in the dendrogram, indicating genetic similarity, although cluster F clearly lies separate in the diagram.

4.2 Methodological considerations

4.2.1 Amplified Fragment Length Polymorphism (AFLP)

AFLP is widely considered as quick, reliable and reproducible and has the capacity to simultaneously screen many different DNA regions distributed randomly throughout the genome (Mueller & Wolfenbarger, 1999).

Two rare cutters, *Eco*RI and *Mlu*I were used with the frequent cutter *Mse*I. The selective PCR products of the 23 Napier grass individuals analysed with the *Eco*RI/*Mse*I combination were visualized on PAGE (Fig. 3.1 and 3.2) and the same 23 individuals analysed with *Mlu*I/*Mse*I combination on the ABI 3130*x*l Genetic Analyser. (Fig. 3.13 and 3.8) The polymorphism index (PI) for the *Eco*RI/*Mse*I combination was 2.7, much lower than the 5.2 for the *Mlu*I/*Mse*I combination. This higher PI value is due to the higher number of polymorphic bands that the automated sequencer detected and it seemed that the nucleotide sequences that *Mlu*I recognizes and cuts (A↓CGCGT) were more frequent in the *Pennisetum* genome than that of *Eco*RI (G↓AATTC). When the other 122 individuals were added to the analysis, the PI value decreased to 0.2 since they formed more groups than the 23 individuals did with much less polymorphic bands and, since these individuals were not analysed with the *Eco*RI/*Mse*I combination, this value cannot be compared to that of the *Eco*RI/*Mse*I combination.

The cost of AFLPs often limits the number of primers that can be used which in turn have an influence on the amount and quality of the variation uncovered (Robertson & Harris, 1999). Five *Mse*I + 3 primers were used with only one *Eco*RI +3 primer (*Eco*RI-AAC, *Mse*I-ACA, *Mse*I-ACC, *Mse*I-CGT, *Mse*I-CCG and *Mse*I-TAC). Each primer combination generated between 43 and 67 bands. Due to the fact that the analysis on the automated sequencer is much more expensive than PAGE, not all five *Mse*I primers could be used with both *Mlu*I-TCA and *Mlu*I-TAA primers (*Mlu*I-TCA and *Mlu*I-TAA; *Mse*I-ACA, *Mse*I-ACC, *Mse*I-CCG and *Mse*I-TAC). Each primer combination generated between 240 and 295 bands.

The reproducibility of the bands on both PAGE (Fig. 3.7) and the automated sequencer (Fig 3.22 a-c) were tested and found to be reproducible, confirming the validity and quality of the results obtained and should be reproducible when repeated in another laboratory.

4.2.2 Random Amplified Polymorphic DNA (RAPD)

The Random Amplified Polymorphic DNA technique was developed in order to overcome the limitations of the restriction fragment length polymorphism (RFLP) method (Karp *et al.*, 1996) and makes use of the PCR process in order to amplify DNA fragments specified by primers of arbitrary nucleotide sequences (Williams *et al.*, 1990).

The technique however has its limitations and disadvantages such as the high number of primers used in order to generate polymorphisms. In the present study, 17 primers were screened of which only four primers revealed bands that could be scored, of which the resulting polymorphisms were not enough to distinguish between the individuals. This could support the AFLP findings that the individuals are genetically very similar.

4.2.3 AFLP versus RAPD

The AFLP technique outperformed the RAPD technique in the present study. Although AFLP is technically more demanding than RAPD, it was able to generate informative bands with only four primer combinations while RAPD could not after seventeen primers were screened.

4.3 Correlation between the oviposition preference and larval survival of *Chilo partellus* on Napier grass and results of the AFLP and RAPD analysis

Since the RAPD technology was not developed any further, no evaluation of the relationships between the oviposition preference and larval survival with plant relatedness could be made.

No pattern in oviposition preference on the different cultivars was observed when the oviposition preference and larval survival was plotted on the AFLP dendrogram (Fig 3.24), indicating that there appears to be no relationship between genetic similarity of cultivars and moth oviposition preference.

CHAPTER 5: CONCLUSIONS

The AFLP data revealed that the *Pennisetum purpureum* and related cultivars studied either have genetic diversity due to the fact that Napier grass is open pollinated or that the primers employed could not sufficiently distinguish between major groupings.

A major drawback to the optimum use of Napier cultivars as animal forage and a pest management tool is the confusion surrounding their true identity. Due to their morphological similarities, cultivars can and are easily confused with one another and consequently are often known by an incorrect or dubious name. Poor nursery management and lack of pedigree information, especially when plants are exchanged, add to this confusion.

The present study included cultivars mainly from Africa, USA and one from China, all of which presumably originated from Africa. The history and origins of these samples are however, not well documented and based on personal communication with nursery managers.

The cultivars did not cluster according to geographical origin, and in spite of their diverse origin, they are all related to one another. Duplicate individuals of the same cultivar did also not always cluster together, indicating diversity within the cultivar or misidentifications. An example of a misidentification is the cultivar Green Gold being no other than cultivar Harare. The different cultivars and hybrids could therefore not be identified with certainty.

As the stemborers did not have preferences for specific Napier grass cultivars, the different cultivars however, are adapted to specific climates and habitat conditions. It is therefore essential to evaluate each cultivar for its habitat preference in order to provide smallholder farmers with the Napier grass that will yield optimum results in their specific conditions.

This is the first report of Napier grass being fingerprinted with the AFLP technology but poor germ plasm management negates its potential to be a powerful tool for this grass' DNA fingerprinting and molecular characterization. It was, however, able to

generate a high number of genetic information using just four primer combinations opposed to the RAPD method. In the RAPD analysis, 17 primers were screened of which only four primers revealed bands that could be scored, of which the resulting polymorphisms were not enough to distinguish between the individuals. This could support the AFLP findings that the individuals are genetically very similar.

Four *Eco*RI/*Mse*I and *Mlu*I/*Mse*I restriction primer combinations were used in the AFLP analysis. The UPGMA trees based on twenty-three individuals analysed with these primer combinations are to a large extent congruent and the main clusters are supported by their respective bootstrap support. The main clusters also result in the absence of bands with low frequencies. In the extended analysis of 145 individuals, using the *Mlu*I/*Mse*I restriction primer combination, nine main clusters were derived with no boostrap support except for one clade.

Principal Coordinate Analysis of the twenty-three individuals analysed with the *Eco*RI/*Mse*I primer combinations grouped the individuals that formed a cluster in the UPGMA trees together, each group distinct from one another. This pattern faded in the analysis with the *Mlu*I/*Mse*I primer combination, especially in the analysis with the 145 individuals, indicating either that *Mlu*I/*Mse*I primer combination could not sufficiently distinguish the groups or that the individuals are genetically similar.

The following points should be noted in order to better utilise *P. purpureum* cultivars in a push-pull system in Africa:

- Cultivars should be examined genetically and morphologically. The genetic analysis is necessary to distinguish between the different cultivars. When the different cultivars are distinguished, they will have to be described morphologically to provide farmers with the correct visual description of the different cultivars.
- An appraisal of other techniques such as co-dominant markers (e.g. microsatellites) or gene sequencing. As the amount of primers can have an influence on the amount and quality of variation uncovered, the number of primers used should only be limited by the available funding. If cost and

access allows, the profiles should rather be generated by an automated sequencer, as it is much more sensitive and faster than PAGE.

- Before any genetic analysis is attempted, hybrids should first be distinguished from *P. purpureum* using either chromosome counts or gene sequencing. The hybrids and *P. purpureum* should then be examined separate from one another and together.
- A minimum of 5 individuals per cultivar should be analysed and localities should have GPS reading, as it will facilitate the interpretation of results. Care should be taken to give each cultivar a correct, unambiguous name. This might necessitate a broad study of countless individuals worldwide.
- Proper management by nursery managers cannot be stressed enough, as this will prevent exasperating the situation. Complete pedigree information, if such exists, should always be provided when plants are exchanged.

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