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The use of AFLP to determine if a slimes-tolerant indigenous species shows local adaptation to slimes dam soils

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INTRODUCTION

1. ADAPTATION

1.1. Adaptation to novel environments

The natural environment is dynamic and constantly changing, yet plant populations show an ability to survive and adapt under such varying conditions (Clausen et al., 1948; Joshi et al., 2001; Hangelbroek et al., 2003; Reed et al., 2003; Eckhart et al., 2004). Individuals within populations respond differently to environmental change, due to intrapopulation genetic variation. If the change is a negative one, for example a toxicant, the individuals may respond by adapting and thereby increasing their fitness, or not adapting and therefore decreasing their fitness or dying. An organism's fitness refers to its ability to survive and reproduce (Clausen et al., 1948; Lopes et al., 2004).

Understanding the variables that affect an organism's ability to adapt to a changing environment is a critical issue in many research fields. Adaptation has therefore been widely studied (Reed et al., 2003). Variation in the environment that plants are exposed to has been shown to be associated with genetic variability. This indicates a genetic basis for the ability to adapt to varying environments (Lesica and Allendorf, 1999; Joshi et al., 2001; Kittelson and Maron, 2001; Hangelbroek et al., 2003; Rossum et al., 2004).

Adaptation is not always genetically based, however, often being associated with a high degree of physiological variation within a single genotype. This is termed phenotypic plasticity and is an example of adaptation without genetic variation (Lesica and Allendorf, 1999; Joshi et al., 2001; Kittelson and Maron, 2001; Price et al., 2003; Eckhart et al., 2004; Ernande and Dieckmann, 2004). The environment only alters the phenotypic expression of the genotypes, for example by increasing enzyme activities, and does not alter the genotypes themselves (Lopes et al., 2004). This ability may, however, be genetically controlled. Most highly adaptable plant species show some degree of phenotypic plasticity together with a high degree of

genetic variation (Lesica and Allendorf, 1999; Joshi et al., 2001; Eckhart et al., 2004; Ernande and Dieckmann, 2004).

Phenotypic plasticity usually confers fitness advantages, through allowing adaptation to harsh environments that would otherwise be uninhabitable (Price et al., 2003; Ernande and Dieckmann, 2004). For example, wild plants that were adapted to low-nutrient soils and subsequently grown in phosphorus-deficient soils grew better and had higher phosphorus levels in their shoots than crop plants grown in the same phosphorus-deficient soil. This was found to be due to a slower growth rate in the wild plants, rather than an increased uptake of phosphorus from the soil by these plants (Marschner, 1986). Adaptive plasticity is characterised by phenotypic variation in the direction of optimising character traits for the particular environment, for example smaller leaves in drier areas (Eckhart et al., 2004). Non-adaptive plasticity may also occur. For example, plants may show reduced growth when fewer nutrients are present, thereby reducing competitive ability as phenotypes move away from the optimum for the environment (Hangelbroek et al., 2003; Eckhart et al., 2004).

Plant strains that have adapted to a chosen environment outperform strains newly transplanted to the same conditions, indicating that adaptation leads to increased fitness in the local environment (Joshi et al., 2001; Hangelbroek et al., 2003; Reed et al., 2003; Eckhart et al., 2004). However, adapted genotypes show decreased fitness in environments that they are not adapted to, indicating that some degree of specialisation is necessary for adaptation to occur (Hangelbroek et al., 2003). This response has also been shown to occur in animals (Reed et al., 2003; Lopes et al., 2004). In *Drosophila* species, populations that were inbred in a variable environment were shown to be more adaptable and have higher fitness and stress tolerance than those that were inbred in a constant environment (Reed et al., 2003).

Local adaptation to particular environmental conditions may also occur within populations, within short distances. Local adaptation has been reported at small geographical scales in response to abiotic factors such as heavy metals, wind, and elevation, and to biotic factors such as interspecific competition and herbivory

(Hangelbroek et al., 2003). For example, within-population adaptation has been shown to occur in plants that have colonised different levels of sand dunes, as a response to varying competition and water-availability levels (Knight and Miller, 2004).

1.2. How does adaptation occur?

If a population does not adapt to a changing environment, it is likely to become extinct. Therefore what conditions will lead to local adaptation rather than extinction? In many cases, local adaptation follows colonisation of a novel environment through dispersal of seeds from surrounding areas (Bush and Barrett, 1993; Campbell et al., 2003; Price et al., 2003; Jacquemyn et al., 2004). Different types of plants have different potentials for colonisation of a new environment, for example those that have wind-dispersed seeds have a higher immigration rate, and therefore a higher rate of colonisation, than those that have seeds that are dispersed over short distances (Campbell et al., 2003). The probability of colonisation also depends on the distance between the two areas (Jacquemyn et al., 2004). The prior environment experienced by the colonising individuals can have a large influence on the ability of plants to adapt to novel conditions (Reed et al., 2003; Jacquemyn et al., 2004).

Population genetic diversity is a fundamental component of biodiversity and is crucial for ensuring the long-term sustainability and survival of a species, as well as influencing the rate of evolutionary change (Faugeron et al., 2001; Bagley et al., 2002; Wimp et al., 2004). Genetic diversity in plant populations has been shown to directly influence diversity in dependent animal species, further emphasising the far ranging implications of genetic diversity (Wimp et al., 2004). The role of genetic diversity in determining the ability of plants to adapt to novel environments will be discussed further.

After colonisation of a new environment, some plant species form ecotypes, i.e., evolve ecological adaptations suited to the new environment, but not reproductive barriers (Clausen et al., 1948; Gibson and Pollard, 1988; Fiedler, 1992; Macnair,

1992). If ecotypes evolve further to form reproductive barriers, a new species has formed (Macnair, 1992). These ecological races approach a dynamic equilibrium with the new environment, an equilibrium that is maintained by the balance between the forces of genetic variation and natural selection (Clausen et al., 1948; Clausen, 1951). Genetic diversity in traits that will assist the organism in the new environment is therefore vital for adaptation to occur (Clausen et al., 1948; Clausen, 1951; Bagley et al., 2002; Reed et al., 2003).

Any particular environment contains a dynamic balance between forces that increase genetic diversity, primarily mutation and gene flow, and those that reduce it, including genetic drift and directional selection (Clausen, 1951; Eckhart et al., 2004; Knight and Miller, 2004; Rossum et al., 2004; Solé et al., 2004). Gene flow refers to the transfer of genes, gametes, or individuals between populations. The extent of gene flow that occurs is influenced by the breeding, pollination, and dispersal systems of the species (Lesica and Allendorf, 1999). A large amount of gene flow will reduce or even prevent adaptation through the continuous influx of non-tolerant genotypes (Antonovics et al, 1971; Faugeron et al., 2001).

In every population there are random changes in reproductive success from one generation to the next. These changes cause genetic drift: shifts in allele frequency from generation to generation. Genetic drift results in the genes transmitted to offspring not being representative of the allele frequency present in the previous generation. Genetic drift becomes greater as the population size decreases due to the reduced number of reproductive adults available (Lesica and Allendorf, 1999). The long-term effective population size is therefore important as it determines the levels of genetic drift the population is subjected to (Bagley et al., 2002; Reed et al., 2003). The effective population size is the size of an ideal population, i.e., one that meets all the Hardy-Weinberg assumptions (Storz et al., 2001). A population in Hardy-Weinberg equilibrium is one where random mating occurs and where there are no disturbing forces such as selection, mutation, and migration (Weir, 1996).

Genetic drift causes a loss of genetic diversity during colonisation due to the small number of individuals present, and later results in changes in allele frequency and

causes genetic differences between subpopulations (Lesica and Allendorf, 1999; Tribsch et al., 2002; Snäll et al., 2004). These allele frequency changes take the form of both a loss of alleles, which is affected by the size of the colonising population, and reduced heterozygosity, which is affected by the length of time that the population remains small following colonisation (Bush and Barrett, 1993). Gene flow levels also change, becoming restricted due to geographic isolation of the colonising population (Bush and Barrett, 1993).

Controversy exists over the relative importance of natural selection versus genetic drift and gene flow in the adaptation process (Kittelson and Maron, 2001). Genetic drift and gene flow are not directly influenced by the environment, and therefore result in changes in diversity throughout the genome. These factors therefore influence adaptive ability through increasing or decreasing overall genetic diversity. In contrast, natural selection influences the selected genome: the diversity of genes that are selected by the environment, or genes closely linked to those genes. Natural selection therefore influences adaptation by influencing diversity in genes directly responsible for survival in the new environment (Clausen et al., 1948; Clausen, 1951).

Following colonisation, a combination of directional selection with at least short-term opportunity for population growth has been suggested to be necessary for adaptation to occur (Reznick and Ghalambor, 2001; Price et al., 2003). Other studies, however, suggest that population growth may be more important, as without it directional selection may cause population decline and local extinction (Reznick and Ghalambor, 2001). Natural selection has various possible effects on populations. Strong selection pressures may result in deterioration in some aspects of performance, along with an increase in desirable traits. Rapidly fluctuating selection can cause a reduced phenotypic expression of genetic variation (genetic canalisation) (Valladares et al., 2002). Natural selection may also reduce the effective population size, increase genetic drift, and cause an increased rate of loss of genetic variation (Lesica and Allendorf, 1999; Bagley et al., 2002; Reed et al., 2003).

In summary, local adaptation can occur when selection pressures are intense and gene flow is of a sufficiently low level that it does not overwhelm selection. In small populations, where gene flow is likely to be significant, local adaptation may occur only if the strength of selection is also intense (Hangelbroek et al., 2003; Knight and Miller, 2004). Sharp boundaries can exist between two sites in close proximity to each other, for example sand dunes and adjacent grasslands, due to dramatic changes in conditions. These boundaries create strong gradients in selection that tend to result in local adaptation (Kittelson and Maron, 2001).

The distribution of genetic variation within and among populations is influenced by the characteristics of the species (Hamrick, 1989). Each plant species has a unique combination of life-history traits, historical factors, and habitat preferences that can influence patterns of genetic variation within and among populations (Vellend and Waterway, 1999). For example, the mating system of the species determines the extent of gene flow in the population, which in turn influences the amount of genetic variation within and between populations. Marked differences in genetic variation can therefore occur over short distances in populations, resulting in a non-random distribution of genetic variation (Hamrick, 1989). These forces all interact to influence the amount of genetic variation in a population. Since genetic diversity affects the level of adaptation a species is capable of, measuring that diversity provides insights into the likely reaction of the species to environmental change (Bush and Barrett, 1993; Bagley et al., 2002).

1.3. Studies on adaptation

Local adaptation has most often been demonstrated through reciprocal transplant experiments (Joshi et al., 2001; Eckhart et al., 2004). This technique was used most often in older studies, for example in the extensive studies done on *Achillea* spp. by Clausen and colleagues (1948). These experiments involved growing plants taken from varying environments in constant and in differing conditions, and studying morphological variation. Morphological and physiological characteristics directly influence the success of plants. By studying these characteristics in plants obtained from different environments that are grown under sets of known conditions, the

experimenter can study the physiological adaptations necessary in order for a plant to survive in its natural environment (Clausen et al., 1948; Clausen, 1951).

Morphological and physiological characteristics show a high correlation with the climate in which the plant population originated. Characteristics that are shown to be inherited and correlated with the environment can be viewed as the morphological manifestation of a genetic response to environmental change (Clausen et al., 1948; Clausen, 1951). Physiological variation is found in nearly all populations, and typically no single gene accounts for this variation (Hartl and Clark, 1989). Reciprocal transplant experiments do not study genetic variation at the molecular level, but rather infer conclusions about the genetic structure of a population through studying morphological and physiological characteristics. These experiments therefore view the products of the selected genome, without viewing the genes themselves.

Serpentine soils offer restrictions on plant growth due to their unique characteristics, for example these soils often contain high levels of nickel and chromium (Antonovics et al, 1971). The serpentine plant *Mimulus nudatus* shows adaptation to drought when compared to the closely related species *M. guttatus,* from which it is thought to have evolved. *Mimulus nudatus* was found to grow better on serpentine soil than on high nutrient compost. This was seen as evidence that *M. nudatus* had adapted to grow on serpentine soil (Macnair, 1992). Generally, species found growing both on and off a particular substrate are thought to have undergone recent adaptation, while species in which growth is confined to one substrate are thought to have undergone adaptation longer ago (Fiedler, 1992).

The earliest studies involving the analysis of genetic variation at the molecular level involved the use of allozymes, often coupled with measures of physiological variation. Allozymes are different allelic forms of a protein, detected through variation in migration rates (caused by molecular weight, shape, and charge differences) during electrophoresis (Weir, 1996). *Spartina patens* populations seemingly adapted to three different, but adjacent, areas (marsh, swale, and dune) were studied. Differences in morphometric traits, for example seed production,

were found between areas. Physiological differences were also found between different areas with regard to salt tolerance, nutrient responses, and drought tolerance. These differences are adaptively significant, allowing the plant to adjust to the local environment (Silander and Antonovics, 1979).

In the molecular component of the study, allozyme variation was found between the different sites, indicating genetic variability within and among the different subpopulations. Population divergence and differentiation were therefore concluded to have occurred, over distances of as little as 200m. The differentiation observed was thought to be due mostly to intense and disruptive selection pressures. Low levels of gene flow, coupled with inbreeding due to a difference in flowering time between the areas, could also have promoted subpopulation isolation (Silander and Antonovics, 1979).

The serpentine endemic *Berkheya rehmanni* var. *rogersiana* was shown to be morphologically very similar to the adjacent but non-serpentine *Berkheya rehmannii* var. *rehmannii*. Enzyme electrophoresis, however, showed var. *rehmannii* to be genetically variable, and genetically distinct from var. *rogersiana*. Unique alleles were found in var. *rogersiana*, providing evidence that negligible levels of gene flow were occurring between this variant and var. *rehmannii*. This led the authors to suggest that these two variants should in fact be different species (Williamson et al., 1997). This is an example of the increased levels of genetic variation that can be detected with molecular methods when compared to morphological and physiological study methods.

Sometimes, however, no evidence of genetic adaptation to a novel environment can be found. *Silene dioica* plants on serpentine and non-serpentine soils were found to differ physiologically, but enzyme electrophoresis showed that the serpentine populations did not form a group that was genetically distinct from the non-serpentine populations. It was therefore concluded that there was no evidence of genetic adaptation to the serpentine soil at the loci studied (Westerbergh and Saura, 1992). This is an example of phenotypic plasticity that does not appear to be genetically controlled.

1.4. Adaptation to metal-contaminated soil

The colonisation of metal-contaminated soils by plants is a good example of strong selection pressures being imposed in a novel habitat (Macnair, 1983; Bush and Barrett, 1993). Heavy metals are toxic to all life forms when present in large quantities, causing disease or death (Antonovics et al., 1971). Some plants are capable of growth and reproduction on soils that have been contaminated with heavy metals. These plants either possess or are able to evolve heavy metal tolerance (Antonovics et al, 1971; Macnair, 1983; Rossum et al., 2004).

Many species have been shown to be able to develop tolerance to one or more metals, with tolerance to multiple metals arising either in separate races or simultaneously in the same race (Antonovics et al, 1971). A few plants show constitutive tolerance to heavy metals (Gibson and Pollard, 1988), for example the copper mosses, which have even been shown to require high levels of copper for optimum growth (Antonovics et al., 1971; Shaw, 1994). Most plant species, however, are constitutionally non-tolerant and instead are capable of evolving tolerant races (Antonovics et al., 1971; Gibson and Pollard, 1988). The ability to evolve tolerance varies between individuals within a population. The level of this variation indicates the ability of the species to adapt to a new environment, as if variation is high different individuals within a population will be able to adapt to different environments (Shaw, 1994).

The evolution of metal tolerant plant populations occurs rapidly. Most heavy metal contaminated mine waste dumps are between 50 and 100 years old, and yet carry well-established populations (Antonovics et al, 1971; Bush and Barrett, 1993). This rapid development of tolerance occurs due to the strong selection pressures present, which are intense enough to result in adaptation even though gene flow levels are often high due to the short distances between contaminated and non-contaminated areas (Antonovics et al, 1971; Kittelson and Maron, 2001). Following colonisation, individuals that have the capability to evolve tolerance survive, while those that do not die within a short space of time (Bush and Barrett, 1993). This results in a low number of survivors among founders following colonisation, causing a loss of

genetic variation due to genetic drift, as discussed earlier (Bush and Barrett, 1993; Lesica and Allendorf, 1999; Snäll et al., 2004).

Mechanisms of metal tolerance vary depending on the metal/s present, environmental conditions, and the organism itself. Mechanisms that may be involved include reducing the uptake of metal ions, removal of metal ions by depositing them in a vacuole or removing them from the cell, converting the metal into a non-toxic form, increasing the requirement of enzyme systems for metal ions, decreasing the permeability of the cell or subcellular units to metal ions, hyperaccumulating metal ions in metal storage organs in the leaves which are then shed, or chelating the metal to the cell wall (Antonovics et al, 1971; Witkowski and Weiersbye, 1998). In several species, for example in the monkey flower *Mimulus guttatus*, tolerance to a metal, in this case copper, seems to be controlled by one major gene system (Macnair, 1983; Macnair et al., 1993; Shaw, 1994). In other species polygenic control is indicated (Macnair, 1983). Tolerance seems to be largely metal specific, with tolerance to one metal not appearing to confer tolerance to another (Antonovics et al, 1971; Wu and Antonovics, 1975; Shaw, 1994).

Heavy metal tolerant plants are widely studied as they are of interest for phytoremediation, where heavy metal tolerant plants are used to limit erosion on toxic soils, thereby preventing the contamination by the metal of surrounding areas (Rossum et al., 2004). The study of species found on contaminated soils is difficult as it is often not possible to define the limits of the contaminated area, to assess ecological factors that may influence results, or to define the amount of toxicity in the area (Antonovics et al, 1971). Adaptation to heavy metals in populations growing on mine waste is therefore of particular interest for study. These sites have very high levels of heavy metal contamination, and are usually made up of toxic zones surrounded by relatively non-toxic areas (Antonovics et al, 1971; Shaw, 1994; Rossum et al., 2004).

In the original studies of growth on toxic soils done by Bradshaw and colleagues, the levels of genetic variation in populations from contaminated and uncontaminated sites were not measured directly through molecular methods, but rather indirectly by

comparing morphological differences in plants grown in reciprocal transplant experiments (Antonovics et al, 1971; Bush and Barrett, 1993). Plants growing on metal-contaminated soils are often characteristic of such soils, usually being smaller, more prostrate, and adapted to nutrient shortages and the exposed conditions that result from sparse colonisation. Most of the higher plants found on toxic soils are perennial herbs. Perennial plants colonise novel areas better than annuals due to persistence and slower rates of growth, and low-growing plants are better adapted to exposed conditions (Antonovics et al, 1971).

Toxin-tolerant ecotypes seem to be highly specialised for growth on toxic soil. They seem to be restricted to toxic soil as they are competitively inferior when grown on normal soil, and are rarely found in surrounding non-toxic areas (Clausen, 1951; Antonovics et al, 1971). There is evidence that metal-tolerant plants are metabolically different from non-tolerant plants. They seem to have a higher requirement for trace metals, probably due to increased metal inactivation ability. Tolerance to any metal also seems to provide plants with increased tolerance to low phosphorus or high sulphur levels (Antonovics et al, 1971). Some species seem to be unable to evolve tolerance even though they are abundant in the areas surrounding metal contaminated soils. Certain characteristics may be needed by a species to 'pre-adapt' them to toxic soil. For example, mine wastes contain restrictions on plant growth other than heavy metal tolerance, for example low nutrient levels. The possession of tolerance to these factors may assist the development of metal tolerance (Antonovics et al, 1971).

In early studies, the presence of tolerance to heavy metals was determined by measuring root length of plants grown in solutions with or without the metal of interest (Wu and Antonovics, 1975; Macnair, 1983). This approach was used to quantify tolerance to lead, zinc, copper, and nickel in the 1950s and 1960s (Antonovics et al, 1971). Root-length experiments have, however, been shown to be inaccurate. Since root growth is under polygenic control, some of the genes influencing it may not be involved in metal tolerance. This results in inaccurate measures of the extent of metal tolerance present in the plant (Macnair, 1983). Reciprocal transplant experiments therefore became the preferred method of study.

For example, high levels of tolerance to copper and zinc were found in mosses, associated with high levels of genetic diversity (Shaw et al., 1987; Shaw, 1994).

The advent of molecular markers based directly on variation in DNA has allowed much more in depth studies on genetic variation to be performed. Polluted soils have been shown to have an effect on genetic diversity in plant populations in many studies on European plant species. Studies show discrepancies as to whether polluted soils cause a decrease or increase in genetic diversity of plant populations growing in them. In *Silene paradoxa* populations, chloroplast microsatellites have shown that heavy-metal tolerant phenotypes evolve at an unexpectedly rapid rate. In several cases the populations retained a high level of variation, while in a few decreased variation was observed (Mengoni et al., 2001).

In Scots pines, heavy metal pollution was found to cause an increase in genetic diversity (Prus-Glowacki et al., 1999). In *Pinus sylvestris*, however, zinc smelter pollution was found to cause a decrease in genetic variation (Prus-Glowacki and Godzik, 1991). Decreased variation was thought to be due partially to an especially strong selection pressure found in extreme conditions, as only specialized genotypes are able to survive in such conditions (Prus-Glowacki and Godzik, 1995; Mengoni et al., 2001).

Research into the toxic effects of heavy metals spans all levels of biological organisation, due to the risks to populations and even ecosystems. Pollutants cause changes in the genetic structure of the species. Studies on both plants and animals have shown that distinct changes in gene pools occur following growth in contaminated habitats, caused by natural selection for increased tolerance to the toxins present. Physical linkages between genes may result in subsequent changes in other traits not directly influenced by selection. For example, decreased competitive abilities have been noted in plant strains that have developed toxin tolerance (Shaw, 1994). The effects of heavy metal pollution on plant populations are therefore widespread and far-reaching.

2. MINING

2.1. Mine wastes

Over 40% of the land surface of the earth has been disturbed in some way. Although the proportion of this disturbance that is due to mining activity is relatively low, consisting of approximately 0.2 Mha in South Africa, this proportion is increasing, with a progressive increase in the production of most metals and minerals over the last few decades (Cooke and Johnson, 2002).

The effects of mining activities are usually more severe than those caused by other forms of land disturbance. The mining process is made up of extraction, milling, processing, refining, and waste disposal. The production and disposal of waste (rock, fine tailings, and slimes) generally cause the most severe and long-lasting disturbance as they involve large areas of land, with waste produced by mining for heavy metals being the most toxic (Bradshaw, 1995; Leteinturier et al., 1999; Cooke and Johnson, 2002). Mining also destroys ecosystems through their removal or burial under waste sites (Cooke and Johnson, 2002).

The metal levels in mine waste tend to be decreasing as a result of improvements in mining technology (Cooke and Johnson, 2002). Modern wastes commonly contain approximately 1000ppm (0.1%) of the metal that was extracted. This number is still very high, but is an improvement on the levels of over 1% that can be found in older waste (Bradshaw, 1995; Leteinturier et al., 1999). High toxicity levels mean that mining wastes often remain uncolonised and are therefore easily eroded through wind and water. Water courses below mine wastes sites have been shown to be contaminated, with alluvial mineralised deposits being found in some parts (Antonovics et al, 1971; Leteinturier et al., 1999). High levels of arsenic, cadmium, cobalt, chromium, lead, copper, nickel and zinc have been found in soils downslope of gold mine tailings in the North West province, South Africa (Schoeman and van Deventer, 2004).

2.2. Slimes dams on the Witwatersrand

Tailings deposits (slimes dams) consist of the pulverised rock slurry left after mineral extraction during gold or uranium mining. On the Witwatersrand alone, they cover 8000ha and comprise $3x10^9$ tons of rock slurry. Slimes dams are divided into the slopes of the dams, made up of the slimes themselves, retaining walls, which are often partially covered in slimes, and toepaddocks surrounding the dams (Figure 1) (Witkowski and Weiersbye, 1998).

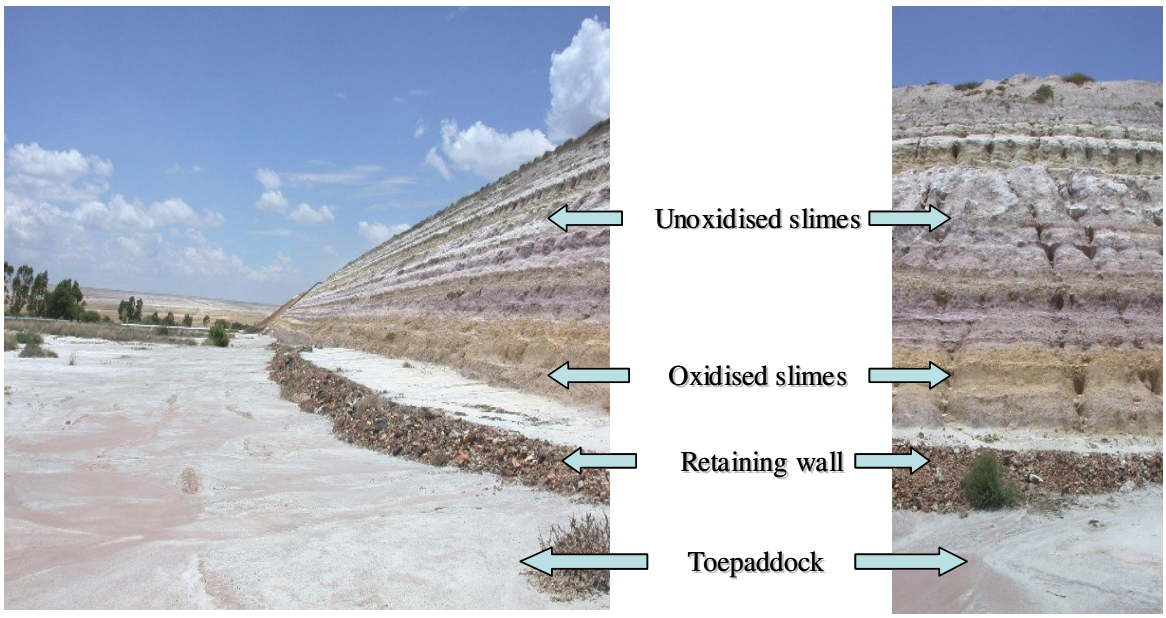


Figure 1: The different zones that make up a typical slimes dam. Photographs were taken at the South Complex slimes dam near Orkney (North West Province).

Slimes dams are formed by soil and rocks from surrounding areas being used to form the initial retaining wall. Slimes slurry is then piped into this shallow dam. The slimes on the outer surfaces dries, allowing the dam to be extended upwards (Witkowski and Weiersbye, 1998). Unoxidised slimes, at the top of the wall, have a high pH due to the presence of liming agents added during extraction of the metal. On exposure to air, pyrite components in the slimes are oxidised and the slimes becomes acidic (Weiersbye et al., 2005). A second soil wall, the toepaddock wall, is made in the grassland a distance from the dam in order to contain runoff, forming

the toepaddock (Witkowski and Weiersbye, 1998). Toepaddock walls are also made in the toepaddock perpendicular to the slimes dam in some places.

The retaining wall, toepaddock wall, and toepaddock are the areas of the dam where most plant growth occurs. This is due to soil from the surrounding grassland being present in or under the slimes in these areas. This results in these areas having a different and more diverse distribution of particle size, more similar to a typical soil, which assists in anchoring seeds so germination can occur. These areas also have increased nutrient levels, and higher numbers of nutrient-cycling microbes. These microbes have evolved tolerance to the relatively acidic and nutrient-poor soil of the surrounding areas, and are therefore capable of surviving on the dams. These areas are also not sloped, which allows better water retention and increases the chances of seeds germinating (Witkowski and Weiersbye, 1998).

Plant growth is influenced by many soil characteristics. These include organic matter content, pH, nutrient availability, and particle density (Hangelbroek et al., 2003). Extensive studies have been done on the slimes dams of the Witwatersrand in order to determine the characteristics of the soil in these areas. The following table shows the soil physical and chemical characteristics for the South Complex slimes dam at AngloGold's Vaal River mine (Table 1) (Witkowski and Weiersbye, 1998).

Table 1: Soil physical and chemical characteristics (mean ± standard deviation) for the South complex slimes dam. Values are means and based on the soil dry mass. Old vegetated refers to sites that were vegetated more than 3 years previous to the study (Witkowski and Weiersbye, 1998).

Feature	Vegetated	Oxidised slimes	Toepaddock
pH (KCL)	Non-vegetated	3.33±0.28	3.60±0.10
	Old vegetated	3.50±0.60	3.60±0.00
Pb (µg/g)	Non-vegetated	110±18	41±9
	Old vegetated	85±2	53±2
As (µg/g)	Non-vegetated	172±52	57±8
	Old vegetated	103±5	57±3
Ni (µg/g)	Non-vegetated	121±48	75±34
	Old vegetated	175±34	43±2
N mineralisation	Non-vegetated	6.13±1.37	1.34±3.47
(µg/g per 7 days)	Old vegetated	-1.74±0.31	9.01±0.01
Total N (µg/g)	Non-vegetated	174±11	270±23
	Old vegetated	146±62	124±41
Total P (µg/g)	Non-vegetated	121±15	70±16
	Old vegetated	100±9	70±11

This compares with average adjacent grassland values, taken 50-100 metres beyond the toepaddock wall, in the Klerksdorp area (Vaal reefs and Afrikander leases mines) of 23 µg/g Pb, 12 µg/g As, and 46 µg/g Ni. Grassland nitrogen mineralisation values were 18.3 µg N/g per 7 days. Average total nitrogen was 774 µg/g and average total phosphorus was 273 µg/g in grassland samples. The grassland pH was found to be an average of 6.3. Mg, Na, Ca, and Cr were also found to be higher in the slimes dam areas than in the grassland. Organic matter was much higher in the grassland. Grassland samples are still affected by the slimes, and therefore represent less contaminated soils, rather than uncontaminated soils (Witkowski and Weiersbye, 1998). These general trends persist between mining regions, as well as between the mine complexes within those regions, although values vary (Witkowski and Weiersbye, 1998).

From these values it can be seen that slimes and slimes-contaminated soils are a very harsh environment for plant growth. Although many heavy metals are necessary plant nutrients, at high levels they become toxic to both plants and soil microbes (Rout and Das, 2003). It is generally not the total levels of these metals that is toxic to plants but the available levels of the metals in solution. The pH of slimes soil is low, due to the oxidation of pyrite to sulphuric acid, and results in increased levels of solubilisation of metals. These metals may therefore be at toxic levels in solution, even though the total amounts present in the soil are low (Witkowski and Weiersbye, 1998). Low pH may also severely inhibit nodulation in legumes, resulting in a decreased nitrogen cycling ability (Marschner, 1986).

Zinc and aluminium are naturally abundant in most soils. At high levels, however, these elements may become toxic. Total zinc levels are generally found to be higher in the grassland than in the slimes. The low pH of the slimes soil, however, results in such high solubilisation levels that zinc may reach toxic concentrations (~45 μg/g in slimes vs. 0.05-20 μg/g in grassland soil) (Witkowski and Weiersbye, 1998). Zinc is assimilated early during the development of plants and can therefore be highly phytotoxic, generally causing stunted growth (Rout and Das, 2003). Aluminium also becomes soluble at lower pH levels. It causes inhibition of root elongation, also resulting in growth inhibition (Sledge et al., 2002). High levels of nickel cause stunted growth in plants (Antonovics et al., 1971). Other components of slimes-contaminated soil affect vegetation, for example low levels of nutrients, including nitrogen and phosphorus, and high levels of sulphur. The organic matter content of the soil is of extreme importance as it forms stable complexes with metal ions, so making them unavailable for uptake by plants (Antonovics et al, 1971).

The concentrations of heavy metals found in mine wastes are therefore sufficient to cause growth inhibition or death in plants that lack the necessary mechanisms of tolerance to the metals present (Bush and Barrett, 1993). Individually, these toxins and other growth-inhibitory factors are not present at the extreme levels found in some natural, non-polluted ecosystems. It is the fact that so many chemical and physical factors act in combination that makes slimes-contaminated soil so inhospitable to plant growth (Witkowski and Weiersbye, 1998).

High levels of pollutants and heavy metals can also be toxic to microorganisms and animals that live on the dams. For example, cadmium has been found to cause lesions in the liver and kidney of mammals (Cooke and Johnson, 2002). Serious disorders and death have been seen in ruminants that graze on forage with elevated Mo, Ni, Co, and Se levels. Therefore even if an area has been successfully vegetated, and has formed a stable ecosystem, grazing by animals should be prevented until the vegetation has been tested to be safe for animal consumption (Witkowski and Weiersbye, 1998).

2.3. Vegetation of slimes dams

The goal of vegetating slimes dams is to assist in some level of restoration of the area. Restoration refers to the process whereby an attempt is made to restore ecological integrity to the area. It involves re-establishing the damaged land's capability to capture and retain resources fundamental to normal ecosystem function, including energy, water, nutrients, and species (Bradshaw, 1995; Cooke and Johnson, 2002; Campbell et al., 2003). It is also important to preserve the native adjacent communities (Lesica and Allendorf, 1999).

Plants play an important role in changing the nature of toxic habitats. Plant populations that will be viable in the long term will prevent erosion (water and wind) through the soil-stabilising effect of root systems, and improve the quality of the soil (Witkowski and Weiersbye, 1998; Lesica and Allendorf, 1999). The contribution of organic matter and humus by these plants increases the nutrient status of the soil, forms complexes with heavy metals and makes metal ions unavailable to plants, and improves soil texture (Antonovics et al, 1971).

If left to natural succession, the development of viable plant populations would occur extremely slowly due to the low resilience of ecosystems contaminated by slimes (Bradshaw, 1995; Cooke and Johnson, 2002). Slimes dams generally contain no topsoil. The fine texture of the slimes (mostly silt) and the lack of organic matter result in high soil bulk density and soil compaction, low aeration, low water infiltration rates, and surface waterlogging (Cooke and Johnson, 2002). Added to

these slimes-specific problems are the fact that Witwatersrand slimes dams are generally located in harsh climatic conditions, with low rainfall (600 to 700mm mean rainfall in the areas of sampled dams) and an annual range of temperatures of –5°C to over 30°C. These factors make vegetation of slimes dams extremely difficult (Witkowski and Weiersbye, 1998).

Attempts have been made to vegetate the slimes dams in the past. This involved liming and fertilising the dams, and introducing mainly northern hemisphere grass species. These efforts were expensive and largely unsuccessful. The grasses used require a continuous supply of large amounts of nutrients and water (Weiersbye and Witkowski, 1998). Vetiver, a grass used in vegetation efforts, needs to be supplied with nitrogen, phosphorus, and water, and can only be propagated by breaking up clumps and replanting (Knoll, 1998). Exotic grass species are also likely to compete with surrounding indigenous species and affect local ecosystem processes, for example fire, as well as causing the loss of locally-adapted genotypes (Lesica and Allendorf, 1999).

Lime (calcium carbonate) is added to the soil in an effort to raise the pH. It does not, however, penetrate to the lower levels of the soil (Sledge et al., 2002), and together with the addition of fertiliser rapidly destroys any naturally tolerant plant and microbial populations already present on the slimes dams and surrounding areas (Weiersbye and Witkowski, 1998; Sledge et al., 2002). Soil microorganisms, such as bacteria and fungi, are responsible for most of the biological activity that occurs in soil. Some of the roles of microorganisms include nitrogen cycling, for example mineralisation, denitrification, and nitrogen fixation, and the decomposition of organic matter. Fungal hyphae and bacterial and fungal exocellular polysaccharides bind soil particles together to form aggregates, thereby stabilising the soil (Haynes and Graham, 2004).

In contrast, vegetation of slimes dams using tolerant indigenous species is a more cost-effective and ecologically-sound approach to rehabilitation (Antonovics et al, 1971; Weiersbye and Witkowski, 1998). Indigenous perennial plants have been shown to out-compete and out-persist introduced grass species in vegetation trials

(Weiersbye et al., 2005). Local genotypes have been shown to be useful when the disturbance is large, as they will have less effect on the gene pools of local and surrounding populations. The use of indigenous species will also maintain the biodiversity of animal and insect species that require native species for food, as well as provide specific nesting habitats that may be required by some animals and birds (Lesica and Allendorf, 1999). Locally adapted populations will also contain a great deal of adaptive genetic variation correlated with local climatic, topographic, edaphic (soil), and biotic differences. This variation is crucial for long-term survival of the plant populations in such harsh conditions (Clausen et al., 1948; Clausen, 1951; Lesica and Allendorf, 1999).

Indigenous legumes are particularly desirable for vegetation due to their symbiosis with nitrogen-fixing rhizobia located in root nodules on these plants. These rhizobia convert atmospheric nitrogen into a usable form, at a rate of up to 400 kg N/ha/year (Bradshaw, 1995; Weiersbye and Witkowski, 1998; Morris, 1999; Doignon-Bourcier et al., 2000). Leguminous species should be able to produce all the nitrogen needed for their growth, unless nitrogen fixation is inhibited by toxic soils (Antonovics et al, 1971). In addition, many legumes are well adapted to arid conditions, and are so successful at nitrogen fixation that they are used in West Africa as an alternative to nitrogen fertilisers (Doignon-Bourcier et al., 2000).

The vegetation that has naturally colonised retaining walls and toepaddocks varies greatly between dams, as it is influenced by the steepness of slopes, aspect, soil physical and chemical characteristics, disturbances, biotic factors, and the overall climate of the area. The slopes of the dams themselves are generally uncolonised due to the steep gradient which constrains seed germination and seedling establishment (Weiersbye and Witkowski, 1998). The majority of plants naturally colonising slimes dams are semi-woody to woody perennials, even if these types of plants are not common in the area. This suggests that these types of plants are naturally more suited for growth on slimes dams (Weiersbye et al., 2005).

3. THE AMPLIFIED FRAGMENT LENGTH POLYMORPHISM TECHNIQUE

3.1. Advantages of AFLP

Molecular techniques have revealed a higher level of genetic variation in plant populations than was previously thought to have existed (Clegg, 1997). Molecular markers have several advantages over the traditional phenotypic markers, that are difficult or time-consuming to select by plant-breeders, as they are not directly influenced by environmental conditions, they are detectable at all plant growth stages and in all tissues, and they have simple inheritance patterns (Lu et al., 1998; Bagley et al., 2002; Najimi et al., 2002). Molecular markers have proved to be powerful tools in the assessment of genetic variation both within and between plant populations (Muluvi et al., 1999).

Several molecular techniques have been used for the analysis of molecular variation. Restriction fragment length polymorphisms (RFLP) are based on detecting variation in restriction endonuclease restriction sites caused by base substitutions or small deletions/insertions. This technique requires large amounts of DNA and the use of Southern blotting, and is therefore time-consuming and expensive. Randomly amplified polymorphic DNAs (RAPD) detect sequence changes within polymerase chain reaction (PCR) oligonucleotide primer binding sites. These markers tend not to be reproducible due to the low specificity of binding of short primers. Microsatellites (simple sequence repeats or SSR) are stretches of DNA that consist of tandem repeats of a simple sequence of nucleotides, with the number of repeats varying between individuals. They require prior sequence knowledge of the study organism, whereas RAPD does not (Hillis et al., 1996). Although microsatellites allow the detection of many alleles per locus, they lack power due to the small number of loci analysed in each reaction (He et al., 2004).

The AFLP (amplified fragment length polymorphism) technique is a relatively new technique, first described by Vos et al. (1995). The technique is based on the amplification of fragments created by digestion with restriction enzymes.

Amplification is carried out through PCR, by which a region of DNA may be amplified over a million fold by the use of DNA synthesis initiated by two specific primers and carried out by a thermostable DNA polymerase (Vos et al., 1995). AFLP is an example of a technique that uses neutral markers. Neutral markers are genetic differences that are assumed to not be affected by natural selection, although they may be influenced by selection at linked loci (Faugeron et al., 2001; Snäll et al., 2004). These types of markers are very powerful in exploring genetic variation (Faugeron et al., 2001).

Like other fingerprinting techniques, for example the RAPD technique, AFLP requires no prior sequence knowledge of the DNA of interest (Vos et al., 1995; Muluvi et al., 1999). The AFLP technique has, however, many advantages over other molecular techniques. Since it is PCR based, it can be performed with very small amounts of DNA (Castiglioni et al., 1999; Najimi et al., 2002). It is also a quick process, providing results in a relatively short space of time (Castiglioni et al., 1999; Najimi et al., 2002). AFLP can detect polymorphisms simultaneously in more loci of the genome than are inspected by other PCR-based techniques (Thomas et al., 1995; Miyashita et al., 1999; Han and Ely, 2002). This high resolution makes it possible to study genetic variation throughout the entire genome (Miyashita et al., 1999; Gobert et al., 2002).

The AFLP technique is capable of producing a large number of informative bands from a single PCR reaction (Castiglioni et al., 1999; Miyashita et al., 1999; Vanhala et al., 2004), many more than can be produced with the study of allozymes (Terashima and Matsumoto, 2004). In barley, melon, potato, *Arabidopsis thaliana*, cassava, *Eucalyptus*, hops, maize, and wheat, AFLP detected a larger number of polymorphisms than, for example, RFLP, RAPD and SSR techniques (Miyashita et al., 1999; Beardsley et al., 2003; Terashima and Matsumoto, 2004).

In addition, AFLP markers are highly reliable and reproducible due to the stringent reaction conditions used during primer annealing (Vos et al., 1995; Jones et al., 1997; Miyashita et al., 1999; Gobert et al., 2002; Vanhala et al., 2004). The reproducibility of RAPD, AFLP and SSR markers was tested by duplicating studies

over time in several different labs. RAPD markers were difficult to reproduce, while SSR markers (microsatelites) showed small size differences on repeat analyses. AFLP markers were found to be highly reproducible, with only a single band difference occurring following one repetition of the experiment (Jones et al., 1997).

3.2. Examples of uses of the AFLP technique

3.2.1. Measuring genetic variation

Variation within and between populations used to be measured primarily by pedigree-based measures, or by measuring phenotypic diversity. Molecular markers produce diversity levels comparable to pedigree-based methods (Barrett et al., 1998), and higher levels than those produced by phenotypic diversity measures (Vanhala et al., 2004). This is assumed to be due to the few traits that are usually measured during phenotypic methods, as well as the fact that many phenotypic traits are directly influenced by the environment. Molecular markers are numerous and are influenced only indirectly by the environment, if at all (Vanhala et al., 2004).

Since AFLP has the capacity to inspect a much greater number of loci for polymorphism than other techniques, it results in the detection of higher levels of genetic variation (Thomas et al., 1995; Muluvi et al., 1999; Yee et al., 1999). In *Vigna angularis*, the mean level of molecular genetic variation detected through the use of AFLP was significantly higher than that detected with RAPD (Yee et al., 1999). AFLP analysis has been used successfully to assess the amount and pattern of genetic diversity in, for example, *Brassica juncea* (Gobert et al., 2002), *Elymus repens*, an economically important grass species (Szczepaniak et al., 2002), and *Avicennia marina*, a wide-ranging mangrove tree species in Vietnam (Giang et al., 2003).

The AFLP technique was used in garlic to see if phenotypic differences observed between accessions are due to genotypic differentiation or phenotypic plasticity (Volk et al., 2004). It has also been used to study genetic diversity differences

between recently founded and established plant populations (Solé et al., 2004). In *Arabidopsis thaliana*, nucleotide diversity was estimated from AFLP data by using a method discussed by Innan et al. (1999) and was used to construct a phylogenetic dendrogram, which was consistent with dendrograms constructed from sequencing and RFLP data (Miyashita et al., 1999). It has been used to study genetic diversity in endangered (the wild apple) and widely utilised (*Moringa oleifera*) species, in order to assist in the development of conservation and management programmes (Muluvi et al., 1999; Coart et al., 2003).

3.2.2. Studies between species and strains

The AFLP technique has been used to assess genetic differentiation between species, strains, and populations. For example, AFLP has been used to assess distinctness, uniformity, and stability of sugar beet varieties (de Riek et al., 2001), and to assess the relatedness between inbred faba bean lines (Zeid et al., 2003). In *Saponaria pumila,* an alpine plant species, AFLP fragments were found in most individuals in selected groups but not in members of other groups. This indicated that the selected group originated from a separate, isolated glacial refugium than that the other groups originated from (Tribsch et al., 2002). A similar study was done with the alpine herb *Rumex nivalis* (Stehlik, 2002). The AFLP technique has also been used to determine phenetic relationships among species in *Dactylorhiza* (Gobert et al., 2002). Variation between species of monkey flower was examined in order to determine the evolution and origin of hummingbird pollination (Beardsley et al., 2003).

AFLP band patterns are species-specific and may therefore be used to identify closely related species or strains that would be difficult to identify by traditional methods (Han and Ely, 2002). For example, AFLP was used to distinguish between wild and cultivated gene pools in chicory (Van Cutsem et al., 2003) and adzuki bean (Xu-xiao et al., 2003) in order to study gene flow and strain evolution, respectively. Since AFLP detects variation at many loci in the genome, species identification of this kind is not based on the presence of one particular allele (Han and Ely, 2002).

Identification of species or strains by this method also does not involve the use of mitochondrial DNA (mtDNA) haplotypes, which means that AFLP can be used to identify hybrids, an ability which is not possible with mtDNA analysis (Han and Ely, 2002). Natural, interspecific hybridisation occurs at high frequency in the section *Mentha*. This hybridisation was detectable through AFLP analysis, and AFLP profiles allowed classification that supported the previously established taxonomic classification for members of *Mentha* (Gobert et al., 2002).

3.2.3. Other uses

Genetic linkage mapping is a powerful tool for the localisation and isolation of genes controlling both simple and complex traits (Lu et al., 1998). The AFLP technique has been used to construct genetic linkage maps in barley, melon, potato, *Arabidopsis thaliana* (Miyashita et al., 1999), rye (Bednarek et al., 2003), and maize (Castiglioni et al., 1999). Linkage maps are the basis for marker-assisted selection, where markers are identified that are closely associated with a desirable trait, for example nematode resistance in peaches (Lu et al., 1998). In tomatoes, AFLP analysis was used to construct a detailed linkage map, and subsequently allowed identification of DNA markers that are tightly linked to *Cf-9,* a gene that provides resistance to *Cladosporium fulvum* (Thomas et al., 1995). This analysis has also been used to identify markers closely linked to Hessian fly resistance in wheat (Najimi et al., 2002) and powdery mildew resistance in the pea (Coyne et al., 2000).

The AFLP technique has also been used for studies on organisms other than plants. In bacteria, AFLP analysis was used in the genus *Bacillus* to determine relatedness between two species (Ticknor et al., 2001), and was used to characterise novel rhizobia from agriculturally important, previously un-studied leguminous species in Senegal (Doignon-Bourcier et al., 2000). In fungi, AFLP was used to study clustering isolates of *Beauveria bassiana* (de Muro et al., 2003), to investigate the genetic structure of the plant pathogen *Phytophthora capsici* (Lamour and Hausbeck, 2001), and to type strains of shiitake mushrooms (Terashima and Matsumoto, 2004). In fish, a modified AFLP protocol involving the use of one

restriction enzyme was used in the study of genetic variation between Morone and Thunnus species (Han and Ely, 2002).

In summary, even though it is a relatively new technique, AFLP analysis has been used extensively in the study of genetic structure in many different organisms. In plants, the AFLP technique has been widely used with many applications. In this study, the AFLP technique will be used together with morphological studies to investigate genetic variation in populations of plants exposed to slimes-contaminated soil.

4. RATIONALE, OJECTIVES, AND PREDICTIONS

4.1. Rationale for the study

There are a large number of slimes dams in South Africa. This number can be expected to increase in the future, due to the fact that revenue from mining still contributes significantly towards the country's economy. The presence of slimes dams results in unique growth conditions being imposed on the plants growing in the vicinity of the dams. It is necessary to understand the full effect that these growth conditions have on plants in order to be better able to plan subsequent use and/or rehabilitation of the slimes dams and surrounding areas.

The aim of this study was to determine if plant populations show local adaptation to the adverse substrate conditions emanating from slimes dams, by investigating genetic and morphological variation between adjacent populations of selected plant species growing at different distances in relation to slimes dams.

4.2. Objectives

The broad objectives of the study were as follows:

- Select suitable plant species to be used in the study. These species should show sufficient population numbers in different areas both close to and far away from the dams
- Perform sampling of leaf tissue for DNA analysis and take pertinent morphological measurements of plants
- Develop an AFLP protocol and perform AFLP analysis on all individuals sampled
- Statistically analyse the genetic and morphological data obtained. This analysis fell into two categories, namely analysis according to distance from the slimes dam, and analysis according to estimates of soil toxicity
- Interpret the results of the statistical analysis and discuss their biological significance. Discuss whether the species studied show local adaptation to the adverse substrate conditions emanating from slimes dams

- In light of the study results, make recommendations on the suitability of these species as candidates for persistence on slimes dams, and make suggestions for further study

4.3. Expectations

It was expected that morphological and genetic differences would be found between populations on and off slimes dams. Lower genetic diversity would probably be noted on the slimes-inundated soil, due to relatively recent colonisation of these soils by plants from the surrounding areas. Alternatively, a lack of genetic or morphological differentiation between areas could indicate that slimes contaminated soils did not have much of an adverse effect on the plants studied, or that colonisation occurred long ago and its affects have since been negated, or that gene flow levels were high enough between the areas to dilute any effect slimes contaminated soil may have had.

METHODS AND MATERIALS

1. SPECIES SELECTION

In 2003, two slimes dams were selected for the study: the 26 year old (constructed 1979) South Complex slimes dam (26° 54.07-54.52´S; 26°44.46-45.38´E) at AngloGold Ashanti Ltd's Vaal River mine, near Orkney, North West Province, and the 34 year old (constructed 1971) New North Complex slimes dams (26°25.57-25.83´S; 27°20.99-21.60´E) at AngloGold Ashanti Ltd's West Wits mine, Carletonville, North West Province. These dams were chosen because surveys of vegetation (Weiersbye and Witkowski, 2002) and chemical characteristics (Weiersbye and Witkowski, 1998) had been performed on these and the surrounding dams.

The selection of plant species to be used in this study was performed in January 2003 at the Afrikander Leases dam, west of Klerksdorp. Several species were sampled on and off slimes dams in order to assess plant distribution and DNA yield. The purpose of this was to determine the presence of species on the various areas or landscape units of the dams, in order that a suitable species may be chosen for study. Samples of young leaves were taken for DNA extraction, in order to determine the suitability of the selected species for study using the AFLP technique. Samples of three *Indigofera* species (*Indigofera adenoides*, an identified species with grey leaves, and an unidentified species with sticky leaves) were obtained from the toepaddock and retaining wall, as well as off-site samples approximately 50 metres from the dam. Samples of *Asparagus laricinus* and a *Lippia* species were obtained from the toepaddock. *Asparagus laricinus* samples were obtained off-site, while off-site *Lippia* species samples could not be found.

DNA extraction was performed on all five species as described in section 3. AFLP analysis was then performed for all species using the method described in section 4. This initial analysis resulted in two *Indigofera* species being chosen as suitable for further study, due to criteria that will be discussed in the results section. *Indigofera*

adenoides Baker f., family Fabaceae, was chosen for sampling at the South Complex dam, which is dry, and relatively unvegetated. This species could not be found at the New North Complex dam, which is wetter and more vegetated. *Indigofera zeyheri* Spreng. ex Eckl. & Zeyh, family Fabaceae, was therefore chosen for sampling at this dam. Slimes dam, species selection, and species characteristics are discussed further under the results section.

2. SAMPLING

Approximately 20 young leaves for DNA extraction were taken from plants growing on several aspects of each slimes dam, both on and off the dam. This was done to provide a sufficient number of samples to allow meaningful analysis of the results. An aspect refers to a side of the dam, as each dam was made up of several straight walls placed at angles. All plants that could be located in the toepaddock, retaining wall, and toepaddock wall were sampled, due to the small population sizes of the selected species in these areas.

Off site samples were taken from beyond the toepaddock wall. The soil in these areas is often contaminated through slimes being blown onto these areas, as well as through contaminated ground water. These areas therefore constitute less contaminated areas, rather than uncontaminated areas. Far off site samples were taken from sites far enough away from the dams to be considered relatively uncontaminated. Off site and far off site areas showed larger population sizes of the selected species than toepaddock areas. Therefore plants in these areas were sampled by taking approximately ten plants from each population, attempting to sample a range of sizes plants from large to small in each population.

In order to perform morphological comparisons, measurements of each plant sampled were taken. These were plant height, canopy width at the widest point, and a second measurement of canopy width taken at 90° to the first measurement. Leaf measurements were performed by measuring lengths and widths of five mature leaves taken at random from each plant. Mean leaf length and leaf width values were then calculated. Resolution was to the nearest centimetre for plant

measurements, and to the nearest 0.1cm for leaf measurements. Sampling numbers are discussed under the results section. Global positioning system (GPS) coordinates were obtained for approximately every fifth plant.

3. SEED GERMINATION

Ten seed pods were taken from ten plants, selected at random, for each species. These were to be germinated so that breeding system studies could be made. The AFLP profile of the offspring was to be compared to that of the parent. The degree of similarity between the profiles can be used to determine whether the species are self fertilising only, obligate out-crossers, or a combination of the two. Seeds were germinated by soaking them in a solution of 0.1 mg fungicide (Benlate) per ml and then placing them on damp filter paper in the dark at 25°C.

4. DNA EXTRACTION

A sub-sample of 0.1 g of young leaf tissue, which produces the highest yield of DNA, was taken from each sample and ground under liquid nitrogen. DNA was extracted using the Qiagen DNeasy® plant mini kit, as this has produced good results with plant material in the past, giving a high quality and yield of DNA from many different species. DNA was eluted the first time with 100 µl of elution buffer, and the second time with 50 µl of elution buffer. The two products were combined. This produced an average DNA concentration of 35 µg/ml. In order to determine the presence, and approximate the purity, of the DNA, 2 µl of extracted DNA was added to 1 µl of 6X loading dye (80% w/v sucrose, 10 mM tris-HCl, 10 mM EDTA, 0.3% bromophenol blue) and run on a 0.8% agarose, 1X TBE (0.089 M tris, 0.089 M boric acid, 0.002 M EDTA, pH 7.8) gel in 1X TBE. The concentration and purity of a random subset of samples was also checked by spectrophotometry.

5. AFLP ANALYSIS

5.1. AFLP protocol

The principle of AFLP analysis is discussed below, and is summarised in Figure 2. The DNA of interest is digested with two restriction enzymes, and adapters containing PCR primer sites are ligated onto the fragments. These fragments are then amplified through the polymerase chain reaction (PCR). A subset of the resulting fragments is then amplified further by using primers with a few selective nucleotides added, in order to reduce the number of fragments amplified and generate a fingerprint with a 'readable' number of bands (Vos et al., 1995).

AFLP analysis was performed as described by Vos et al. (1995), with some changes. MilliQ® water (Millipore) was used throughout. The restriction enzymes MseI (a frequent cutter) and ApoI (a rare cutter) were used. Both were obtained from New England Biolabs. All digestion, ligation, and PCR steps were performed in a thermocycler (GeneAmp® 2700, Applied Biosystems). 20 µl ApoI digestions were performed containing 5 U of ApoI and 2 µl extracted DNA (approximately 70 ng) in 100 mM NaCl, 50 mM tris-HCl (pH 7.9), 10 mM $MgCl_2$, and 1 mM dithiothreitol. Digestions were incubated at 50° C for two hours. A 10 µl volume of 5 U MseI in 100 mM NaCl, 50 mM tris-HCl (pH 7.9), 10 mM $MgCl_2$, and 1 mM dithiothreitol was then added to the ApoI mixture and the mixture incubated at 37° C for two hours.

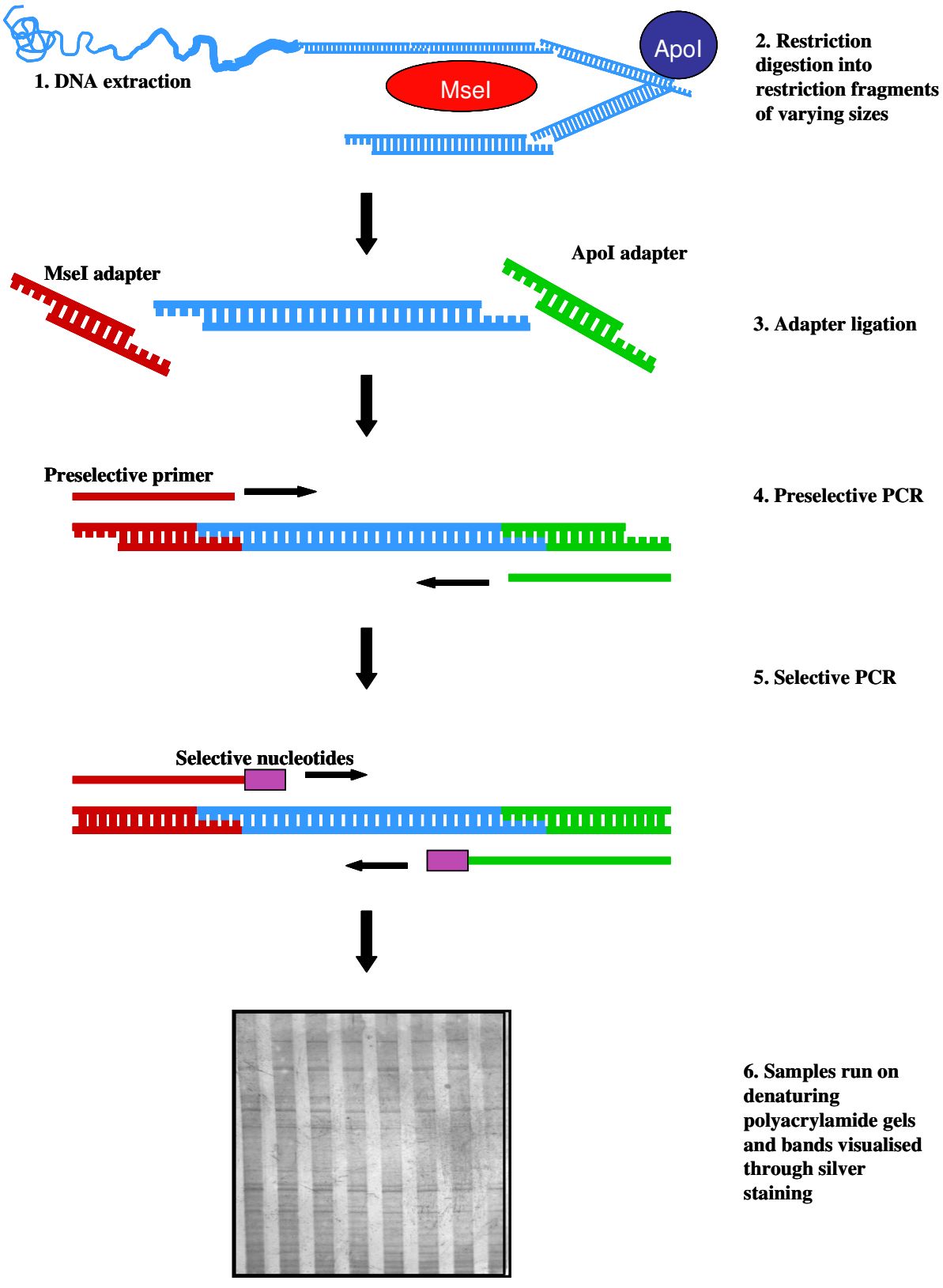


Figure 2: Summary of the AFLP technique as developed by Vos et al. (1995)

The double stranded adapter sequences used were as follows:

ApoI adapter: 5'-CTCGTAGACTGCGTACC
CATCTGACGCATGGTTAA-5'

MseI adapter: 5'-GACGATGAGTCCTGAG
TACTCAGGACTCAT-5'

Double stranded adapter working solutions were prepared by adding the two adapters that make up each pair to adapter buffer (200 mM tris-HCl (pH 7.4), 2 mM $MgCl_2$, and 5 mM NaCl) to a final concentration of 5 µM for the ApoI adapter and 50 µM for the MseI adapter. The mixture was then incubated at 95°C for 5 minutes, cooled slowly to room temperature in a thermocycler, and stored at -20°C. Ligations of 10 µl, containing 5 U T_4 DNA ligase (Fermentas) and 1 µl of each adapter solution in 40 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, and 0.5 mM ATP, were added to the digested DNA mix and incubated at 15° C overnight. Digested and ligated solutions were stored at -20° C until needed.

The pre-selective ApoI primer contained no selective nucleotides, while the MseI primer contained 1 selective nucleotide. The sequences used were as follows:

ApoI primer: 5'-CTCGTAGACTGCGTACCAATT
MseI primer: 5'-GACGATGAGTCCTGAGTAA

Pre-selective PCRs of 50 µl were performed containing 100 ng of each pre-selective primer, 0.9 U Taq polymerase (Promega), 0.25 mM of all four dNTPs, and 20 µl of the digested/ligated DNA mixture, in 10 mM tris-HCl (pH 9.0), 0.1% Triton X-100, 50 mM KCl, and 1.5 mM $MgCl_2$. The PCR profile used is shown in Table 2. Pre-selective PCR products were diluted ten fold in MilliQ® water and used for selective amplification.

Table 2: PCR profile used for pre-amplification of samples.

Number of Cycles	Temperature	Time
1	72.0	5.0 min
40	94.0	30 sec
	52.0	30 sec
	72.0	1.0 min
1	72.0	5.0 min

Various combinations of selective primers were tested in order to determine which set gave the most readable, reproducible, and meaningful band pattern. Selective PCR primers chosen contained two selective nucleotides for the ApoI primer and three selective nucleotides for the MseI primer. Only one set of primers was used as this was found to produce sufficient numbers of polymorphic bands to allow meaningful analysis. The sequences used were as follows:

ApoI primer: 5'-GACTGCGTACCAATTCC
MseI primer: 5'-GATGAGTCCTGAGTAACTC

30 µl selective PCRs were performed containing 15 ng ApoI selective primer, 30 ng MseI selective primer, 0.4 U Taq polymerase, 0.25 mM of all four dNTPs, and 3 µl of the diluted pre-selective PCR product in 10 mM tris-HCl (pH 9.0), 0.1% Triton X-100, 50 mM KCl, and 1.5 mM $MgCl_2$. The PCR profile used for selective amplification is shown in Table 3.

Table 3: PCR profile used for selective amplification of samples. The annealing temperature was reduced every cycle for the first 13 cycles.

Number of cycles	Temperature (°C)	Time
	94.0	30 sec
13	65.0, reduced by 0.7 each cycle	30 sec
	72.0	1.0 min
	94.0	30 sec
23	56.0	30 sec
	72.0	1.0 min

5.2. Denaturing polyacrylamide gel electrophoresis

The gels used for fragment separation were 10% denaturing polyacrylamide gels, made up of 10% acrylamide, 0.25% methylene bisacrylamide, and 7.5 M urea in 1X TBE (0.089 M tris, 0.089 M boric acid, and 0.002 M EDTA, pH 7.8). Gel mixes were made in advance by dissolving the urea in the other ingredients for several hours at room temperature, and storing the solution at 4° C for up to one month. Gels were cast by adding 300 µl 10% ammonium persulphate solution and 50 µl TEMED to 50 ml of gel solution (sufficient for two gels) and casting the gels in 18 cm x 16 cm x 1 mm gel apparatus, adding 10 well, 0.8 cm x 1.5 cm well size combs, and leaving the gels to polymerise at 4° C overnight.

The following day, the wells were flushed with 1X TBE to remove unpolymerised acrylamide. Gels were pre-run at 1500 V for 15 minutes to remove the polymerising agents TEMED and ammonium persulphate, as these are very strongly charged and may therefore interfere with the migration of the DNA fragments. Following the pre-run the wells were flushed a second time. The entire 30 µl selective PCR reaction product was run on the gel, in order to maximise the visibility of the bands. Reaction products were mixed with an equal volume of formamide solution (98% formamide, 10 mM EDTA (pH 8.0), 0.1% xylene cyanol as tracking dye) and heated

at 90° C for 3 minutes in order to denature the DNA. Samples were cooled quickly on ice before being loaded.

The outer two lanes of the polyacrylamide gels were not used for samples, as they tend to run slightly slower due to temperature differences within the gel, creating bent lanes (smiling) and making accurate scoring more difficult. A molecular weight marker (GeneRuler 100bp DNA ladder, Fermentas) was run in the first lane as a size standard. Gels were run at 50 V for 15 minutes to allow stacking of the fragments, and then run at 500 V for approximately 150 minutes until the tracking dye was 1 cm from the bottom of the gel. Following electrophoresis, gels were removed from the plates immediately and stained with silver.

5.3. Silver staining

Several silver staining methods were tested until one was found that allowed visualisation of the fainter bands. The method used was adapted from Blum et al. (1987) and is shown in Table 4. All solutions were made up with MilliQ® water as this produced darker and sharper bands, and fewer artefacts, than other types of purified water tested.

Gels were visualised on the UVP BioDoc-It™ system. Bands were scored directly from the viewing screen, as this provided the best band intensity and clarity. Bands were identified both by being compared to the molecular weight marker, and by being identified from a reference sheet developed that diagrammed all of the bands obtained, each labeled with a number. This facilitated easier and more accurate scoring. To test for reproducibility, a subset of 16 samples was randomly chosen and analysis was repeated, including DNA extraction, AFLP analysis, and band scoring. This has been suggested to be a necessary test for reproducibility in some studies (Zeid et al., 2003).

Table 4: Silver staining protocol, adapted from Blum et al. (1987). The original protocol has been shown in italics where steps have been modified.

Steps	Solutions	Time of treatment
1. Fix	50% methanol, 12% acetic acid	1.5 hrs to overnight (≥*1 hr*)
2. Wash	50% ethanol	3 x 20 minutes
3. Pretreat	0.02% (W/V) $Na_2S_2O_3.5H_20$	1 minute
4. Rinse	Water	1 x 5 sec, 2 x 20 sec (*3 x 20 sec*)[1]
5. Impregnate	0.2% (W/V) silver nitrate, 0.075% (V/V) formaldehyde	20 minutes in the dark
6. Rinse	Water	2 x 5 sec (*2 x 20 sec*)[1]
7. Develop	6% (W/V) sodium carbonate, 0.0008% (W/V) $Na_2S_2O_3.5H_20$, 0.05% (V/V) formaldehyde	2 hrs in the dark (≥*10 min in dark*)[2]
8. Wash	Water	Not performed (*2 x 2 min*)[2]
9. Stop	Fix solution	Not performed (*10 minutes*)[2]
10. Wash/store	50% methanol	Overnight (≥*20 min*)[3]

[1] The duration of rinses was decreased to retain more pre-treatment and impregnate solutions in the gels, thereby increasing band staining. This process also increased background staining, but not to an unacceptable level.

[2] The gels were developed for longer and the development reaction was not stopped. This ensured that bands developed to their maximum and gave the clearest results.

[3] The gels were scored after storage overnight at 4° C in 50% methanol. This shrank the gels, thereby concentrating the bands, making them easier to view.

6. DATA ANALYSIS

6.1. Morphological analysis

6.1.1. Plant and leaf measurements

Measurements taken for each plant included plant height (*Ht*), canopy diameter at the widest point (*CD*), canopy diameter at 90° to the first measurement (*CD*90º), leaf length (*LL*), and leaf width (*LW*). These measurements were used to calculate several measures of overall plant size, leaf size, and leaf shape. Selected variables that did not follow a normal distribution were converted to $\log_{10}$ values, in order to normalise the distributions of the variables and facilitate more accurate statistical analysis.

Canopy area (ellipsoid shape) was calculated according to the formula:

$$CA = \pi \frac{CD}{2} \frac{CD90^0}{2}$$

The canopy volume (*CV*) measurement calculated estimates the ellipsoid volume the plant fills. It was calculated according to the formula:

$$CV = \frac{4}{3} CA \frac{Ht}{2}$$

Leaf area (*LA*) (ellipsoid shape) was calculated according to the formula:

$$LA = \pi \frac{LW}{2} \frac{LL}{2}$$

The leaf length to breadth ratio was determined. This was termed leaf shape (*LS*) as it gives an indication of the overall shape of the outline of the leaf. The higher the value, the longer and thinner the leaf shape. *LS* was calculated according to the formula:

$$LS = \frac{LL}{LW}$$

The distributions of these measurements were plotted and compared between areas. Correlations between various variables were also determined and compared between sites.

6.1.2. Statistical analysis

Original measurements (*Ht*, *CD*, *CD*90°, *LL*, *LW*) and calculated plant measurements (*CA*, *CV*, *LA*, *LS*) were used for statistical analysis using the SAS system (SAS Institute, 2000). Several techniques were used to compare plant measurements between areas. This involved firstly comparing each measurement between areas, and then testing composite variables as a whole.

Analysis of variance (ANOVA) was performed as a necessary precursor to Tukey and canonical discriminant analysis, in order to determine if differences in mean values for each variable are significant between groups. The null hypothesis:

H_0: $\mu_1 = \mu_2 = \ldots = \mu_k$

is tested, where k is the number of groups being analysed. Equal sample sizes are not necessary for ANOVA tests. The test is performed by calculating the ratio of group mean squared deviations from the mean (MS) to error MS values. If this value is greater than, or equal to, the critical value for the test, the null hypothesis is rejected and the means are shown to be unequal (Zar, 1984).

The Tukey test allows further examination of the differences in means detected through ANOVA tests, through testing multiple pairs of means. This allows a study of between which groups the detected mean differences are significant.

It considers the null hypothesis:

$$H_o : \mu_B = \mu_A$$

versus the alternate hypothesis:

$$H_A : \mu_B \neq \mu_A$$

where the subscripts denote any possible pair of groups.

Pairwise mean differences are calculated, and divided by *SE* to produce a *q* value.

$$SE = \sqrt{\frac{s^2}{2}\left(\frac{1}{n_A} + \frac{1}{n_B}\right)}$$

where s^2 is the error mean square from the analysis of variance and *n* is the number of samples in groups *A* and *B*. If the calculated *q* value is equal to or greater than the critical *q* value, the null hypothesis is rejected (Zar, 1984).

A two way comparison T-test was used to detect differences in variable means between *I. zeyheri* groups. This test, as in the ANOVA test, compares the null hypothesis:

$$H_o : \mu_B = \mu_A$$

versus the alternate hypothesis:

$$H_A : \mu_B \neq \mu_A$$

A *t* value for testing the hypotheses is determined by the difference between the two means over the standard error of the difference between the means. The null hypothesis is rejected if *t* is either greater than, or equal to, the critical *t* value, or if the *t* value is less than or equal to the negative critical *t* value (Zar, 1984).

Canonical discriminant and discriminant analyses are techniques that allow the simultaneous comparison of all variables and all groups, thereby exposing which groups are significantly morphologically differentiated from each other. Canonical discriminant analysis determines whether one set of variables, taken as a whole, is different from another set of variables, and if so, discovers which variable/s is/are responsible for the difference. The analysis is based on the canonical correlation, which is the maximum correlation between linear functions of the two sets of variables (Blackith and Reyment, 1971; Cooley and Lohnes, 1971). Variables that are shown not to account for significant variation indicate that the differences among group means for that variable are small in relation to the differences among individuals within a group (Blackith and Reyment, 1971).

Canonical discriminant analysis was also used to calculate a distance matrix based on pair-wise squared distances between groups. This was used to construct a dendrogram using the KITSCH programme in PHYLIP (Felsenstein, 2004) and viewed using TREEVIEW (Page, 1996). The KITSCH programme uses a method of constructing rooted trees similar to the method used by the programme UPGMA. An evolutionary clock is assumed (expected amount of evolution in any lineage is proportional to elapsed time). It is more accurate than UPGMA but is not often used due to it being more computationally intensive and therefore time consuming.

Discriminant analysis allows the allocation of individuals to specific groups based on a set of variables for those individuals. The approach involves locating a line in the space of the attributes of the individual plants for which the separation of the groups is optimised when the individual points of the different groups of plants are projected onto it. A test of the statistical significance of the separation of the groups is then performed (Cooley and Lohnes, 1971).

6.2. AFLP analysis

6.2.1. Scoring of gels

Any position on the gel which contained at least one band was termed a fragment. Monomorphic fragments are those that contained a band in each sample, while polymorphic fragments lacked a band in one or more samples. AFLP profiles were scored by determining the presence (1) or absence (0) of bands between approximately 50-75 and 1500 base pairs in size. Bands smaller than 50 base pairs were not scored as they were found to have no detectable variability. This is presumably because of the amplification of large numbers of fragments of a very similar, small size. These bands will run close to each other under electrophoresis, causing them to appear as one band and be scored as such.

6.2.2. Statistical analysis

AFLP data were analysed both within and among sample groups. The number and percentage of loci that are polymorphic regardless of allele frequencies was determined for each group using POPGENE software v1.31 (Yeh et al., 1997). POPGENE software was also used to calculate Shannon's information index (*I*) and Nei's (1973) gene diversity statistic (*h*) for each group, as well as Nei's (1973) gene diversity statistics for the overall population.

Shannon's information index (*I*) provides an estimate of the relative degree of genetic variation within each population. Its advantage is that it is relatively insensitive to skewing of data that occurs due to the inability of AFLP analysis to detect heterozygotes (Jover et al., 2003).

It is calculated for each locus by the formula:

$$I = -\sum p_i \log_2 p_i$$

where p_i is the frequency of the presence/ absence of the band at each locus. The group mean diversity is estimated by averaging the I values obtained for all loci in that group (Lynch and Milligan, 1994).

Nei's (1973) gene diversity statistics were calculated. These included the measure of gene diversity within each group (h), similar to I, the total genetic diversity of the entire population (H_T), and the amount of total genetic diversity that is due to diversity within the different subpopulations (groups) (H_S) (Nei, 1973). H_T indicates what the heterozygosity of the population would be if all subpopulations were pooled together and mated randomly (Hartl and Clark, 1989). H_T is calculated by the formula:

$$H_T = H_S + D_{ST}$$

where D_{ST} refers to the average gene diversity between subpopulations. Therefore the gene diversity of the total population is made up of the gene diversities within and between subpopulations (Nei, 1973).

Wright's fixation index (F_{ST}) was measured using AFLP-SURV (Vekemans et al., 2002). F_{ST} measures the proportion of the total gene diversity that occurs among as opposed to within the groups (Vekemans et al., 2002). It indicates the reduction in heterozygosity that has occurred in a group due to random genetic drift. This value will equal zero if all groups are in Hardy-Weinberg equilibrium with the same allele frequencies (Hartl and Clark, 1989). F_{ST} is calculated by:

$$F_{ST} = (H_T - H_S)/H_T$$

A permutation test for genetic differentiation among groups was also performed. The null hypothesis for this test is that there is no genetic differentiation among the groups. F_{ST} values are calculated repeatedly after each random permutation of individuals. The number of permutations to be performed was set to 1000, allowing significance testing at the 1% level. The distribution of the set of values of F_{ST} obtained by permutations is then compared to the observed F_{ST} value for the groups

being tested. If the observed F_{ST} value is higher than the value of F_{ST} lying at the 1% rightmost part of the distribution, the null hypothesis is rejected, and it can be concluded that the groups being tested are more genetically differentiated than random assemblages of the individuals making up those groups (Vekemans et al., 2002).

An estimate of the amount of gene flow between groups (*Nm*) was determined by POPGENE. *Nm* is a measure of the number of migrants per generation and is represented by the formula:

$Nm = (1/G_{ST}-1)/4$

where G_{ST} is the multiple allelic form of F_{ST} (Nei, 1973; Yeh et al., 1997)

Nei's genetic distance (*D*) was calculated in order to construct distance matrices of pairwise genetic distances between groups. The probability that a randomly chosen allele from each of two different groups will be identical, relative to the probability that two randomly chosen alleles from the same group will be identical, is determined. A lack of divergence of two groups will result in a genetic distance between them of zero (Hartl and Clark, 1989). Distance matrices produced during this analysis were used to construct a dendrogram using the PHYLIP KITSCH programme (Felsenstein, 2004), which was subsequently viewed in TREEVIEW (Page, 1996).

Group allocations were performed where individual samples were assigned to a specific group based on an individual's similarity to the individuals making up each group. This procedure gives an indication of the degree of genetic distance between individuals and groups, as well as an indication of gene flow between groups. Low percentages of correct assignment will be seen if high levels of gene flow occur between groups.

Population assignment was performed through the use of the programme AFLPOP (Duchesne and Bernatchez, 2002). This involves the allocation of individuals to

populations based on a minimal log likelihood difference measure. This value was set to 0 due to the close geographic proximity of the groups, as well as a lack of definite boundaries between the areas. With this measure an individual is assigned to a population as soon as the likelihood of an individual belonging to a group is higher than the likelihood of it belonging to any other group (Duchesne and Bernatchez, 2002).

The correlation between distance matrices obtained from genetic and morphological data was evaluated and tested using a Mantel test. This analysis was performed using GenAlEx V5 (Peakall and Smouse, 2001). The correlation *rxy* between morphological and genetic pair-wise distance matrices is determined. A probability (*p*) is determined through random permutation of the distance matrices in order to test the significance of the correlation (Peakall and Smouse, 2001; Szczepaniak et al., 2002). The number of permutations performed was set to 999.

RESULTS

1. SPECIES SELECTION AND DNA EXTRACTION

DNA was extracted from the five species from the three different genera sampled during the initial species selection process. Genomic DNA obtained appeared to be of high quality and was not degraded (Figure 3). The highest DNA yield was obtained for *Asparagus laricinus* and *Indigofera adenoides*. The yield for a second unidentified *Indigofera* species was very low (lane 5). This may be due to the 'stickiness' of the leaves of this species, which could have prevented effective homogenisation of the tissues. This species was therefore excluded from further analysis.

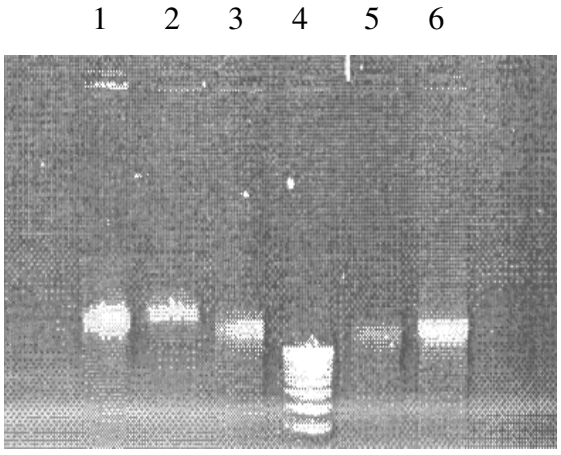


Figure 3: An 0.8% agarose gel showing genomic DNA for selected plant species. The sharpest and most intense band (lane 6) indicates DNA that is both of a high concentration and not degraded. Lane 1: *Asparagus* laricinus, Lane 2: *Lippia* species, Lane 3: *Indigofera* speceis (grey), Lane 4: Molecular weight marker, Lane 5: *Indigofera* species (sticky), Lane 6: *Indigofera adenoides*.

The *Lippia* species was also discarded due to the fact that plants of this species could not be found off site during the initial sampling process. AFLP analysis was therefore performed on *Asparagus laricinus* and the two remaining *Indigofera*

species. All three were found to produce a clear band pattern with a readable number of bands, with *Indigofera adenoides* producing the largest number of bands.

Asparagus laricinus was rejected as a study species due to the fact that seeds of this species are likely to be bird dispersed. This would increase the rate of gene flow between on and off site areas dramatically, thereby reducing the chance of local ecotypes forming in response to slimes-associated contamination. Initial DNA extraction and AFLP analysis therefore resulted in *Indigofera adenoides* being chosen as the study species. This species could not be found during subsequent sampling at the New North Complex slimes dam. *Indigofera zeyheri* was therefore chosen for sampling at this site. *Indigofera* is one of the largest and most widespread genera in South Africa (Allen and Allen, 1981).

2. SAMPLING

2.1. *Indigofera adenoides*

Sampling of *I. adenoides* was performed in late December 2003 at the South Complex slimes dam, Vaal River Mine (Figures 4 and 5).

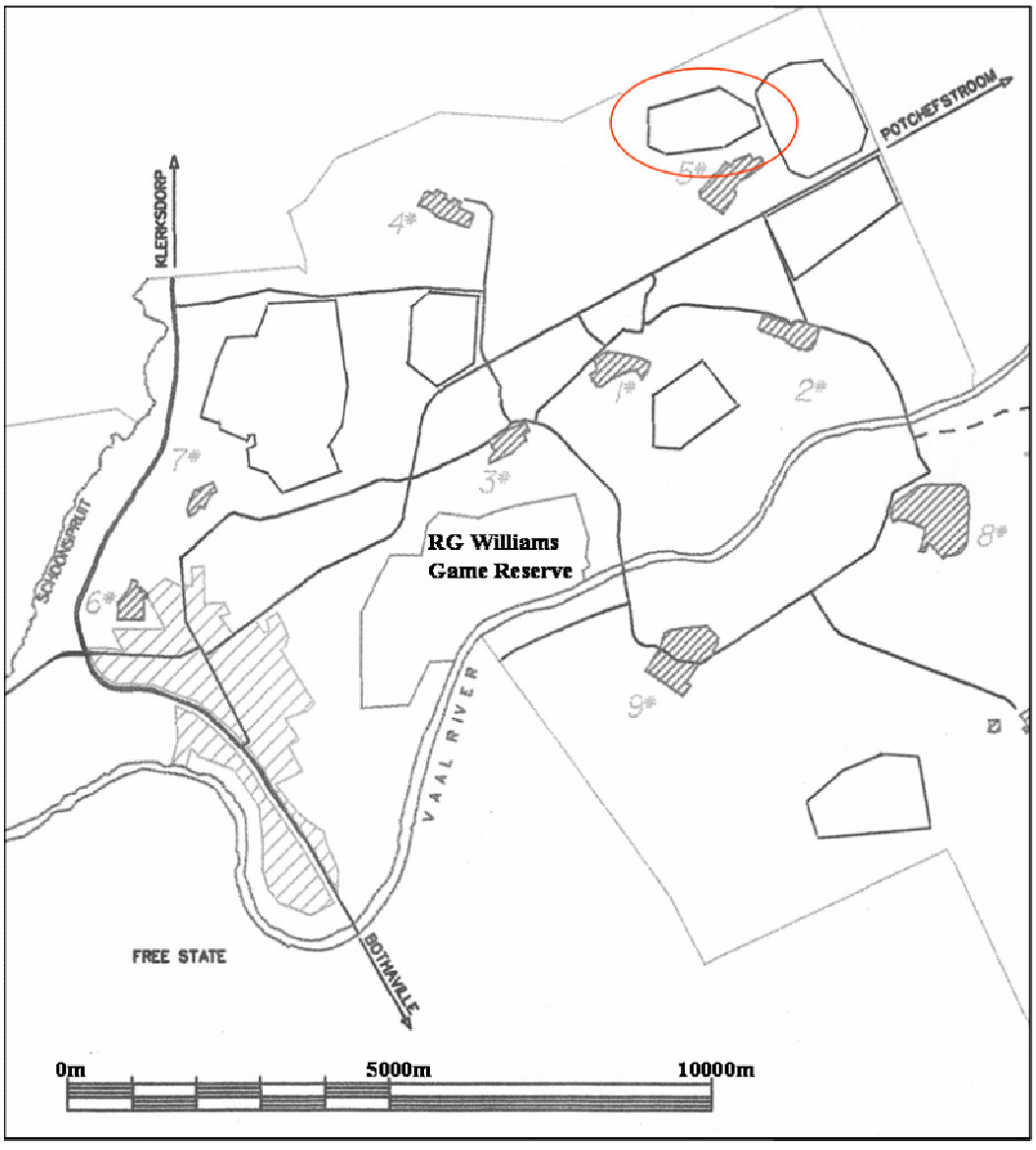


Figure 4: Layout of the areas surrounding the South Complex slimes dam (indicated by a red circle), including the Vaal River, other slimes dams in the area, and the location of the RG Williams Game Reserve. Shaded areas indicate rock dumps and the town of Orkney.



Figure 5: The South Complex slimes dam (top left corner of the photograph) and surrounding areas (a), and the South Complex dam (b), at the Vaal River mine, near Orkney, North West province. The indicated scales are approximate.

Indigofera adenoides is a perennial, woody shrublet with a spreading growth habit. Stems and pods are both covered throughout, but not densely, in glandular hairs. Seed pods are many-seeded, flattened, and cylindrical (Milne-Redhead and Polhill, 1952; van Wyk and Malan, 1998). This species has compound leaves, each consisting of three to six pairs of opposite leaflets. Small, singular flowers are produced, with a pink corolla and petals of 2-4 mm long (Figure 6) (van Wyk and Malan, 1998). The root system is made up of a long tap root with comparatively fine lateral roots. Roots are nodulated, mostly within the first 10 cm of the soil (Allen and Allen, 1981). The species is likely to die back with fire and re-sprout from the root stock.

A total of 144 *I. adenoides* samples were obtained, consisting of 24 from three aspects of the retaining wall and toepaddock wall, 54 from 6 aspects of the toepaddock, 22 from 6 aspects off site, 25 from far off site A, approximately 500-1000 metres away from the slimes dam, and 20 from far off site B (Figures 7 and 8). Each side of the dam is referred to as an aspect. Far off site B is located in the RG Williams Game Reserve, Orkney, approximately eight kilometres away from the studied slimes dam and at least two kilometers away from any slimes dam. Far off site A can therefore be considered to have a low level of slimes-associated contamination, while far off site B can be considered relatively unaffected by slimes-associated contamination.

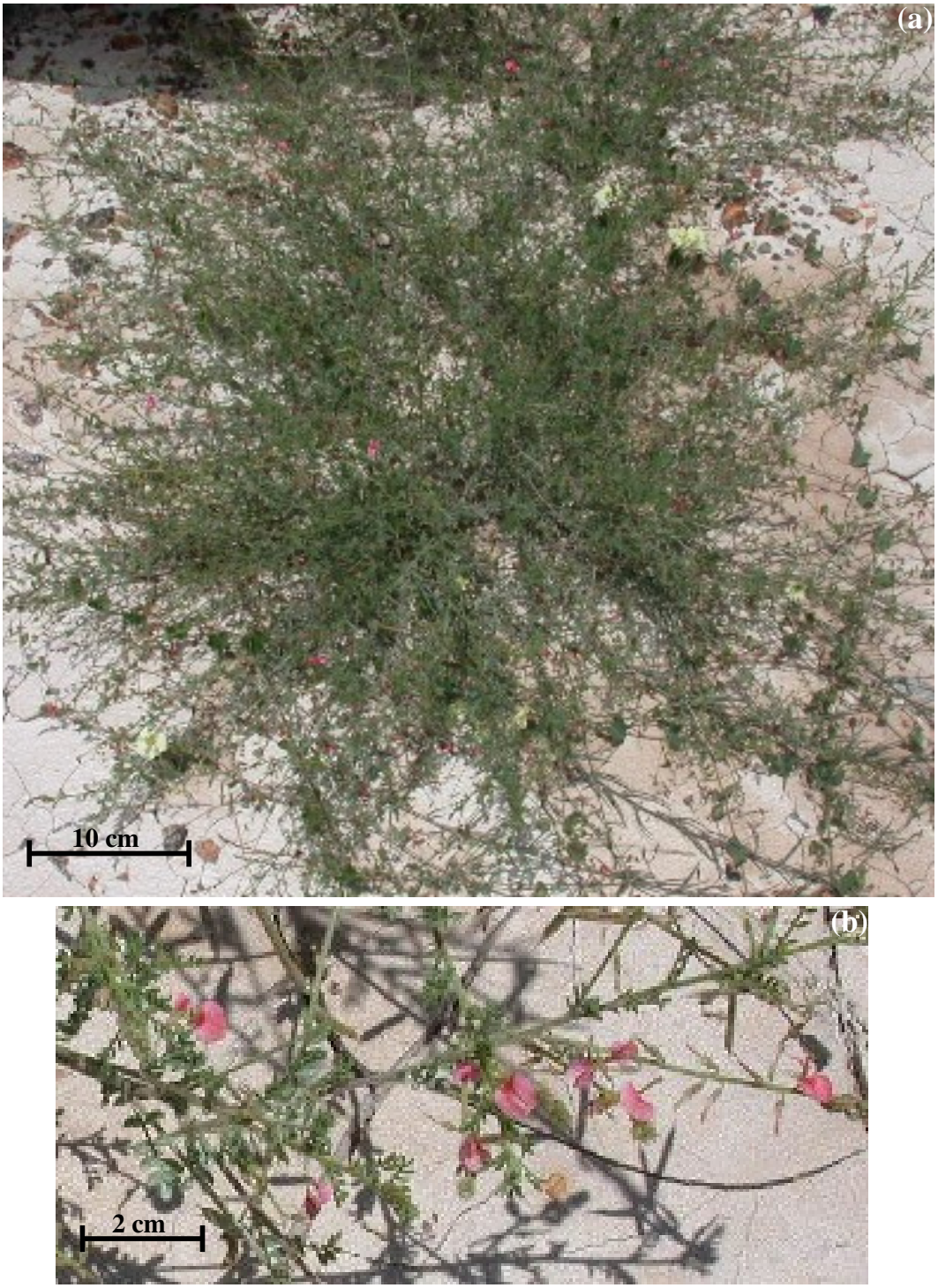


Figure 6: The characteristics of *Indigofera adenoides*, including the overall structure and spreading nature of the plants, which was more pronounced in individuals on the toepaddock (a), and a close up of the leaves and flowers (b). The individual photographed is growing on the toepaddock of the South Complex slimes dam, Vaal River mine, near Orkney, North West province.

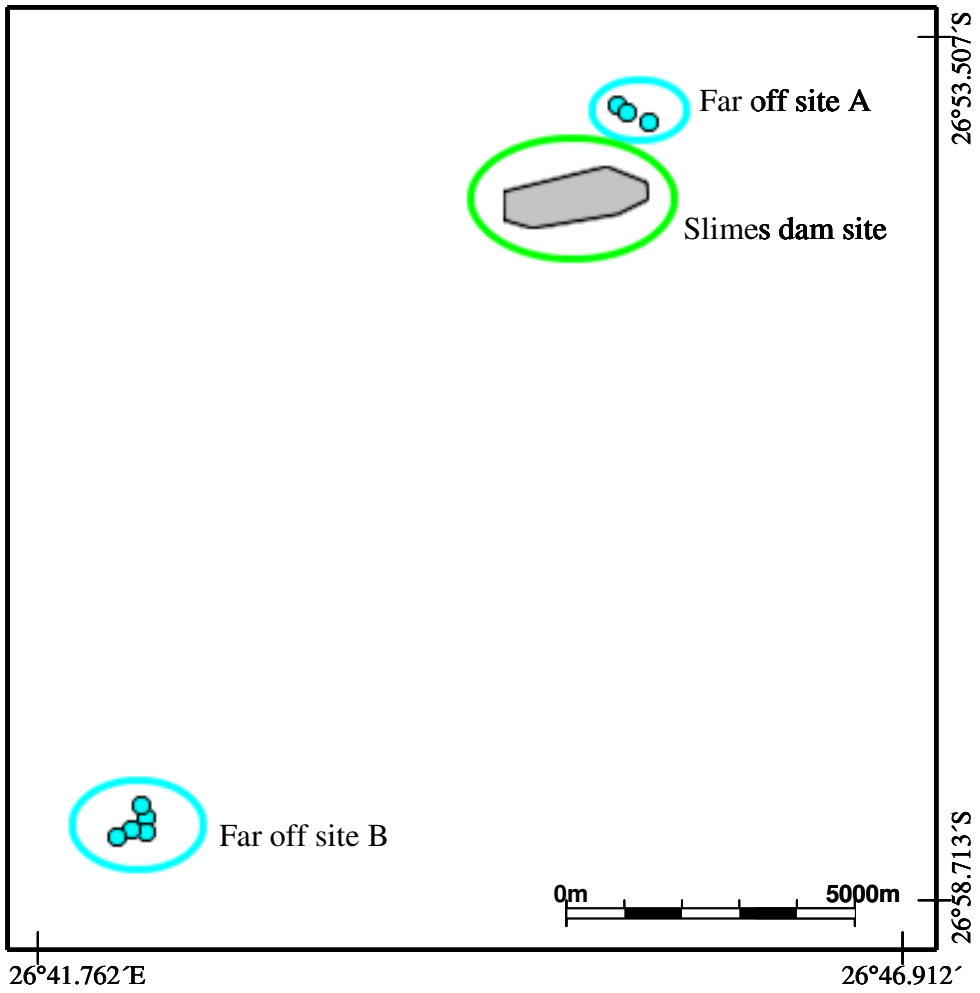


Figure 7: The relative locations of the three *Indigofera adenoides* sampling areas near Orkney, North West province. Note this is a straight plot of GPS coordinates, and is therefore inaccurate with regard to geographic distance due to longitude warping. The indicated scale is approximate. Figure constructed in SBI, a GIS programme written for this study by L. A. du Pisani (2005) and obtained through personal correspondence.

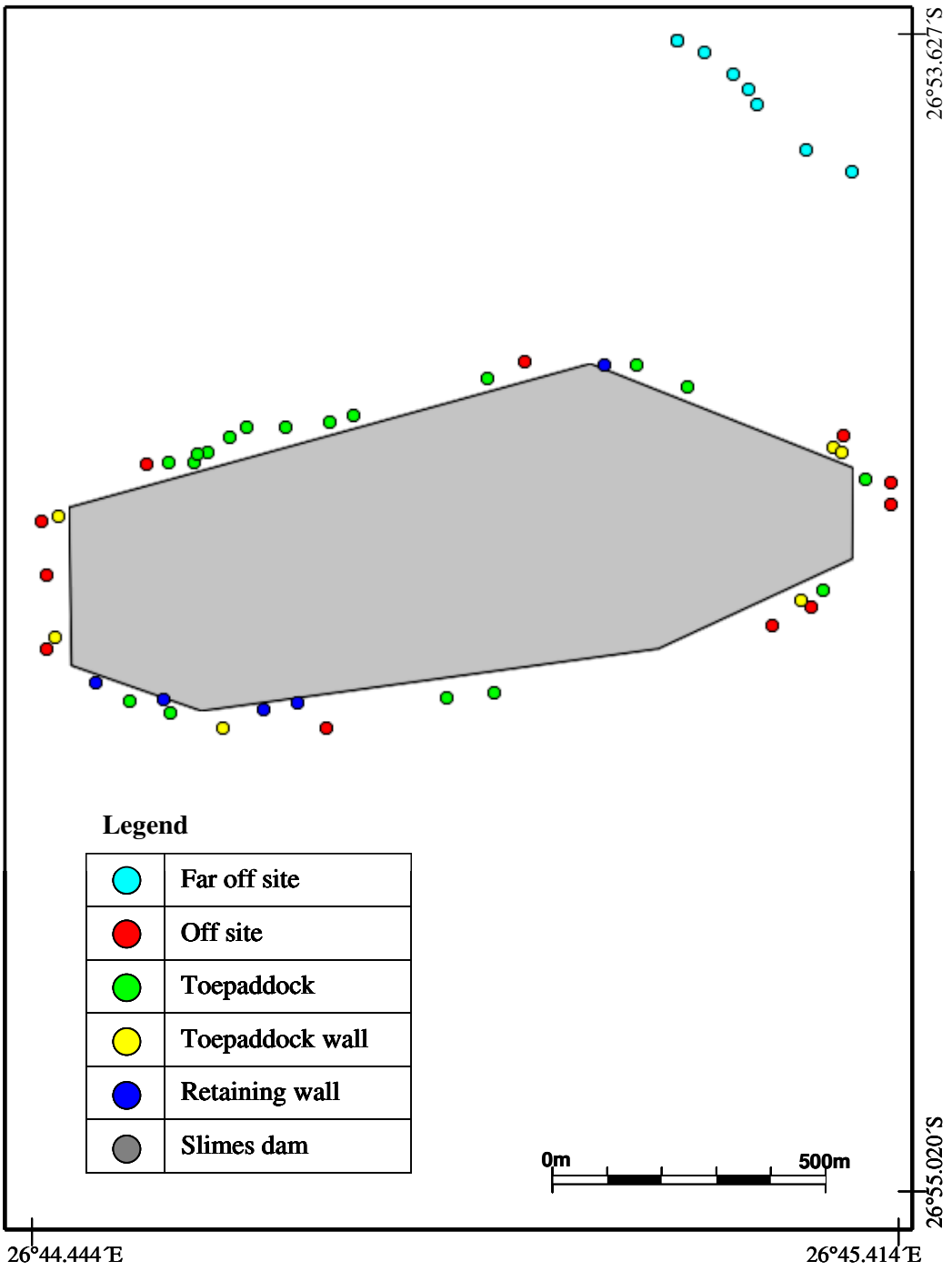


Figure 8: Locations of sampled *Indigofera adenoides* plants on the slimes dam and far off site A areas. Each point refers to approximately three plants, and each point is colour-coded according to the area the plants were sampled from. Note this is a straight plot of GPS coordinates, and is therefore inaccurate with regard to geographic distance due to longitude warping. The indicated scale is approximate. Figure constructed in SBI, a GIS programme written for this study by L. A. du Pisani (2005) and obtained through personal correspondence.

Indigofera adenoides plants obtained from the toepaddock were generally found growing in the uncolonised, slimes-inundated soils, rather than amidst other plants on the slightly higher, less slimes-inundated areas. In contrast, off site samples were found growing amongst other vegetation. The vegetation cover on the toepaddock of the South Complex dam averaged approximately 50% overall, with the slimes inundated areas averaging approximately 30%. The level of overstorey shading was very low due to the general absence of tall shrubs and trees (Figure 9). Differences in flowering time seemed to be evident between the sites. Toepaddock plants had fully formed, mature seed pods and an absence of flowers, while off site plants had mature flowers and a few immature seed pods. Vegetation cover at far off site B, in the RG Williams Game Reserve, was approximately 80%, with little overstorey shading (Figure 10).



Figure 9: Sampling of *Indigofera adenoides* occurring on the toepaddock of the South Complex slimes dam, Vaal river mine, near Orkney, North West Province. The spreading growth habit of this species can be seen, as well as its preference for growing in relatively uncolonised, slimes-inundated areas of the toepaddock.



Figure 10: The topography and vegetation cover of the area of the RG. Williams Game Reserve, Orkney, North West province, used for sampling of the far off site B group.

2.2. *Indigofera zeyheri*

Sampling of *I. zeyheri* was performed in January 2004 at the New North Complex slimes dam, West Wits mine, near Carletonville (Figure 11). *Indigofera zeyheri* is taller and has a sparser crown than *I. adenoides*. It is a slender, sparsely branched perennial herb that appears greyer in colour than *I. adenoides*. The species has an erect growth habit and is covered throughout, but not densely, in hairs (Milne-Redhead and Polhill, 1952). Leaves are compound, consisting of five to seven pairs of opposite leaflets. Flowers are found on inflorescences at the end of long axes, and are small and pinkish to white in colour (Figure 12) (van Wyk and Malan, 1998). The seed pods of this species are flattened, cylindrical, and many-seeded (Milne-Redhead and Polhill, 1952). The root system is made up of a long tap root with comparatively fine laterals. Roots are nodulated, mostly within the first 10 cm of the soil (Allen and Allen, 1981). It is likely that the species dies back during fire, subsequently reprouting from the root stock.

A total of 98 samples were obtained for this species, 63 from 4 aspects of the toepaddock and 35 from 5 aspects off site (Figure 13). Retaining wall samples could not be obtained as the retaining wall had been completely inundated with slimes. Despite a considerable effort to find suitable populations, the species could not be found far enough away from the dam to allow sampling of a far off site population. As was noted with *I. adenoides*, flowering time differences seemed to be apparent between the sites. Toepaddock plants had fully formed, mature seed pods and an absence of flowers, while off site plants had mature flowers and a few immature seed pods.

Vegetation on the New North Complex slimes dam is much denser than vegetation on the South Complex dam (approximately 70% aerial cover on the toepaddock and 90% off site). This could be due to the climate in the area, which appears to be cooler and wetter than that at the South Complex dam. *Indigofera zeyheri* samples were not found growing on slimes inundated soils, again in contrast to *I. adenoides*. Toepaddock plants were found growing only on the highly colonised, relatively slimes free, higher areas formed by low banks of grassland soil.

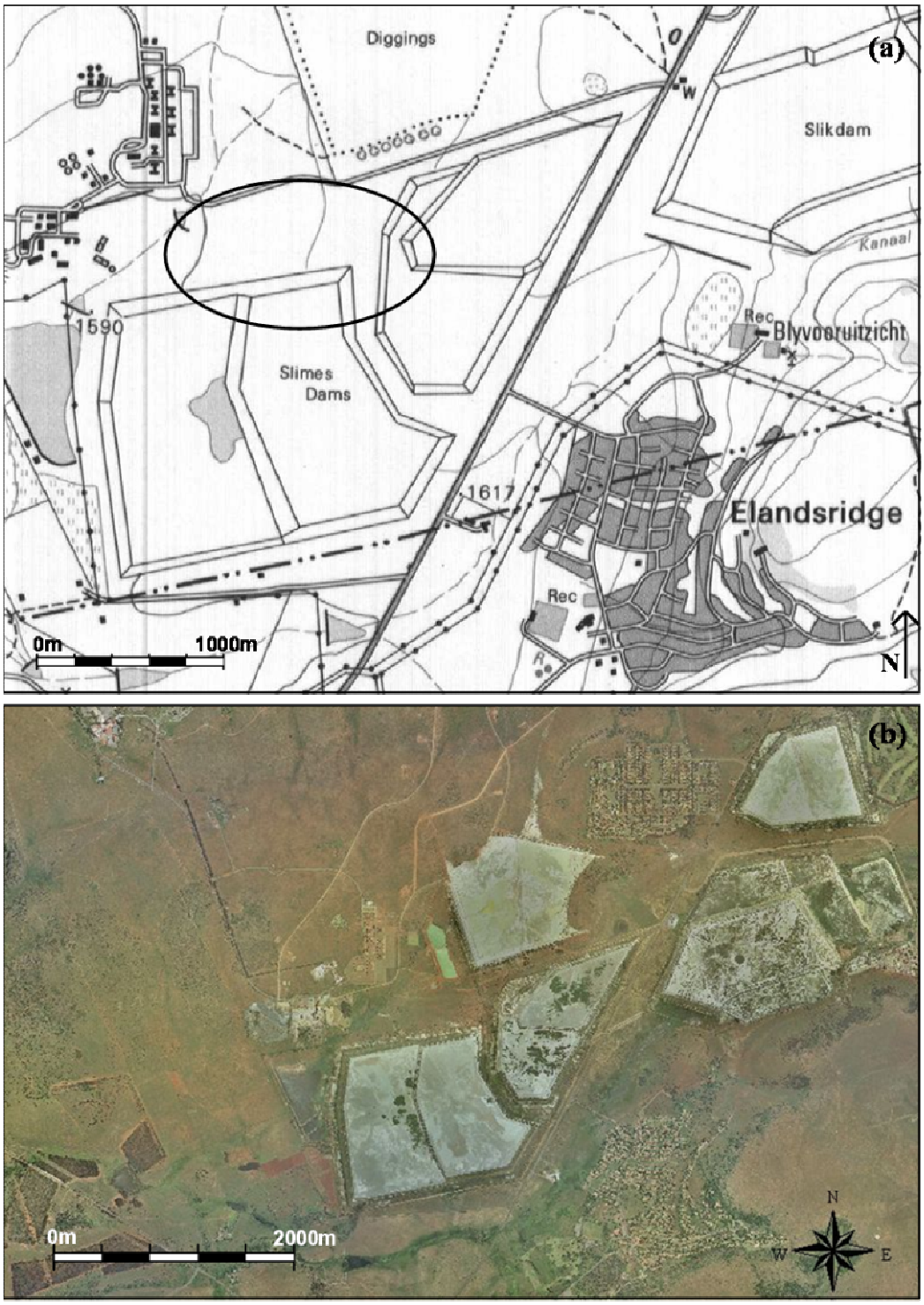


Figure 11: A comparison of a map (a) and an aerial photograph (b) showing the New North Complex slimes dam and surrounding areas, at the West Wits mine, near Carletonville. The circled area in figure (a) indicates the area where sampling of *Indigofera zeyheri* plants occurred. Indicated scale bars are approximate.



Figure 12: The general morphological structure of *Indigofera zeyheri*. The individual shown was growing on the toepaddock at the New North Complex slimes dam, West Wits mine, near Carletonville.

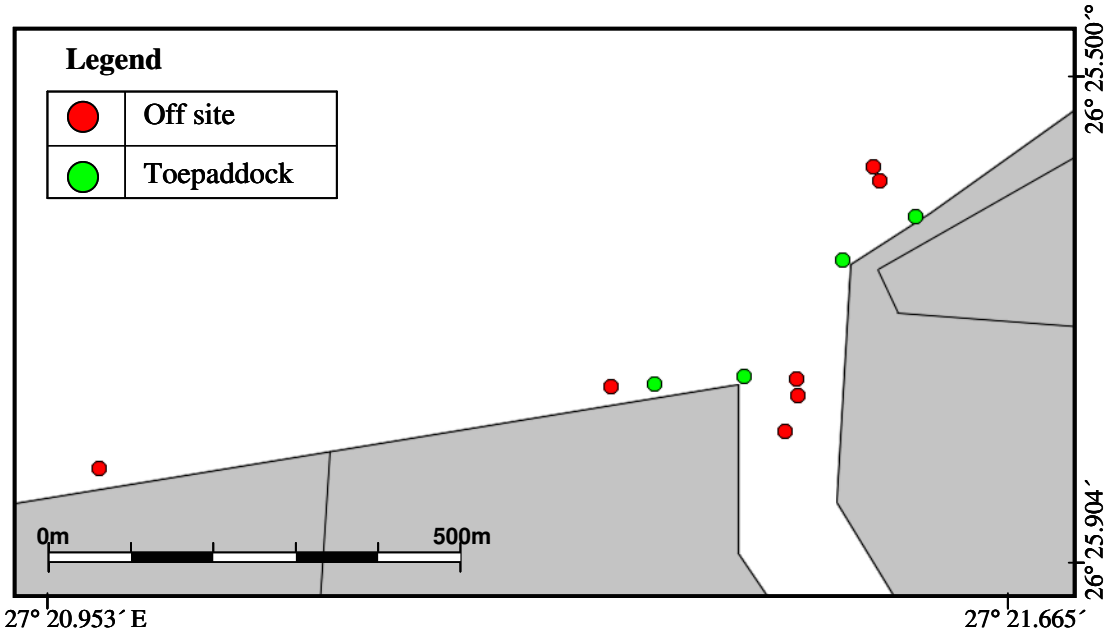


Figure 13: Locations of sampled *Indigofera zeyheri* plants in the toepaddock and off site areas. Each point indicates three to ten plants, and each point is colour-coded according to the area the plants were sampled from. Note this is a straight plot of GPS coordinates, and is therefore inaccurate with regard to geographic distance due to longitude warping. The indicated scale is approximate. Figure constructed in SBI, a GIS programme written for this study by L. A. du Pisani (2005) and obtained through personal correspondence.

3. SEED GERMINATION

The percentage of seeds that germinated was approximately 50%, which is consistent with that obtained in previous studies (Weiersbye and Witkowski, 2002). Unfortunately the majority (70%) of seedlings died soon after germination due to fungal contamination, even after treatment with fungicide. DNA was therefore extracted from the remaining seedlings as soon as roots were long enough to provide sufficient tissue (approximately 1 cm). This was successful, and AFLP analysis was performed. The number of samples available was however not sufficient to allow accurate inference of breeding system from AFLP band patterns. All that could be inferred was that both *I. adenoides* and *I. zeyheri* are not obligate self-fertilisers.

4. AFLP BAND PATTERNS

4.1. *Indigofera adenoides*

A total of 44 bands were obtained for *I. adenoides* samples, varying in size from 75bp to approximately 1500bp (Figure 14). The mean number of bands per individual was 30.3, and the total number of polymorphic bands was 40 (90.9% of the total number of bands obtained). All bands were found to be reproducible. Three bands that were found at a low frequency in the far off site groups were not found at all in off site or toepaddock groups. These were fragments 2, 36, and 42.

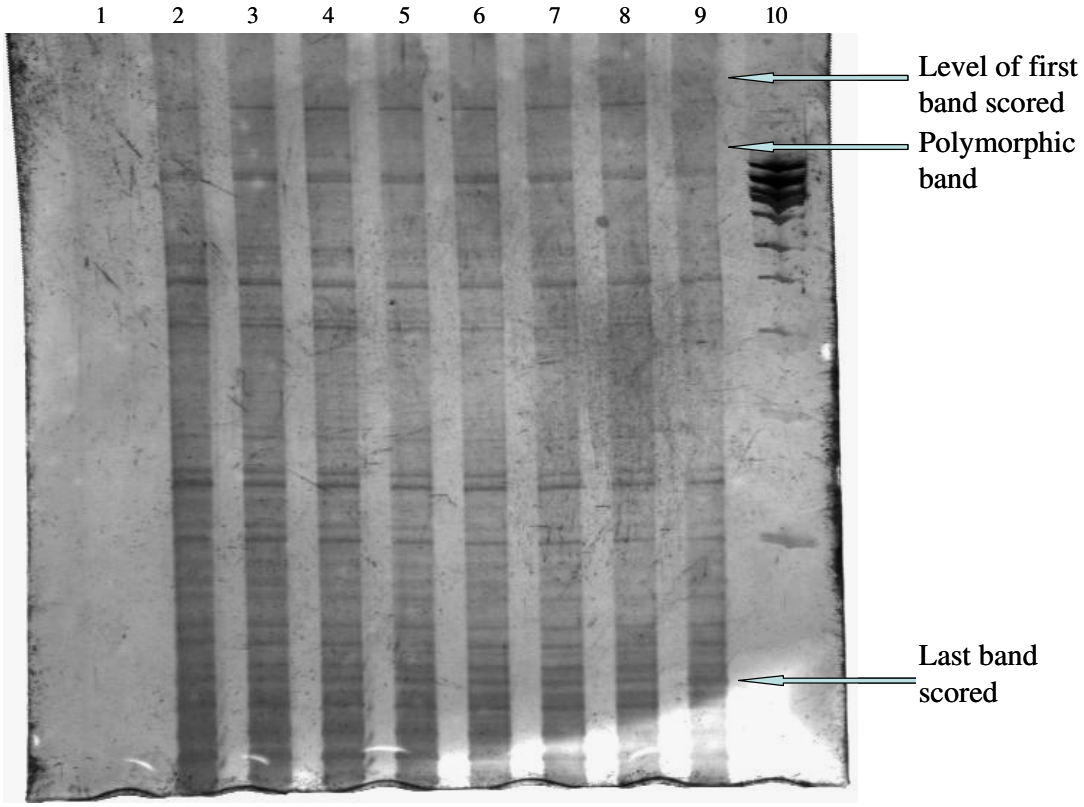


Figure 14: Silver stained gel showing the typical band pattern obtained with *Indigofera adenoides* samples. Levels of the first and last bands scored are shown, as well as an example of a polymorphic band. Samples are present in lanes 2-9, lane 10 contains a molecular weight marker. The largest molecular marker band is 1031 base pairs, while the smallest one visible on the gel is 100 base pairs.

4.2. *Indigofera zeyheri*

The total number of fragments obtained with *I. zeyheri* was 34, varying in size from approximately 40bp to approximately 1600bp while the mean number of fragments per individual was 19.1 (Figure 15). The total number of polymorphic fragments was 30 (88.2% of the total number of fragments). Three fragments were found at a low frequency in the off site group that were not found at all in the toepadddock group. These were fragments 1, 7, and 19.

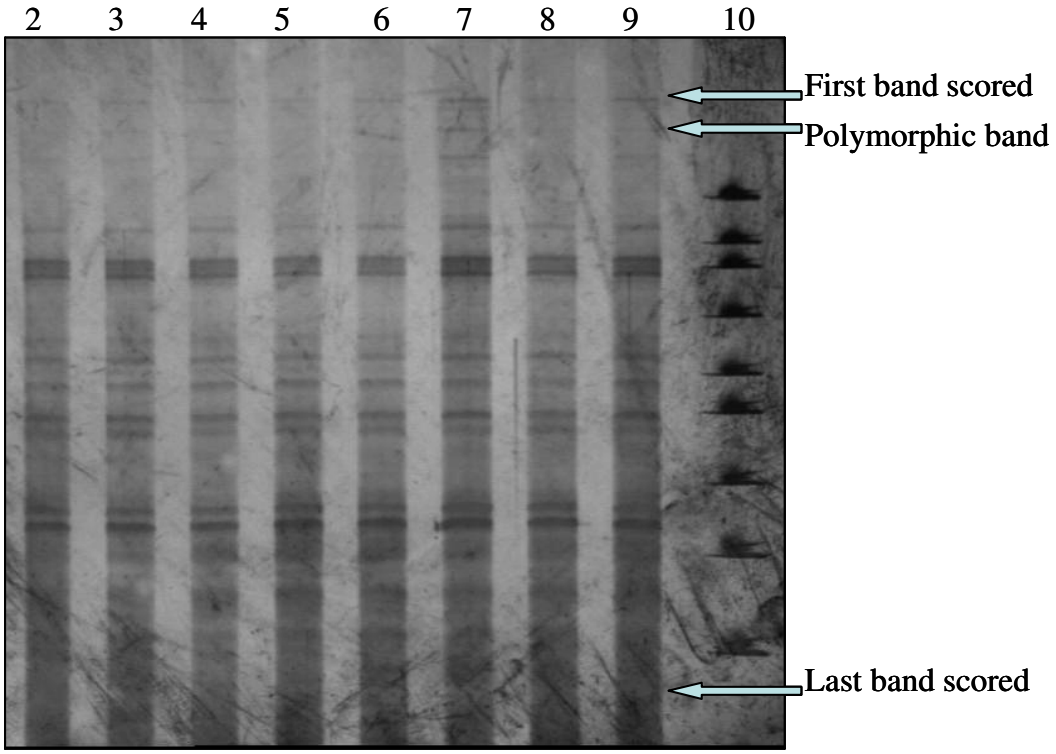


Figure 15: Silver stained gel showing the typical band pattern obtained with *Indigofera zeyheri* samples. Levels of the first and last bands scored are shown, as well as an example of a polymorphic band. Samples are present in lanes 2-9, lane 10 contains a molecular weight marker. The largest molecular marker band is 1031 base pairs, while the smallest one visible on the gel is 50 base pairs.

5. ANALYSIS OF *INDIGOFERA ADENOIDES* ACCORDING TO AREA

Initial analysis of *Indigofera adenoides* data was performed by separating individuals into groups according to the six main areas of the slimes dam and surrounding areas, namely toepaddock, toepaddock wall, retaining wall, off site, far off site A, and far off site B. This was done to determine if genetic and morphological differences could be detected between the plants that have naturally colonised these different areas. Any differences detected would presumably be due to differences in toxicity between the areas and/or differences in local growth conditions, for example increased water availability in the toepaddock due to seepage from the moist slimes continually being added to the slimes dam.

5.1. Morphological analysis

Indigofera adenoides samples were divided into five groups for morphological analysis. Retaining wall and toepaddock wall individuals were grouped together as plants from these groups appeared morphologically similar (average values for plant height and leaf width differed by 1.3 cm and 0.04 cm respectively). These areas also have similar substrate conditions, both consisting of walls of grassland soil that have been placed in the toepaddock. The groups analysed were therefore toepaddock, retaining/toepaddock wall, off site, far off site A, and far off site B. Analysis of variance (ANOVA) showed plants from the five areas to be significantly different from each other, with most of the variables contributing significantly to differentiation between the areas (Table 5).

Table 5: Analysis of variance (ANOVA) performed for all variables (DF=4, error DF = 141). This was done to determine which variables show significant differences between area groups.

Variable	F value	Pr > F
Plant height	10.47	<0.0001
Canopy width	6.40	<0.0001
Canopy width 90°	2.02	0.0948
Leaf length	26.28	<0.0001
Leaf width	71.43	<0.0001

Tukey analysis emphasised the differences in plant height and leaf width that were shown to be significant with ANOVA (Table 6). Differences in these variables lead to differences in leaf area, canopy area, and canopy volume, due to the presence of plant height and leaf width in the formulas used for calculating these composite variables. Differences in other variables were seen, but only between far off site B and the other areas, showing the extent to which plants in this area are morphologically differentiated from those in the other areas.

Table 6: Canopy and leaf dimensions (mean ± standard deviation) for plants in the various area groups. Superscripts with the same letter within a row indicate values that are not significantly different (P<0.05%; Tukey).

Factor	Retaining wall, toepaddock wall	Toepaddock	Off site	Far off A	Far off B
Plant height (cm)	47.2±12.4^{a}	33.7±11.3^{b}	39.7±12.7^{a}	28.3±6.7^{b}	27.4±7.6^{b}
Canopy diameter (cm)	50.1±18.6^{a}	51.2±24.6^{a}	49.3±21.3^{a}	38.0±14.8^{ab}	27.9±8.2^{b}
Canopy diameter90° (cm)	38.4±18.9^{a}	37.8±19.5^{a}	39.1±17.7^{a}	28±12.6^{a}	18.5±5.8^{a}
Leaf length (cm)	1.5±0.3^{a}	1.3±0.3^{a}	1.7±0.4^{a}	1.6±0.4^{a}	1.3±0.2^{a}
Leaf width (cm)	0.5±0.1^{b}	0.4±0.1^{b}	0.6±0.2^{a}	0.6±0.1^{a}	0.6±0.1^{a}
Leaf area (cm^2)	0.6±0.3^{ac}	0.5±0.2^{c}	0.8±0.5^{a}	0.8±0.4^{ac}	0.6±0.2^{b}
Leaf shape (cm)	3.0±0.7^{ab}	3.0±0.7^{a}	3.3±1.0^{ab}	2.8±0.6^{b}	2.4±0.5^{ab}
Canopy area (cm^2)	1697± 1341^{a}	1830± 1637^{a}	1629± 1500^{a}	963± 770^{ab}	429± 212^{b}
Canopy volume (cm^3)	82987± 68799^{a}	65588± 66369^{ab}	63135± 54542^{a}	30254± 28487^{bc}	12363± 8457^{c}
Canopy volume 2 (cm^3)	55324± 45866^{a}	43725± 44246^{ab}	42090± 36361^{a}	20170± 18991^{bc}	8241± 5638^{c}

Following the results of this analysis, graphs were constructed showing the distribution of the variables shown to be significantly different between the areas, i.e., leaf width and plant height. Graphs of mean values for canopy area and canopy volume were also constructed. Leaf width is, on average, larger in plants from the areas less affected by slimes (off site and far off site areas). Leaf width is the most diverse in individuals from the off site areas (Figure 16). Plants from far off site areas are not only shorter on average, they also show less variation in height than individuals in areas affected by slimes (Figure 17). Mean canopy area and canopy volume are highly variable throughout all areas (large standard deviations). Both variables are significantly lower in the far off site populations (Figure 18).

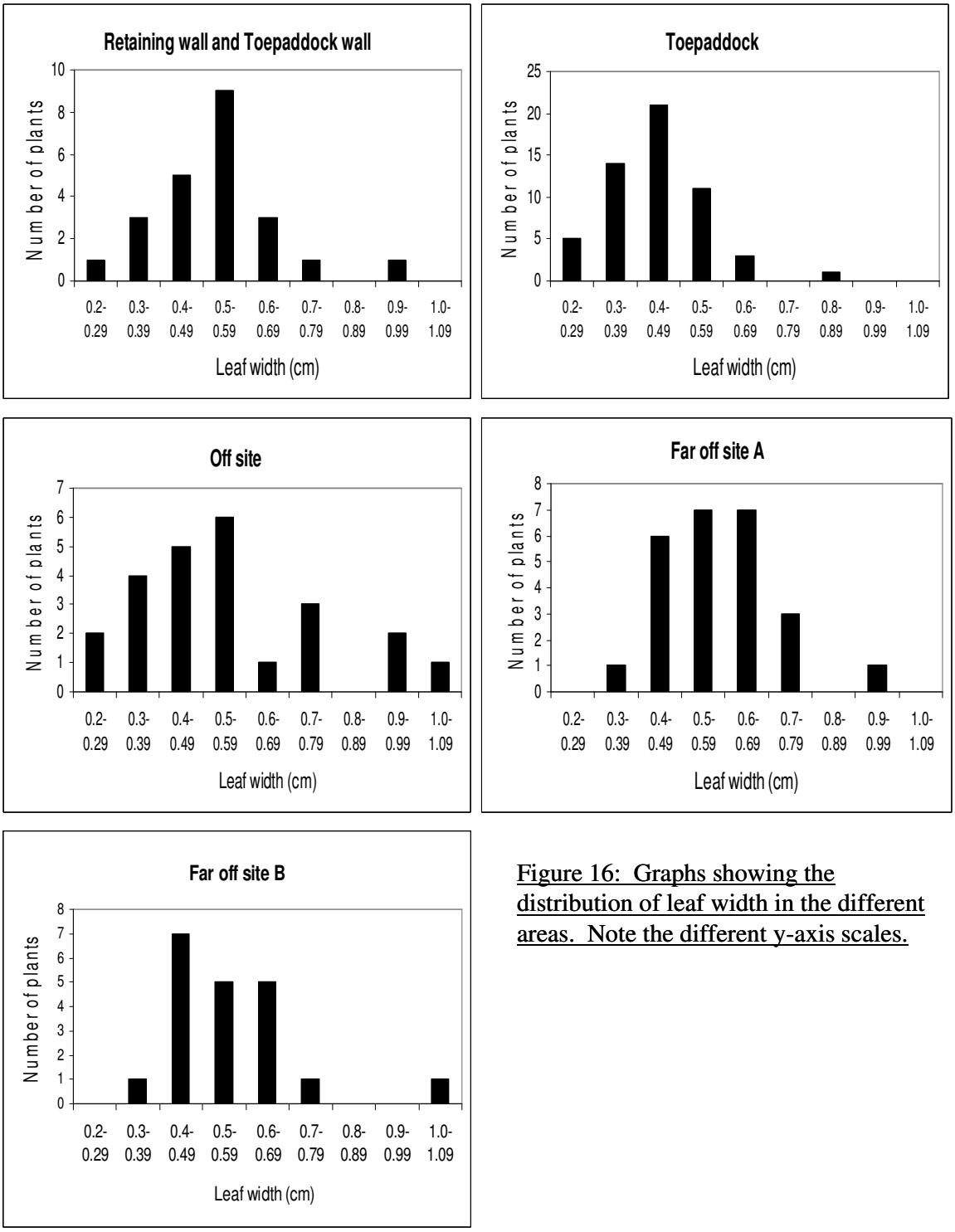


Figure 16: Graphs showing the distribution of leaf width in the different areas. Note the different y-axis scales.

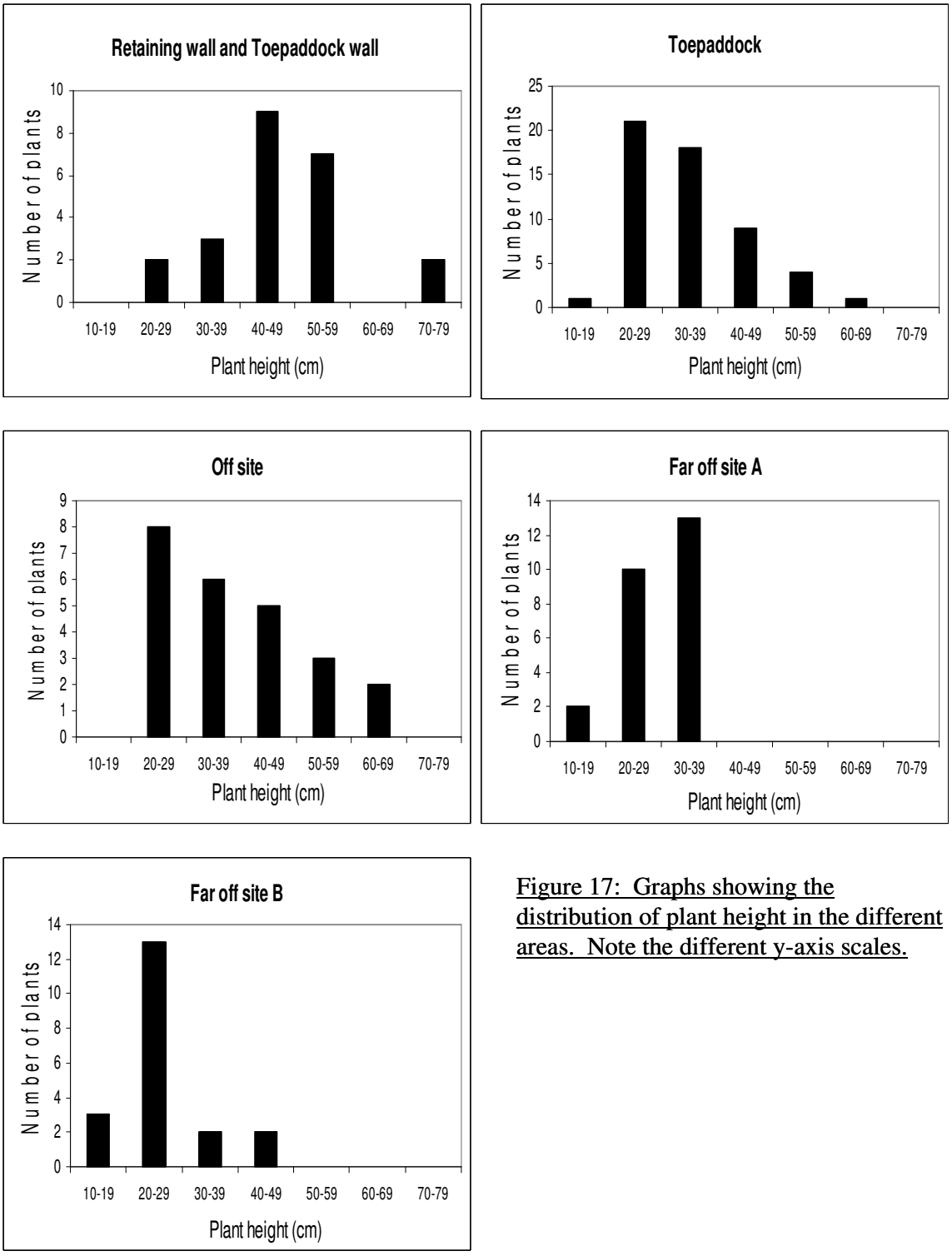


Figure 17: Graphs showing the distribution of plant height in the different areas. Note the different y-axis scales.

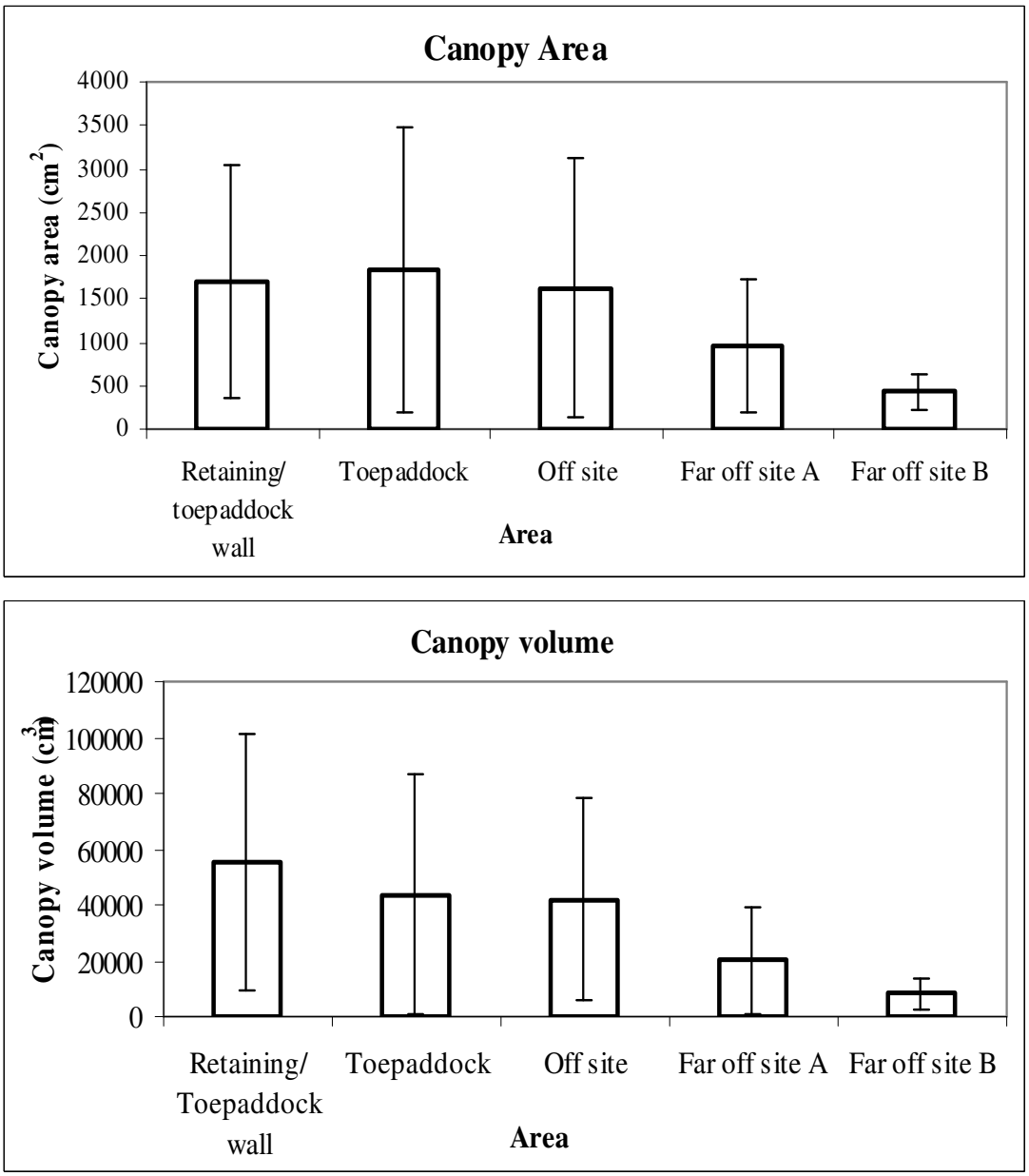


Figure 18: A comparison of canopy area (mean ± standard deviation) and canopy volume (mean ± standard deviation) in plants from the different areas.

The most notable differences in correlation coefficients were found in far off site B canopy area and leaf area, and canopy volume and leaf area correlations. These differences are due to the significantly lower canopy volume and canopy area values in plants from this group, when correlated with a relatively constant leaf area measurement (Table 7).

Table 7: A comparison of correlation coefficients between various variables in plants from the different areas. The asterix (*) indicates a p value of less than 0.0001, showing the two variables to be highly significantly correlated.

Correlation between	Overall	Retaining and toepaddock wall	Toepaddock	Off site	Far off site A	Far off site B
Height and leaf length	0.340*	0.285	0.165	0.146	0.648	-0.211
Height and leaf width	0.272	0.0667	0.161	0.134	0.185	0.200
Height and canopy area	0.374*	0.319	0.260	0.0695	0.645	0.231
Height and leaf area	0.306	0.234	0.150	0.0999	0.561	0.110
Leaf length and leaf width	0.597*	0.226	0.230	0.543	0.115	0.204
Canopy area and leaf area	0.744*	0.798*	0.774*	0.773*	0.705*	0.236
Canopy volume and leaf area	0.722*	0.759*	0.722*	0.738*	0.718*	0.242

Discriminant analysis show toepaddock and far off site B plants to classify most consistently to the correct area group, while plants from the other groups show only a low percentage of correct classifications (Table 8). This indicates that plants in these two area groups are morphologically distinct from plants in all other areas groups.

Table 8: Classification of plants from each area group into the different areas, expressed as a percentage of the total number of individuals in each area group, obtained through discriminant analysis. The total number of plants that classifies to each area group is shown, expressed as a percentage of the total number of plants sampled.

		Area group classified to				
Area group of origin	Sample size	R/TW (%)	TP (%)	OS (%)	FOSA (%)	FOSB (%)
Retaining wall and toepaddock wall (R/TW)	24	40.91	45.45	13.64	0.00	0.00
Toepaddock (TP)	54	7.14	78.57	8.93	5.36	0.00
Off site (OS)	24	8.70	39.13	43.48	8.70	0.00
Far off site A (FOSA)	25	0.00	40.00	20.00	40.00	0.00
Far off site B (FOSB)	19	0.00	20.00	0.00	0.00	80.00
Total		10.27	52.74	15.75	10.27	10.96

Canonical discriminant analysis was used to produce a distance matrix of pair-wise squared distances between the various area groups. The dendrogram constructed from that data shows plants from far off site B to be highly morphologically distant from plants in the other groups. Plants in toepaddock/retaining wall and off site groups are the most morphologically similar to each other (Figure 19).

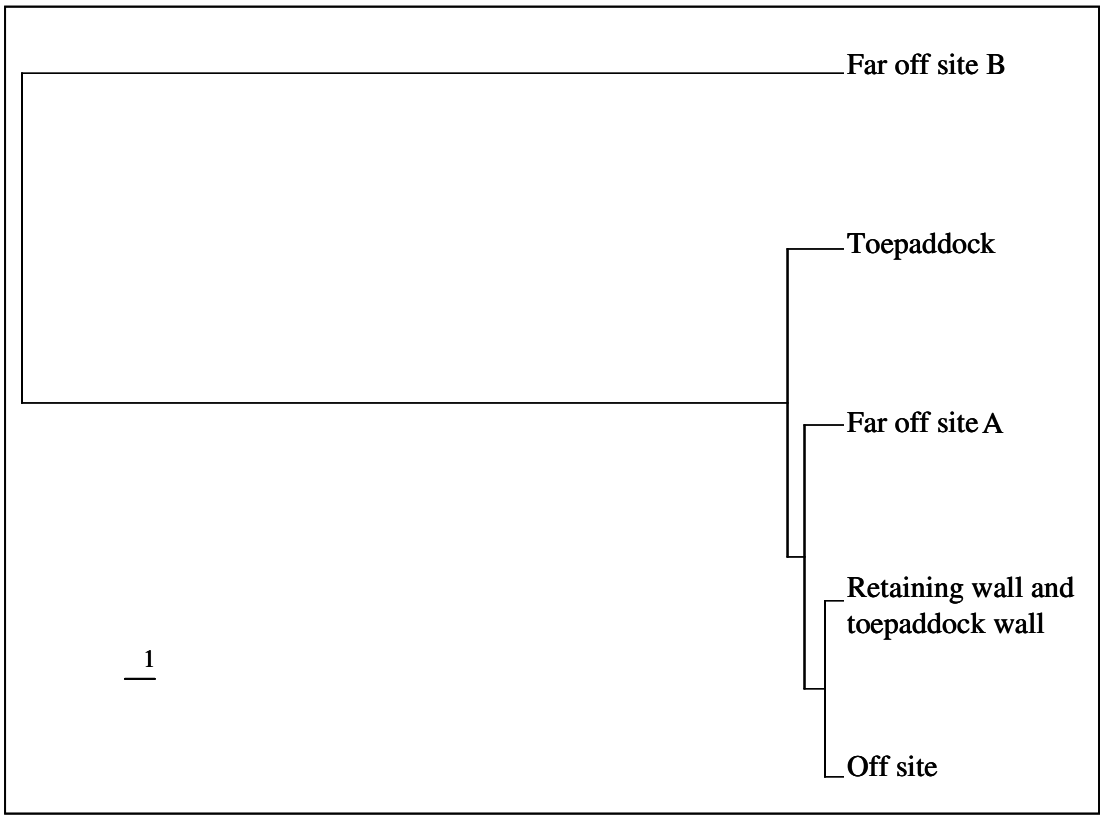


Figure 19: Dendrogram based on pair-wise squared distances of morphological data, obtained through canonical discriminant analysis. The scale bar indicates 1 distance unit.

The diversity of each variable within groups of plants from the different areas was determined by calculating coefficients of variation (Table 9), in order to allow the comparison of morphological variation and genetic variation. Toepaddock and far off site A area groups contain the most variance between individuals. Leaf length, leaf width, and leaf shape show the largest differences in correlation coefficients between area groups.

Table 9: A comparison of coefficients of variation for each variable between groups of plants from the different areas. The highest coefficient of variation for each variable is shown in bold.

Variable	Retaining/ toepaddock wall	Toepaddock	Off site	Far off site A	Far off site B
Sample size	24	54	24	25	19
Height	7.33	**11.79**	8.54	8.16	7.93
Canopy width	37.69	**47.47**	43.17	38.83	29.28
Canopy width 90	48.95	**51.47**	45.29	45.07	31.30
Leaf length	32.90	39.30	28.68	**41.62**	60.80
Leaf width	61.03	**106.08**	50.13	63.29	37.54
Canopy area	11.37	16.09	10.93	**16.22**	11.35
Canopy volume1	8.56	12.39	7.98	**12.70**	8.41
Canopy volume2	8.89	12.89	8.29	**13.24**	8.80
Leaf area	51.58	56.61	**57.26**	51.61	29.09
Leaf shape	45.63	47.86	37.36	**75.30**	28.91

5.2. Genetic analysis

5.2.1. Initial grouping

Initial organisation of groups for genetic analysis involved grouping retaining wall and toepaddock wall plants together. Far off site A and B were also grouped together. This was done to provide a broad overview of genetic structure between different substrate types. Genetic diversity is lower in plants from areas that have a higher level of slimes contamination, i.e., toepaddock area groups (toepaddock, retaining wall, and toepaddock wall). Plants from these areas also have a lower percentage of loci polymorphic (Table 10). The majority of total population variation is due to diversity within groups, indicating a low level of genetic differentiation between groups. Estimated gene flow levels are high (Table 11).

Table 10: Genetic structure of plants from the different areas in terms of sample size, percentage of loci polymorphic, Shannon's information index (*I*) (mean ± standard deviation), and Nei's (1973) gene diversity (*h*) (mean ± standard deviation).

Area	Sample size	Percentage of loci polymorphic	*I*	*h*
Retaining wall/ toepaddock wall	24	61.34	0.335 ± 0.293	0.226 ± 0.206
Toepaddock	54	65.9	0.383 ± 0.294	0.261 ± 0.207
Off site	24	75.0	0.443 ± 0.276	0.304 ± 0.197
Far off site	44	84.1	0.446 ± 0.244	0.300 ± 0.175

Table 11: Nei's gene diversity statistics (mean ± standard deviation) for subdivided populations, showing the genetic structure of the overall population. An estimate of gene flow (*Nm*) is also shown.

Statistic	Total genetic diversity (H_T)	Diversity within groups (H_S)	Gene flow between groups (*Nm*)
Value	0.288 ± 0.031	0.273 ± 0.030	8.975

A permutation test for genetic differentiation among area groups was performed for all groups. The F_{ST} value is 0.0211 ± 0.408 (mean ± standard error). Permutation testing on this F_{ST} value showed the null hypothesis to be rejected at the 1% level, indicating that the area groups are more genetically diverse than a random assemblage of the individuals. Permutation testing of F_{ST} values between each pair of area groups emphasises the diverse nature of the plants in the far off site groups, with these groups being significantly differentiated from each of the other groups (Table 12).

Table 12: Permutation test for genetic differentiation among groups of plants from different areas, based on pairwise F_{ST} values between each pair of groups. Numbers in italics indicate rejection of the null hypothesis at the 5% level, while numbers in bold type indicate rejection of the null hypothesis at the 1% level. If the null hypothesis is rejected, it indicates the two populations are more genetically differentiated than random assemblages of the individuals at the significance level indicated.

	Retaining wall/ toepaddock wall	Toepaddock	Off site
Toepaddock	0.005	/	
Off site	*0.016*	0.013	/
Far off site	**0.029**	**0.024**	**0.037**

Nei's genetic distances (*D*) between plants from the area groups were calculated and used to construct a dendrogram. Plants in the far off site groups are genetically distant from those in the other groups, while plants in the toepaddock and toepaddock/ retaining wall groups are the most genetically similar to each other (Figure 20).

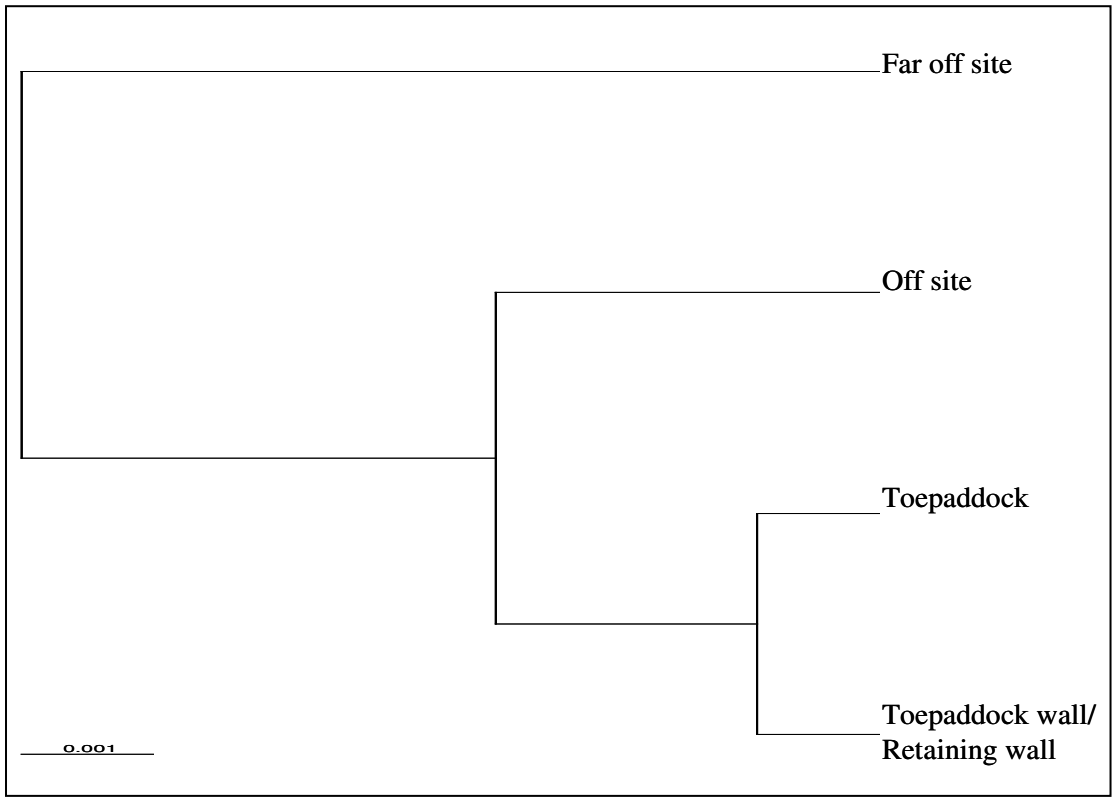


Figure 20: Dendrogram based on pair-wise Nei's genetic distances (*D*) between plant groups from different areas. The scale bar indicates 0.001 genetic distance units.

Plants were assigned to area groups based on genetic similarity (Table 13). Accurate classification is higher in the off site and far off site groups than in the on site groups. Probability (*P*) values are however lower in the off site groups, indicating that classification is less certain in those areas. Individuals were assigned as soon as the likelihood of being assigned to one group was higher than that for any other group, as assignment only when likelihood values were 10 times higher

produced a high percentage (75%) of nil assignments. This indicates the groups are quite similar, as would be expected considering their close geographic proximity.

Table 13: Percentage of plants from each area group that assign to the different groups, based on AFLPOP analysis. Values shown indicate the percentage of individuals from each area group that are shown to assign to each of the groups. Individuals are assigned to groups as soon as the likelihood of belonging to that group is higher than the likelihood of belonging to any other group. Probability (*P*) values are shown for each group indicating the likelihood that assignment was correct.

	Area of origin			
Area group assigned to (%)	R/TW	TP	OS	FOS
Sample size	24	54	24	44
Retaining/ Toepaddock Wall (R/TW)	66.7	20.4	12.5	4.6
Toepaddock (TP)	8.3	61.1	4.2	11.4
Off site (OS)	4.2	13	75	9.1
Far off site (FOS)	20.9	5.6	8.3	75
Average *P* value	0.70	0.61	0.55	0.52

5.2.2. Subgroups

Following initial genetic analysis, groups of plants were subdivided further in order to provide information on genetic differences between plants due to finer scale differences in growth conditions. Toepaddock wall plants were separated from retaining wall plants and the far off site plant group was separated into far off site A for the plants sampled several hundred metres from the dam, and far off site B for the plants sampled from the site several kilometres away from the dam.

As was seen in the initial area groups, genetic diversity is lower in the toepaddock, toepaddock wall, and retaining wall groups than in off site and far off site groups. Lowest values are seen in the retaining wall group. Interestingly, the two far off site groups show different diversities, with far off site A showing a higher diversity than far off site B (Table 14).

Table 14: Genetic structure of plants from the different areas in terms of sample size, percentage of loci polymorphic, Shannon's information index (*I*) (mean ± standard deviation), and Nei's (1973) gene diversity (*h*) (mean ± standard deviation). Note toepaddock and offsite group values are the same as in Table 10, and are reproduced here for comparison purposes.

Population	Sample size	Percentage of loci polymorphic	*h*	*I*
Retaining wall	9	47.7	0.178 ± 0.205	0.264 ± 0.295
Toepaddock wall	15	54.6	0.215 ± 0.215	0.315 ± 0.307
Toepaddock	54	65.9	0.264 ± 0.207	0.385 ± 0.295
Off site	24	75.0	0.304 ± 0.197	0.443 ± 0.276
Far off site A	25	77.3	0.251 ± 0.175	0.383 ± 0.251
Far off site B	19	72.7	0.304 ± 0.203	0.440 ± 0.285

The majority of total variation of the entire population is due to diversity between plants within the areas, indicating a low genetic differentiation between plants from different areas. Estimated gene flow is of a moderate level, lower than that estimated for the initial area groups (Table 15).

Table 15: Nei's gene diversity statistics (mean ± standard deviation) for subdivided populations, showing the genetic structure of the overall population. An estimate of gene flow (*Nm*) is also shown.

Statistic	Total genetic diversity (H_T)	Diversity within groups (H_S)	Gene flow between groups (*Nm*)
Value	0.281 ± 0.031	0.253 ± 0.027	4.448

The overall F_{ST} for all groups is 0.0369 ± 0.1830. Permutation testing for genetic differentiation among groups showed this value to be significant, resulting in a rejection of the hypothesis at the 1% level. Therefore, although differentiation between plants from different areas is low, they are still significantly different from each other. Permutation testing between each pair of groups showed far off site plants to be genetically differentiated from those in most other areas, as was noted in the initial area groupings. The retaining wall group is not significantly differentiated from the toepaddock wall group, supporting morphological analysis where these two groups were grouped together (Table 16).

Table 16: Permutation test for genetic differentiation among groups of plants from different areas, based on pairwise F_{ST} values between each pair of groups. Numbers in italics indicate rejection of the null hypothesis at the 5% level, while numbers in bold type indicate rejection of the null hypothesis at the 1% level. If the null hypothesis is rejected, it means the two populations are more genetically differentiated than random assemblages of the individuals.

	Retaining wall	Toepaddock wall	Toepaddock	Off site	Far off site A
Toepaddock wall	0.0434	/			
Toepaddock	**0.0176**	0.0110	/		
Off site	0.0212	*0.0262*	0.0119	/	
Far off site A	**0.1029**	*0.0277*	**0.0450**	**0.0678**	/
Far off site B	*0.0460*	**0.0348**	*0.0223*	0.0200	**0.0495**

As for the initial area grouping, a dendrogram based on Nei's genetic distances (*D*) shows plants from the far off site areas to be genetically distant from plants in the other area groups. Interestingly, the retaining wall group is not shown to group with the toepaddock wall group, even though these groups are morphologically similar and were not significantly different from each other during F_{ST} permutation testing (Figure 21).

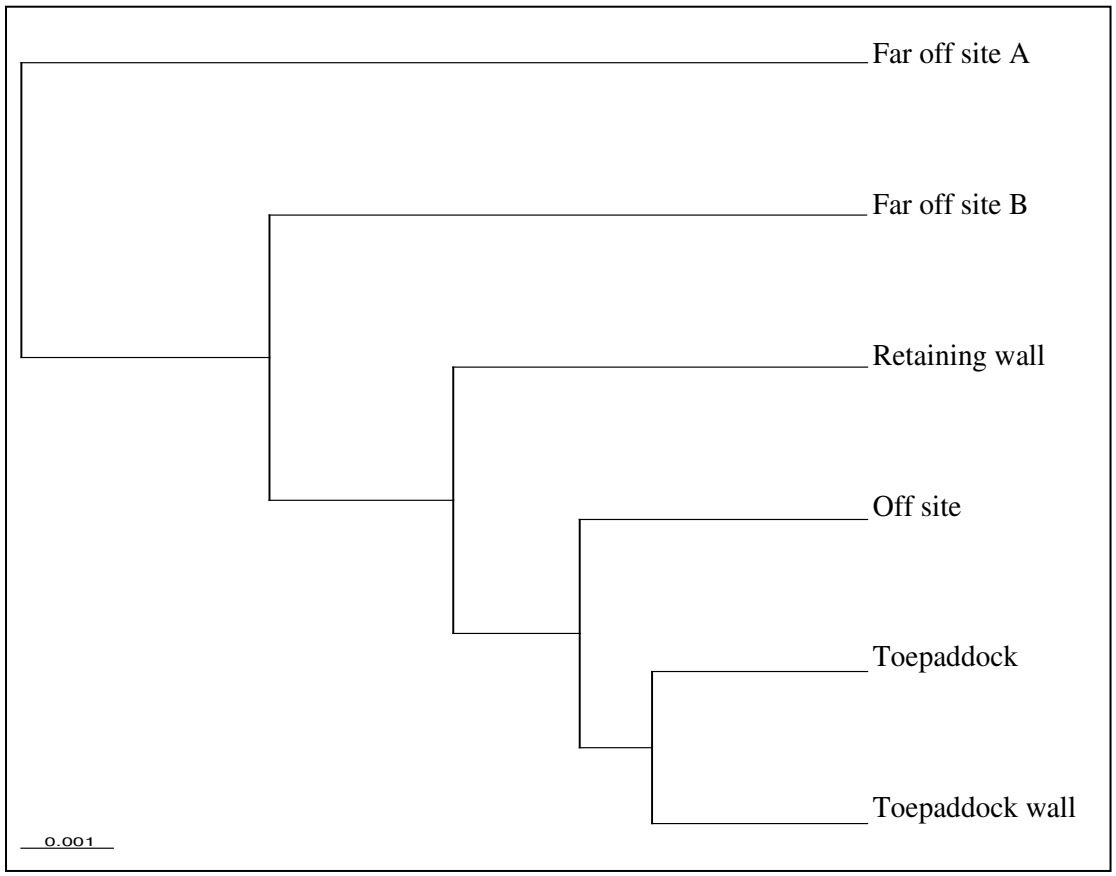


Figure 21: Dendrogram based on pair-wise Nei's genetic distances (*D*) between plant groups from different areas. The scale bar indicates 0.001 genetic distance units.

A genetic distance matrix was constructed showing distances between groups as shown in Figure 21, except that retaining wall and toepaddock wall plants were grouped together. This was in order that a Mantel test could be performed comparing this distance matrix to the one used to construct the dendrogram of morphological distance (Figure 19). This test showed a slight, non-significant (P=0.65), negative correlation (rxy=-0.456) between the two distance matrices. Group assignment showed a higher percentage of accurate classifications in plants from the retaining wall, toepaddock wall, and far off site A areas. Accurate classification was lowest in plants from the toepaddock group (Table 17).

Table 17: Percentage of plants from each area that assign to the different area groups, based on relatedness values between individuals. Values shown indicate the percentage of individuals from each area that is shown to assign to the various groups. Individuals are assigned to groups as soon as the likelihood of belonging to that group is higher than the likelihood of belonging to any other group.

	Population of origin					
Population assigned to (%)	RW	TW	TP	OS	FOSA	FOSB
Sample size	9	15	54	24	25	19
Retaining wall (RW)	77.8	0	16.7	12.5	0	5.3
Toepaddock wall (TW)	0	86.7	16.7	4.2	4	5.3
Toepaddock (TP)	0	0	40.7	8.3	0	10.5
Off site (OS)	22.2	0	11.1	66.7	0	5.3
Far off site A (FOSA)	0	13.3	5.6	4.2	84	10.5
Far off site B (FOSB)	0	0	9.2	4.2	12	63.2
Average P value	0.68	0.70	0.62	0.55	0.58	0.53
Percentage P values less than 0.3	0	6.6	9.3	20.8	20	21.1

6. ANALYSIS OF *INDIGOFERA ZEYHERI* ACCORDING TO AREA

6.1. Morphological analysis

Indigofera zeyheri plants were divided into two groups – toepaddock and off site – as no far off site or retaining wall plants could be obtained. To begin, T-tests were performed on each variable to determine which variables contribute significantly towards differences between plants from the different areas (Table 18). The variables shown to be significantly different between plants from the different areas, canopy area and canopy volume, were represented graphically. Canopy width variables were not represented in a graph as these variables make up the canopy area measurement. In contrast to *Indigofera adenoides*, measurements for both canopy area and canopy volume are significantly lower in plants growing on the toepaddock than in plants in the other areas. All measurements have a very high standard deviation, indicating high variability in the morphology of plants within the different areas (Figure 22). No large differences in correlation coefficients were observed for pairs of variables between the two areas (Table 19).

Table 18: T-test showing which variables are significantly different between plants from the different areas (DF=96).

Variable	T value	Pr > t
Plant Height	1.55	0.1234
Canopy Width	4.23	<0.0001
Canopy Width 90°	4.08	<0.0001
Canopy Area	4.33	<0.0001
Canopy Volume	4.09	<0.0001
Leaf Area	-0.60	0.5486
Leaf Shape	-2.58	0.0115
Leaf Length	-1.34	0.1839
Leaf Width	0.36	0.7230

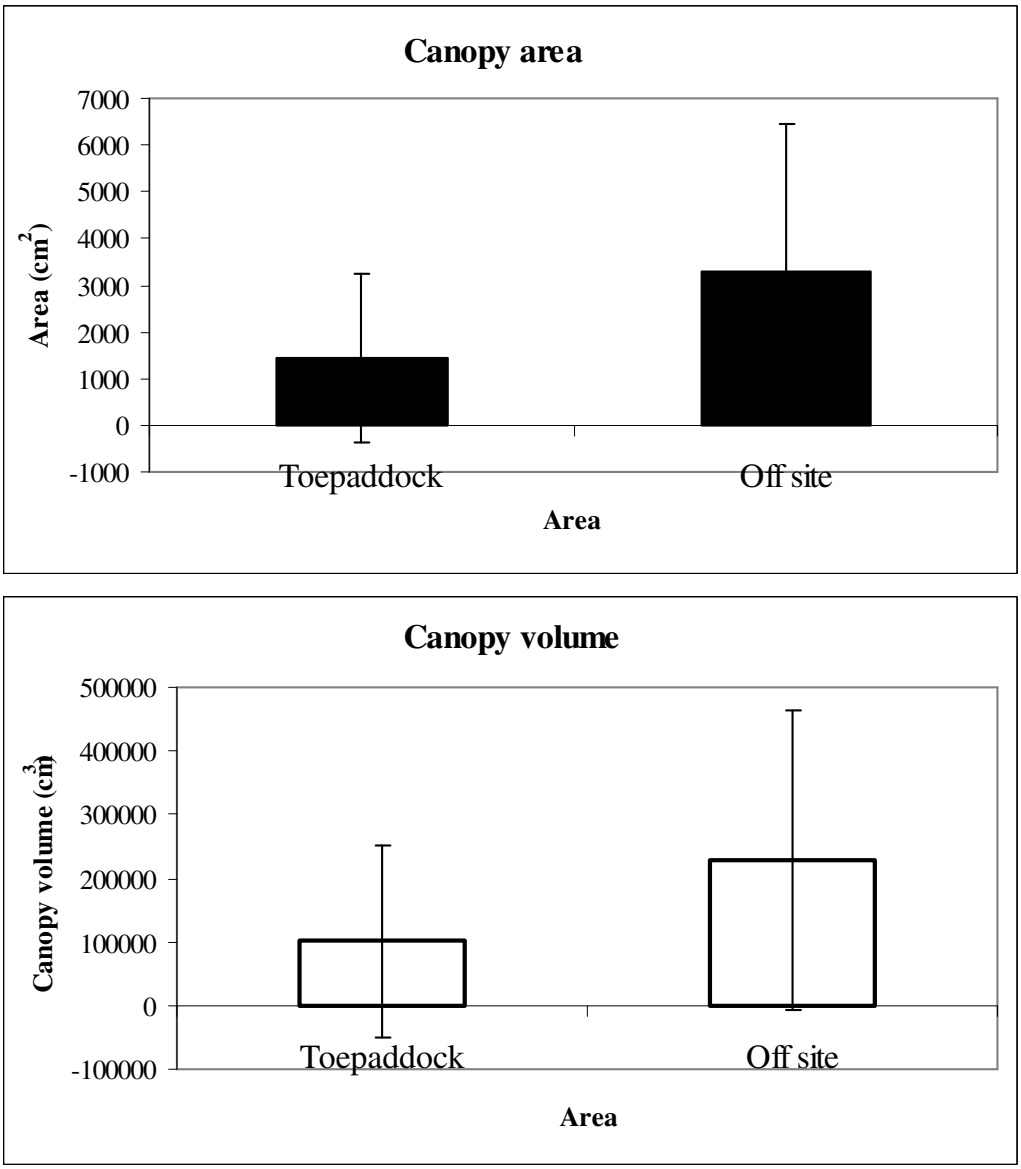


Figure 22: A comparison of canopy area (mean ± standard deviation) and canopy volume (mean ± standard deviation) between plants from the two area groups.

Table 19: A comparison of correlation coefficients between various variables in plants from different areas. The asterix (*) indicates a p value of less than 0.0001, showing the two variables to be highly correlated.

Correlation between	Overall	Toepaddock	Off site
Height and leaf length	0.340*	0.165	0.146
Height and leaf width	0.272	0.161	0.134
Height and canopy area	0.374*	0.260	0.0695
Height and leaf area	0.306	0.150	0.0999
Leaf length and leaf width	0.597*	0.230	0.543
Canopy area and leaf area	-0.161	-0.139	-0.186
Canopy volume and leaf area	-0.145	-0.0953	-0.241

Coefficients of variation show plants from the toepaddock to be more morphologically diverse than plants from the off site areas (Table 20). Discriminant analysis and canonical discriminant analysis could not be performed for *Indigofera zeyheri* as only two groups were available for comparison.

Table 20: A comparison of coefficients of variation for each variable in plants from the two area groups. The higher coefficient for each variable is shown in bold type.

Variable	Off site	Toepaddock
Sample size	35	63
Height	5.16	**7.78**
Canopy Width	42.61	**57.58**
Canopy Width 90	54.29	**68.72**
Leaf Length	19.79	**21.42**
Leaf Width	**22.42**	18.85
Canopy Area	12.43	**21.58**
Canopy Volume	8.81	**14.89**
Canopy Volume 2	9.12	**15.46**
Leaf area	**33.13**	32.58
Leaf shape	12.48	**13.25**

6.2. Genetic analysis

Genetic analysis was performed to compare the genetic structure of the toepaddock group of plants with that of the off site group of plants. A subset of *I. zeyheri* plants were used for AFLP analysis, due to time constraints. The toepaddock group has a lower genetic diversity than the off site group, although the difference between the groups is smaller than was seen with *I. adenoides* (Table 21). Total population diversity is mostly due to diversity within groups, indicating that diversity between the two groups of plants is low. Estimated gene flow levels are very high (Table 22).

Table 21: Genetic structure of the groups of plants from the two areas in terms of sample sizes, percentage of loci polymorphic, Shannon's information index (*I*) (mean ± standard deviation), and Nei's (1973) gene diversity (*h*) (mean ± standard deviation).

Population	Sample size	Percentage of loci polymorphic	*h*	*I*
Toepaddock	26	73.5	0.2531 ± 0.1941	0.3800 ± 0.2711
Off site	16	82.4	0.2542 ± 0.1839	0.3872 ± 0.2538

Table 22: Nei's gene diversity statistics (mean ± standard deviation) for subdivided populations, showing the genetic structure of the overall population. An estimate of gene flow (*Nm*) is also shown.

Statistic	Total genetic diversity (H_T)	Diversity within groups (H_S)	Gene flow between groups (*Nm*)
Value	0.2642 ± 0.0303	0.2536 ± 0.0290	12.0201

The F_{ST} value for the overall population is 0.0067 ± 0.0318. Permutation testing showed this F_{ST} value to not be significant. The null hypothesis was therefore not rejected, indicating that the groups are not more genetically differentiated than a random assemblage of the individuals.

7. ANALYSIS OF *INDIGOFERA ADENOIDES* ACCORDING TO TOXICITY

The analysis performed in the previous chapter involved the relation of the plants to the slimes dams. Following this analysis, plants were separated into groups according to toxicity information obtained from AngloGold Ashanti Ltd. technical data (Labuschagne, 2004), in order to determine if plants show genetic or morphological differences that correspond to differing toxicity levels. *Indigofera adenoides* plants were separated into six toxicity groups based on total dissolved solids (TDS) levels in groundwater, measured from boreholes in the area of the slimes dam, which have been shown to be correlated with soil toxicity (Figure 23).

Total dissolved solids in this data set are made up mostly of sulphates and chlorides. Increased TDS levels increase the toxicity of the groundwater to plants due to increased osmotic stress, a lower pH, as well as due to the toxicity of the individual salts and mobile elements, and the interactions between them (Weiersbye et al., 2002). Zone 2 can therefore be considered to have the least toxic groundwater for the purposes of this study, while zone 7 can be considered to have the most toxic groundwater. The depth to groundwater is important as it determines the extent to which the toxicity of the groundwater will impact on plants growing on the surface. A consistently high depth to groundwater is evident throughout the area surrounding the South Complex slimes dam (14-19 mbgl), with slightly shallower levels being evident in higher toxicity zones (Figure 24).

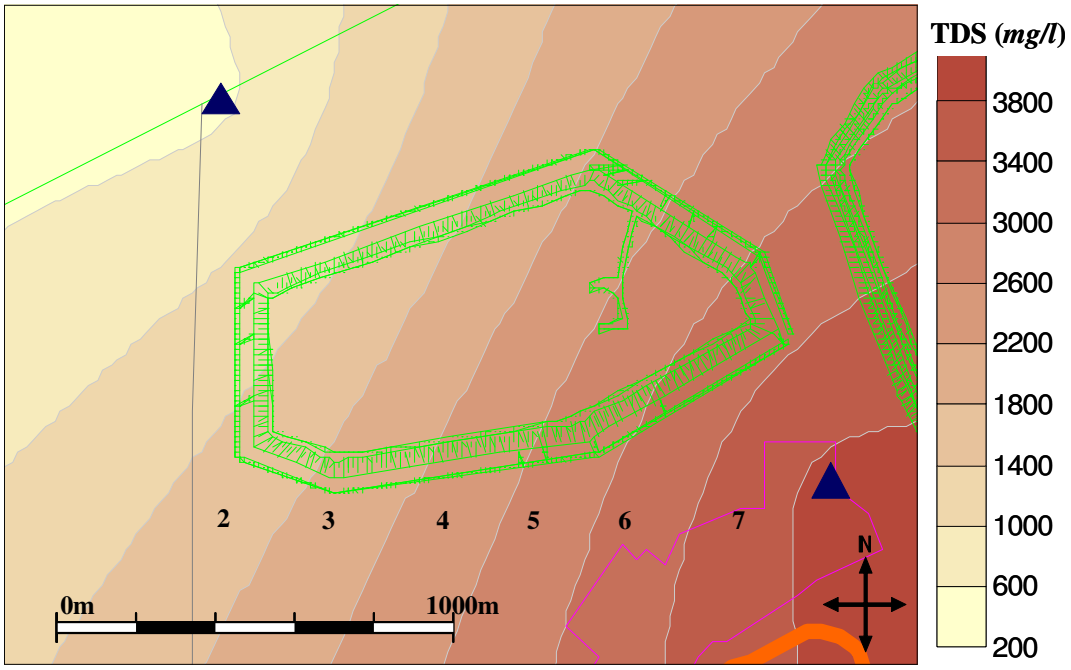


Figure 23: Zones of levels of total dissolved solids (TDS) surrounding the South Complex slimes dam. Adapted from AngloGold Ashanti Ltd. technical data. Plants were divided into the indicated zones 2 to 7.

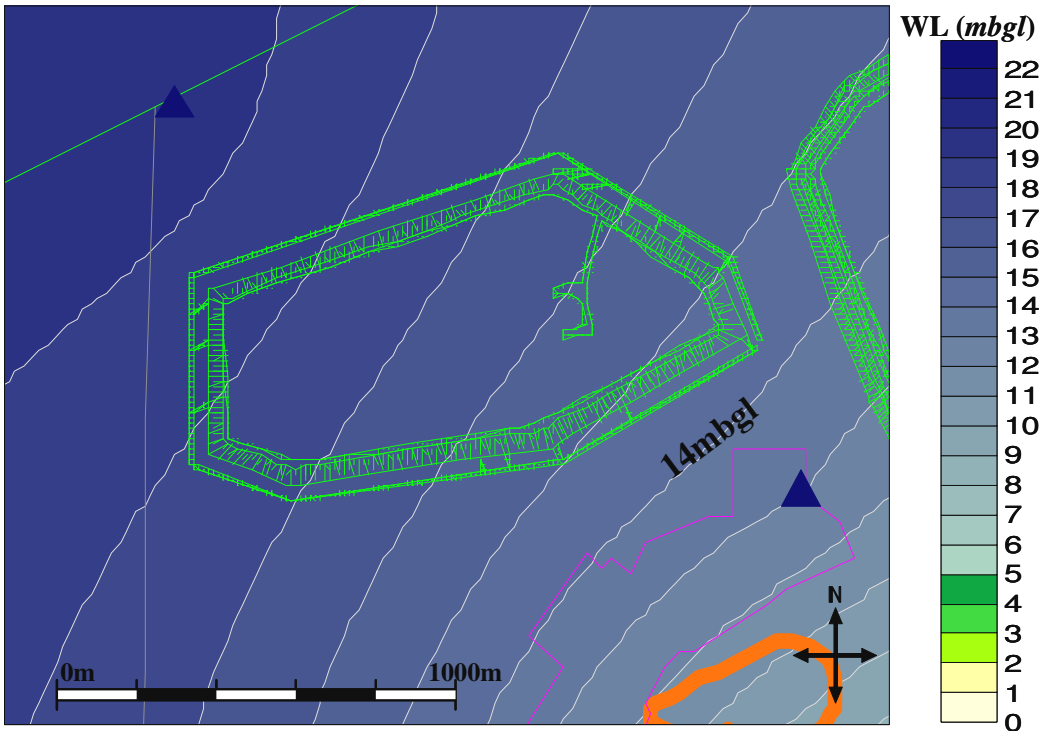


Figure 24: Depth to groundwater in metres below ground level (*mbgl*) for the toxicity zones studied. Adapted from AngloGold Ashanti Ltd. technical data.

7.1. Genetic analysis using plants from all areas

Initial analysis involved separating plants from the retaining wall, toepaddock wall, toepaddock, and off site areas into the six zones (zones 2 to 7) shown in Figure 23. This was done to determine if a significant genetic or morphological difference could be detected between the toxicity zones, regardless of the changes in local growth conditions caused by proximity of the plants to the slimes dam.

Genetic diversity is the highest in the lowest toxicity group (group 2), is fairly constant in groups 3, 4, and 5, decreases in group 6, then increases sharply in the highest toxicity group (group 7). The percentage of loci polymorphic decreases from group 2 to 6, then increases in group 7 (Table 23). The majority of total genetic diversity is due to diversity within toxicity groups, indicating a low level of genetic differentiation between the different toxicity groups of plants. Estimated gene flow levels are moderate (Table 24).

Table 23: Genetic structure of the plants in the toxicity groups in terms of sample size, percentage of loci polymorphic, Shannon's information index (*I*) (mean ± standard deviation), and Nei's (1973) gene diversity (*h*) (mean ± standard deviation).

Toxicity group	Sample size	Percentage of loci polymorphic	*h*	*I*
Group 7	15	65.91	0.2703 ± 0.2137	0.3919 ± 0.3018
Group 6	10	47.73	0.1948 ± 0.2187	0.2829 ± 0.3109
Group 5	10	56.82	0.2362 ± 0.2182	0.3422 ± 0.3115
Group 4	17	61.36	0.2292 ± 0.2041	0.3398 ± 0.2918
Group 3	20	63.64	0.2266 ± 0.2018	0.3378 ± 0.2879
Group 2	30	68.18	0.2590 ± 0.2014	0.3820 ± 0.2864

Table 24: Nei's gene diversity statistics (mean ± standard deviation) for subdivided populations, showing the genetic structure of the overall population. An estimate of gene flow (*Nm*) is also shown.

Statistic	Total genetic diversity (H_T)	Diversity within groups (H_S)	Gene flow between groups (*Nm*)
Value	0.2666 ± 0.0377	0.2360 ± 0.0309	3.8547

The overall F_{ST} is 0.0320 ± 0.2110 (mean ± standard error). Permutation testing showed this value to be significant, resulting in a rejection of the null hypothesis at the 1% level. The groups of plants are therefore more genetically diverse than a random assemblage of the individuals. Permutation testing of F_{ST} values for each pair of groups shows plants in the highest toxicity group (group 7) to be the most genetically differentiated from plants in the other groups (Table 25). F_{ST} data are supported by a dendrogram constructed from Nei's genetic distances, which shows plants in the highest toxicity groups (group 7 and 6) to be the most genetically distant from plants in the other groups (Figure 25).

Table 25: Permutation test for genetic differentiation among toxicity groups, based on pairwise F_{ST} values between each pair of groups. Numbers in italics indicate rejection of the null hypothesis at the 5% level, while numbers in bold type indicate rejection of the null hypothesis at the 1% level. If the null hypothesis is rejected, it means the two populations are more genetically differentiated than random assemblages of the individuals at the significance level indicated.

	Group7	Group6	Group5	Group4	Group3
Group6	*0.0585*	/			
Group5	*0.0345*	0.0269	/		
Group4	**0.0405**	*0.0284*	0.0071	/	
Group3	**0.0491**	*0.0335*	0.0231	0.0114	/

Group2	0.0248	0.0257	*0.0546*	*0.0360*	0.0163

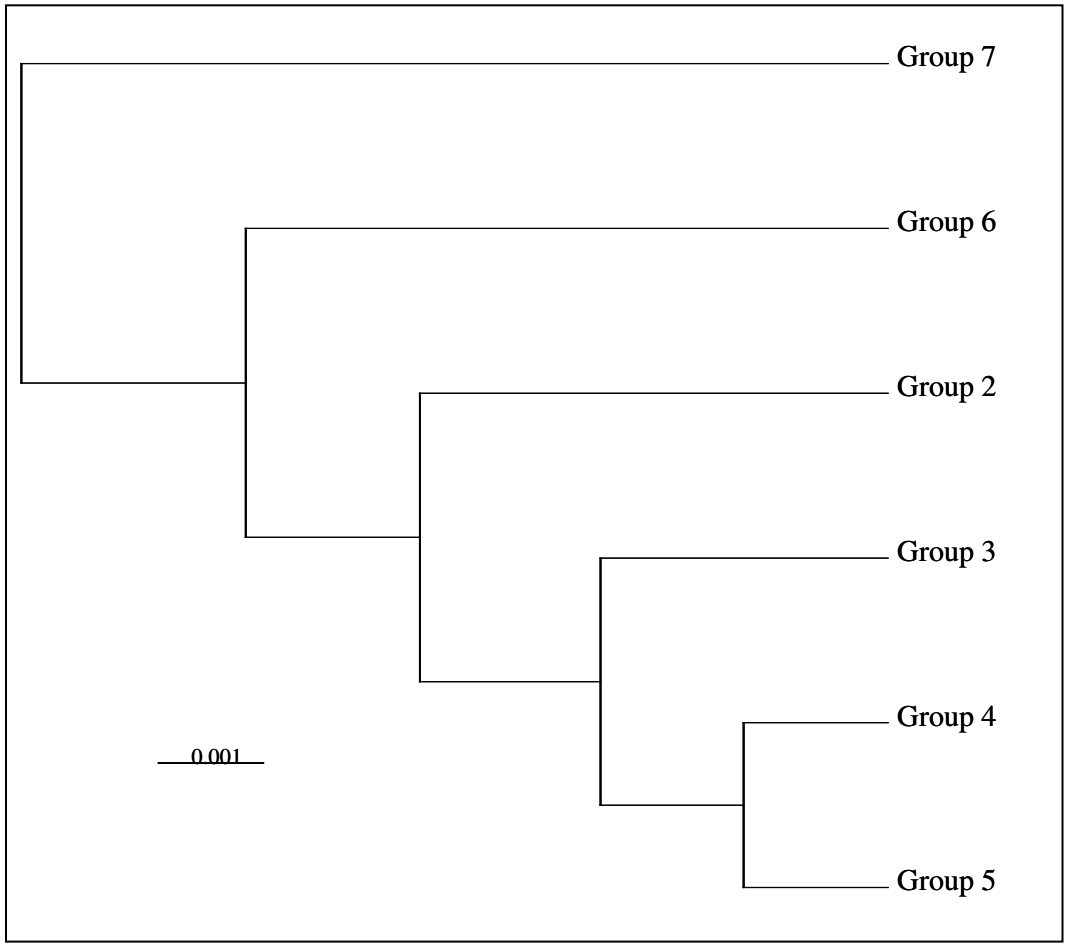


Figure 25: Dendrogram based on pair-wise Nei's genetic distances (*D*) between plants in the different toxicity groups. The scale bar indicates 0.001 genetic distance units.

7.2. Morphological analysis using plants from all areas

Initial morphological analysis was performed with the same groups used in the genetic analysis section 7.1. Toxicity groups of plants are not significantly morphologically different from each other at the $P<0.001$ level. The two measures of canopy diameter, together with leaf shape, are the closest to being significantly different between the groups. Consequently, canopy area is significantly different between the groups at the $P<0.05$ level (Table 26). Canopy diameter, and consequently canopy area, is the only variable shown to be significantly different between plants in the different toxicity groups following Tukey analysis (Table 27).

Table 26: Analysis of variance (ANOVA) for all variables. DF=5, error DF=96. P values indicate whether the variable accounts significantly towards variation between the different toxicity groups of plants.

	F	P > F
Plant height	0.34	0.8861
Canopy diameter	2.31	0.0500
Canopy diameter 90°	2.36	0.0456
Leaf length	1.38	0.2388
Leaf width	1.59	0.1714
Leaf area	1.65	0.1553
Leaf shape	2.14	0.0676
Canopy area	2.83	0.0198
Canopy volume	2.10	0.0724

Table 27: Plant and leaf dimensions (mean ± standard deviation) for plants in each of the toxicity groups. Superscripts of the same letter within a row indicate values that are not significantly different (P<0.05%; Tukey).

Factor	Group7	Group6	Group5	Group4	Group3	Group2
Plant height (cm)	37.8±13.0^{a}	43.7±14.0^{a}	38.1±14.0^{a}	37.5±11.6^{a}	37.2±14.8^{a}	38.2±12.5^{a}
Canopy diameter (cm)	47.9±17.3^{a}	41.0±13.9^{a}	44.2±21.1^{a}	66.2±24.4^{a}	50.0±27.0^{a}	48.8±21.9^{a}
Canopy diameter 90° (cm)	34.4±17.0^{ab}	27.5±12.9^{a}	34.8±18.4^{ab}	50.4±23.8^{b}	37.3±20.3^{ab}	38.9±16.0^{ab}
Leaf length (cm)	1.4±0.4^{a}	1.4±0.2^{a}	1.2±0.2^{a}	1.5±0.4^{a}	1.3±0.4^{a}	1.5± 0.4^{a}
Leaf width (cm)	0.5±0.2^{a}	0.5±0.1^{a}	0.4±0.1^{a}	0.6±0.2^{a}	0.5±0.2^{a}	0.5± 0.1^{a}
Leaf area (cm)	0.6±0.4^{a}	0.6±0.2^{a}	0.4±0.1^{a}	0.7±0.5^{a}	0.6±0.3^{a}	0.5± 0.3^{a}
Leaf shape	3.1±0.9^{a}	2.8±0.3^{a}	2.9±0.7^{a}	2.8±0.5^{a}	3.0±0.9^{a}	3.4± 0.9^{a}
Canopy area (cm^2)	1464± 1305^{ab}	985± 796^{a}	1443± 1170^{ab}	2941± 2103^{b}	1799± 1533^{ab}	1664± 1454^{ab}
Canopy volume (cm^3)	61442± 66560^{a}	40017± 28757^{a}	56634± 52776^{a}	113552± 87756^{a}	73674± 68541^{a}	65238± 60077^{a}
Canopy volume 2 (cm^3)	40962± 44374^{a}	26678± 19172^{a}	37756± 35184^{a}	75701± 58504^{a}	49116± 45694^{a}	43492± 40051^{a}

Several differences in correlation coefficients of plant measurements can be noted between toxicity groups, but these differences do not seem to be located in any particular group, nor follow any particular pattern (Table 28). This separation of plants into toxicity groups was not morphologically analysed further as it was apparent these groups of plants are not significantly differentiated from each other. Genetic analysis of these groups did not show dramatic differences, either (Section

7.1). This is thought to be due to local conditions that differ between off site and toepaddock areas, for example the toepaddock has increased water levels due to seepage from the slimes. Diversity of morphological characteristics of plants within toxicity groups would therefore be high, resulting in little differentiation between groups. Toxicity groups were therefore redesigned.

Table 28: A comparison of correlation coefficients between various variables in plants in the different toxicity groups. The asterix (*) indicates a p value of less than 0.0001, showing the two variables to be highly correlated.

Correlation between	Group 7	Group 6	Group 5	Group 4	Group 3	Group 2
Height and leaf length	-0.122	-0.107	0.467	0.438	0.437	0.085
Height and leaf width	-0.092	0.091	0.043	0.546	-0.231	-0.213
Height and canopy diameter	0.403	-0.297	-0.057	0.314	0.335	0.280
Height and canopy diameter90	0.523	-0.317	0.215	0.100	0.599	-0.001
Canopy diameter and diameter 90	0.795	0.780	0.860	0.748	0.827*	0.643
Leaf length and leaf width	0.712	0.812	0.373	0.838*	0.602	0.523
Canopy area and leaf area	0.141	0.186	-0.445	-0.314	0.043	0.488
Canopy area and canopy volume 1	0.968*	0.793	0.857	0.909*	0.900*	0.927*
Canopy volume and leaf area	-0.015	0.220	-0.302	-0.167	0.036	0.413

7.3. Genetic analysis using plants from toepaddock areas

The previous analysis containing plants from all areas surrounding the slimes dam did not indicate significant differentiation between plants from different toxicity groups. Off site plants were therefore removed from the analysis to see if a significant difference exists between toxicity groups containing individuals from the toepaddock areas (toepaddock, toepaddock wall, and retaining wall). Groups 6 and 7 were combined due to the small sample sizes of the individual groups, to create group 6/7.

Genetic diversity and the percentage of loci polymorphic are similar in all groups, with variation in values seeming not to follow any particular pattern (Table 29). Diversity between plants from different groups is very low, as the majority of total genetic diversity is due to diversity within groups. Estimated gene flow levels are moderately high (Table 30).

Table 29: Genetic structure of the toxicity groups in terms of sample size, percentage of loci polymorphic, Nei's (1973) gene diversity (*h*) (mean ± standard deviation), and Shannon's information index (*I*) (mean ± standard deviation).

Toxicity group	Sample size	Percentage of loci polymorphic	*h*	*I*
Group 6/7	13	52.27	0.2215 ± 0.2253	0.3187 ± 0.3193
Group 5	10	56.82	0.2362 ± 0.2182	0.3422 ± 0.3115
Group 4	13	54.55	0.2099 ± 0.2114	0.3088 ± 0.3015
Group 3	20	63.64	0.2266 ± 0.2018	0.3378 ± 0.2879
Group 2	22	59.09	0.2295 ± 0.2134	0.3362 ± 0.3033

Table 30: Nei's gene diversity statistics (mean ± standard deviation) for subdivided populations, indicating the genetic structure of the overall population. An estimate of gene flow (*Nm*) is also shown.

Statistic	Total genetic diversity (H_T)	Diversity within groups (H_S)	Gene flow between groups (*Nm*)
Value	0.2487 ± 0.0413	0.2247 ± 0.0352	4.6833

The overall F_{ST} value is 0.0175 ± 0.3832. Permutation testing showed this value to be significant, and the null hypothesis was rejected at the 1% level. Therefore although differentiation between plants from different toxicity groups is low, they are still significantly differentiated from each other. Permutation testing between each pair of area groups shows plants from different groups to be highly genetically similar to each other (Table 31).

Table 31: Permutation test for genetic differentiation between plants from different toxicity groups, based on pairwise F_{ST} values between each pair of groups. Numbers in italics indicate rejection of the null hypothesis at the 5% level. If the null hypothesis is rejected, it indicates the two populations are more genetically differentiated than random assemblages of the individuals at the significance level indicated.

	Group7/6	Group5	Group4	Group3
Group5	0.0258	/		
Group4	0.0042	0.0020	/	
Group3	*0.0408*	0.0231	-0.0056	/
Group2	0.0054	*0.0528*	0.0066	0.0119

These analyses show the different toxicity groups of plants to not be significantly differentiated from each other. Further genetic analysis was therefore not performed on these groups. Morphological analysis was also not performed. Toxicity groups were therefore redesigned further.

7.4. Genetic analysis using plants from the toepaddock only

Analysis was performed on toxicity groups containing toepaddock individuals only. This was done to remove the effect of local growth condition changes caused by proximity of the plants to the slimes dam. Two different sets of groups were analysed. One set contained groups 6 and 7 grouped together (group 6/7) while the other contained groups 5, 6, and 7 grouped together (group 5/6/7). This was done due to the small size of group 6/7.

Diversity is lower in group 6/7, although this could be due to the small sample size of this group. Diversity of group 5/6/7 is very high. Diversity in the lower toxicity groups (groups 2, 3, and 4) decreases slightly with increasing toxicity (Table 32). When calculated for the set containing group 6/7, differentiation between groups is higher, and gene flow lower, than that calculated for the set containing group 5/6/7 (Table 33).

Table 32: Genetic structure of toxicity groups in terms of sample sizes, percentage of loci polymorphic, Shannon's information index (*I*) (mean ± standard deviation), and Nei's (1973) gene diversity (*h*) (mean ± standard deviation). Two separate sets of groups are shown: groups 6 and 7 combined, or groups 5, 6, and 7 combined.

Toxicity group	Sample size	Percentage of loci polymorphic	*h*	*I*
Group 6/7	6	40.91	0.1578 ± 0.2083	0.2314 ± 0.2965
Group 5	10	56.82	0.2362 ± 0.2182	0.3422 ± 0.3115
Group 4	12	52.27	0.1988 ± 0.2106	0.2931 ± 0.3004
Group 3	18	61.36	0.2191 ± 0.2054	0.3256 ± 0.2926
Group 2	17	56.82	0.2206 ± 0.2146	0.3232 ± 0.3046
Group 5/6/7	16	61.36	0.2572 ± 0.2141	0.3729 ± 0.3056

Table 33: Nei's gene diversity statistics (mean ± standard deviation) for subdivided populations, showing the genetic structure of the overall population. An estimate of gene flow (*Nm*) is also shown. Values are shown for the two different sets of toxicity groups.

Statistic	Total genetic diversity (H_T)	Diversity within groups (H_S)	Gene flow between groups (*Nm*)
Value 6/7	0.2480 ± 0.0406	0.2065 ± 0.0304	2.4859
Value 5/6/7	0.2457 ± 0.0405	0.2239 ± 0.0347	5.1499

Overall F_{ST} is 0.0556 ± 0.5497 for the set containing group 6/7 and 0.0183 ± 0.3825 for the set containing group 5/6/7. Permutation testing showed the null hypothesis to be rejected at the 1% level for both sets of groups, indicating the groups are more genetically diverse than a random assemblage of the individuals. Permutation testing of F_{ST} values was then performed between each pair of toxicity groups in each set of groups individually. Group 6/7 is significantly genetically differentiated from all other groups. Group 5/6/7 is significantly genetically differentiated from most other groups. The remaining groups are genetically similar to each other (Tables 34 and 35). A dendrogram constructed from Nei's genetic distances (*D*) shows group 6/7 and group 5/6/7 to be genetically distant from the remaining groups in their respective sets (Figure 26).

Table 34: Permutation test for genetic differentiation among toxicity groups of the set containing group 6/7, based on pairwise F_{ST} values between each pair of groups. Values in italics indicate rejection of the null hypothesis at the 5% level, while numbers in italics indicate rejection of the null hypothesis at the 1% level. If the null hypothesis is rejected, it indicates the two populations are more genetically differentiated than random assemblages of the individuals.

	Group 6/7	Group5	Group4	Group3
Group5	*0.0981*	/		
Group4	**0.0858**	0.0070	/	
Group3	**0.1439**	0.0214	-0.0016	/
Group2	**0.1078**	*0.0511*	0.0127	0.0016

Table 35: Permutation test for genetic differentiation among toxicity groups of the set containing group 5/6/7, based on pairwise F_{ST} values between each pair of groups. Values in italics indicate rejection of the null hypothesis at the 5% level, while numbers in italics indicate rejection of the null hypothesis at the 1% level. If the null hypothesis is rejected, it indicates the two populations are more genetically differentiated than random assemblages of the individuals.

	Group5/6/7	Group4	Group3
Group4	0.0109	/	
Group3	**0.0347**	-0.0016	/
Group2	**0.0451**	0.0127	0.0016

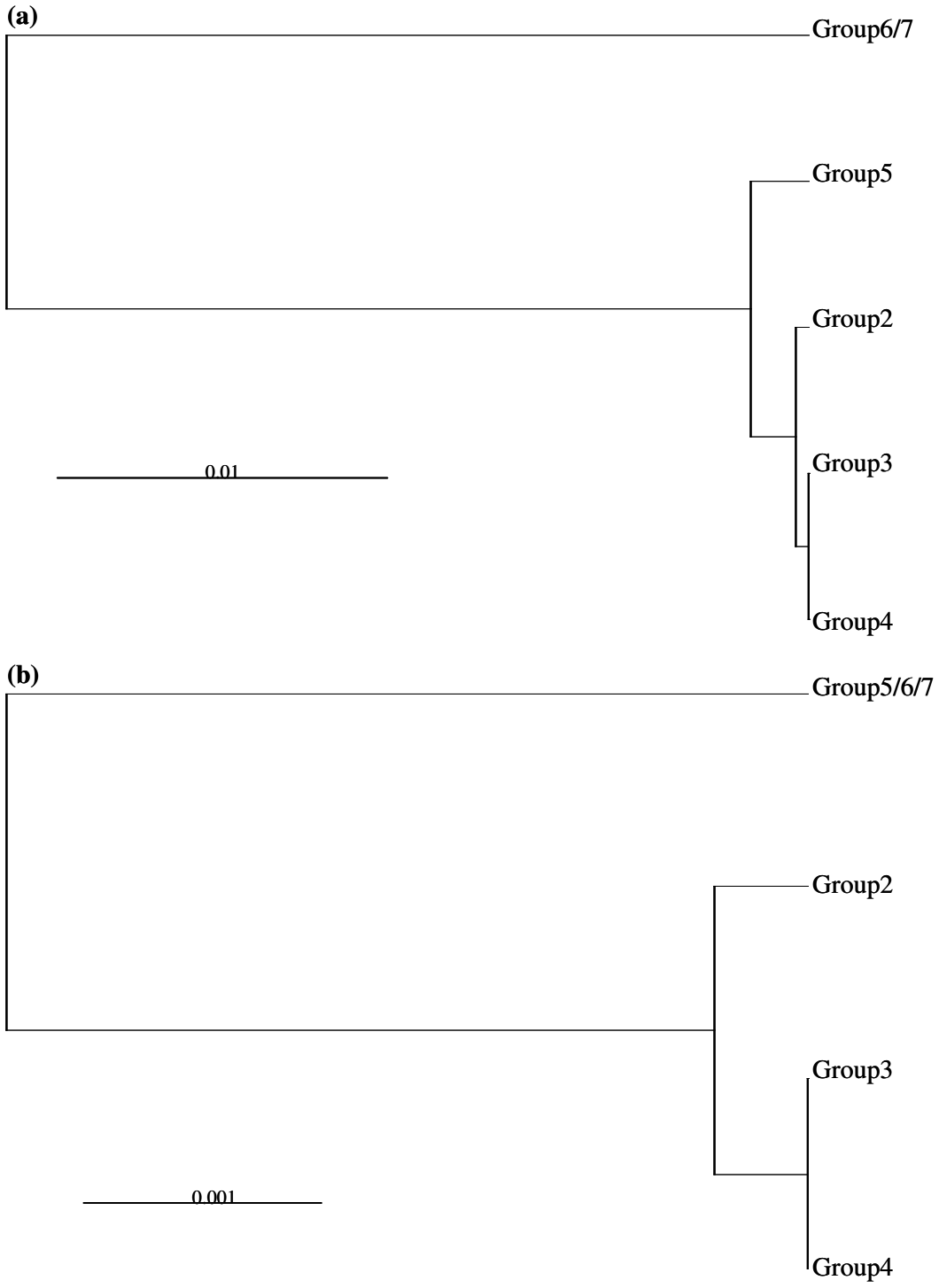


Figure 26: Dendrograms based on Nei's genetic distances (*D*) showing the genetic distance between plants in different toxicity groups. Two sets of groups are shown: the set containing group 6/7 (a) and the set containing group 5/6/7 (b). The scale bar indicates the shown number of genetic distance units.

Assignment of plants to the correct toxicity group is high in all groups, and for both sets of groups. The *P* value is the highest in group 6/7, indicating assignment is the most certain in that group. Individuals are not assigned incorrectly to group 5/6/7 with a high frequency (Tables 36 and 37).

Table 36: Percentage of plants from each toxicity group in the set containing group 6/7 that assign to the different toxicity groups. Values shown indicate the percentage of plants from each group that assigns to the various groups. Plants are assigned to groups as soon as the likelihood of belonging to that group is higher than the likelihood of belonging to any other group. Probability (*P*) values are also shown indicating the average probability of correct classification for each group.

	Group of origin				
Group assigned to (%)	Group 6/7	Group 5	Group 4	Group 3	Group 2
Sample size	6	10	12	18	17
Group 6/7	66.7	0	0	0	5.9
Group 5	16.7	80.0	0	0	0
Group 4	16.7	20.0	75.0	5.6	23.5
Group 3	0	0	8.3	77.8	5.9
Group 2	0	0	16.7	16.7	64.7
Average *P* value	0.87	0.55	0.57	0.57	0.59

Table 37: Percentage of plants from each toxicity group in the set containing group 5/6/7 that assign to the different toxicity groups. Values shown indicate the percentage of plants from each group that assigns to the various groups. Plants are assigned to groups as soon as the likelihood of belonging to that group is higher than the likelihood of belonging to any other group. Probability (*P*) values are also shown indicating the average probability of correct classification for each group.

	Group of origin			
Group assigned to (%)	Group 5/6/7	Group 4	Group 3	Group 2
Sample size	16	12	18	17
Group 5/6/7	75.0	8.3	0	0
Group 4	18.8	66.7	5.6	23.5
Group 3	0	8.3	77.8	5.9
Group 2	6.3	16.7	16.7	70.6
Average P value	0.52	0.60	0.57	0.60

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7.5. Morphological analysis using plants from the toepaddock only

Morphological analysis was performed with groups 6/7, 5, 4, 3, and 2, as these groups were shown to be the most genetically differentiated from each other. No variables are significantly different at the $P<0.001$ level between plants in different toxicity groups. The two measures of canopy diameter, and consequently canopy area and canopy volume, are significantly different between plants in different toxicity groups at the $P<0.05$ level (Table 38).

Table 38: Analysis of variance (ANOVA) for all variables. DF=4, error DF=58. P values indicate whether the variable accounts significantly towards variation between the different toxicity groups of plants.

	F	P > F
Plant height	0.31	0.8697
Canopy diameter	2.83	0.0324
Canopy diameter 90°	4.19	0.0047
Leaf length	0.56	0.6941
Leaf width	0.78	0.5429
Leaf area	0.74	0.5656
Leaf shape	1.03	0.3983
Canopy area	5.33	0.0010
Canopy volume	4.12	0.0053

Tukey results support ANOVA analysis, showing plants in group 4 to be over twice the canopy area and canopy volume of those plants in other toxicity groups (Table 39). Following the results of these analyses, graphs were constructed showing the distribution of canopy area in the various toxicity groups (Figure 27). Canopy area is highest in group 4, and lowest in group 6/7.

Table 39: Plant and leaf dimensions (mean ± standard deviation) for plants in the various toxicity groups. Superscripts of the same letter within a row indicate values that are not significantly different (P<0.05%; Tukey).

Factor	Group7/6	Group5	Group4	Group3	Group2
Plant height (cm)	30.8±7.4[a]	38.1±13.2[a]	37.5±12.1[a]	35.4±14.5[a]	35.0±12.0[a]
Canopy diameter (cm)	54.0±8.3[ab]	44.2±20.0[ab]	71.2±24.3[a]	48.6±28.0[ab]	44.7±21.4[b]
Canopy diameter 90° (cm)	34.0±13.8[ab]	34.8±17.4[b]	58.2±24.0[a]	34.7±19.8[b]	33.5±11.9[b]
Leaf length (cm)	1.4±0.4[a]	1.2±0.2[a]	1.4±0.4[a]	1.3±0.3[a]	1.4± 0.3[a]
Leaf width (cm)	0.4±0.1[a]	0.4±0.1[a]	0.5±0.2[a]	0.4±0.2[a]	0.4± 0.1[a]
Leaf area (cm)	0.5±0.2[a]	0.4±0.1[a]	0.6±0.4[a]	0.5±0.3[a]	0.5± 0.2[a]
Leaf shape	3.4±0.9[a]	2.9±0.7[a]	2.8±0.5[a]	3.0±1.0[a]	3.2± 0.7[a]
Canopy area (cm^2)	1514± 818[b]	1443± 1110[b]	3609± 2155[a]	1672± 1557[b]	1276± 1002[b]
Canopy volume (cm^3)	47706± 29803[b]	56634± 50068[b]	138846± 91048[a]	64310± 63763[b]	50704± 56209[b]
Canopy volume 2 (cm^3)	31804± 19869[b]	37756± 33378[b]	92564± 60699[a]	42873± 42509[b]	33802± 37473[b]

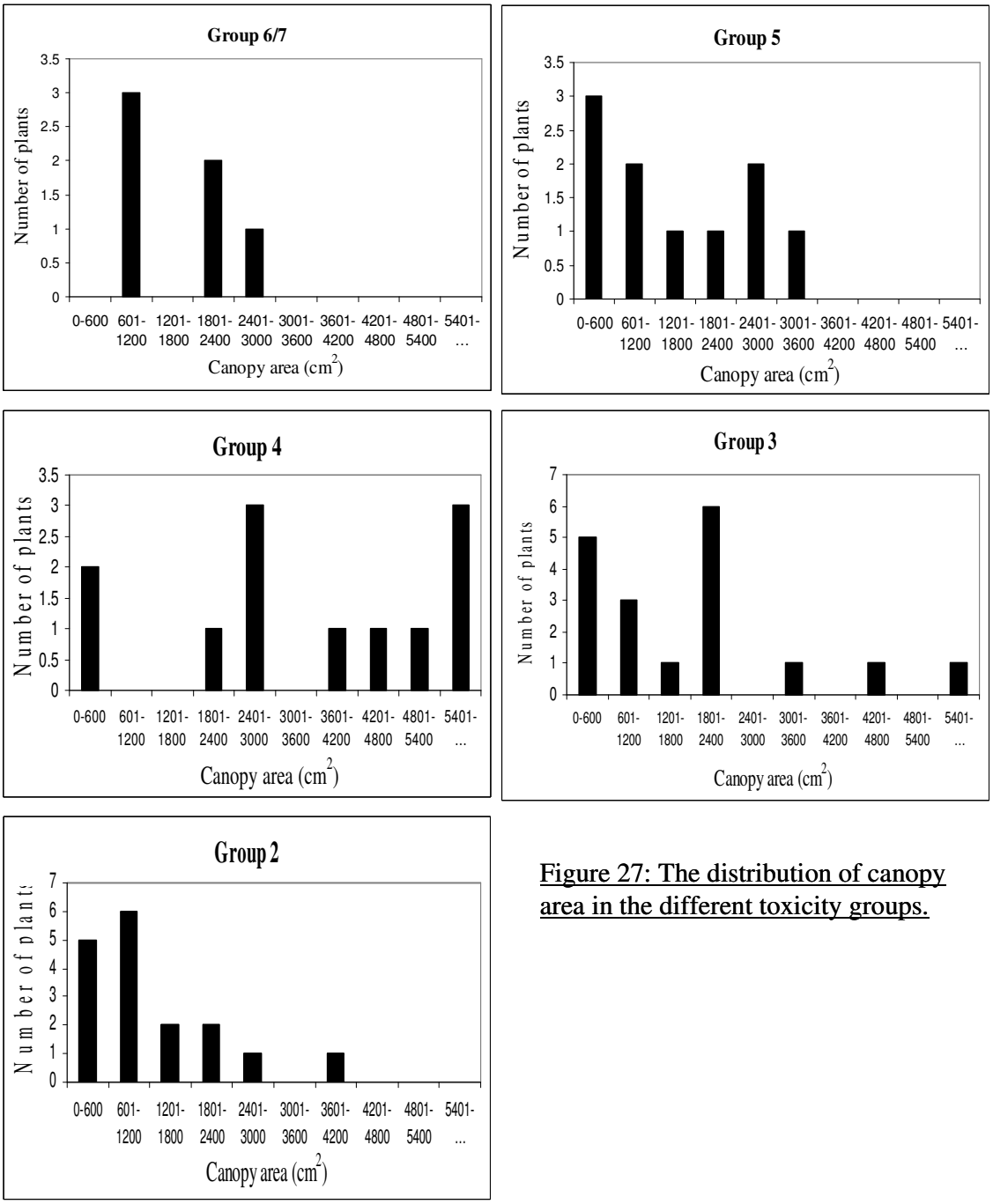


Figure 27: The distribution of canopy area in the different toxicity groups.

The majority of differences in correlation coefficients are seen in plants from the highest (group 6/7) and lowest (group 2 and group 3) toxicity groups, and in correlations containing canopy area or associated variables, further indicating the extent that canopy area varies between plants from different toxicity groups (Table 40). A higher percentage of accurate classifications is seen in plants from higher toxicity groups (groups 4, 5, and 6/7) than in plants from lower toxicity groups (groups 2 and 3) (Table 41).

Table 40: A comparison of correlation coefficients between variables in plants from different toxicity groups. The asterix (*) indicates a p value of less than 0.0001, showing the two variables to be highly correlated.

Correlation between	Group 7/6	Group 5	Group 4	Group 3	Group 2
Height and leaf length	0.542	0.467	0.448	0.334	-0.170
Height and leaf width	0.601	0.043	0.526	-0.437	-0.427
Height and canopy diameter	0.149	-0.057	0.258	0.297	0.504
Height and canopy diameter90	0.185	0.215	0.167	0.530	-0.045
Canopy diameter and diameter 90	0.951	0.860	0.846	0.841*	0.532
Leaf length and leaf width	0.698	0.373	0.785	0.505	0.621
Canopy area and leaf area	0.712	-0.445	-0.151	-0.093	-0.007
Canopy area and canopy volume 1	0.846	0.857	0.885	0.908*	0.970*
Canopy volume and leaf area	0.921	-0.302	-0.018	-0.204	-0.046

Table 41: Classification of plants from each toxicity group into different groups, expressed as a percentage of the total number of plants in each group, obtained through discriminant analysis. The total number of plants that classifies to each toxicity group is shown, expressed as a percentage of the total number of plants.

From area	Group2 (%)	Group3 (%)	Group4 (%)	Group5 (%)	Group6/7 (%)
Sample size	17	18	12	10	6
Group 2	29.41	17.65	11.76	11.76	29.41
Group 3	22.22	5.56	22.22	27.78	22.22
Group 4	0.00	0.00	75.00	16.67	8.33
Group 5	0.00	20.00	10.00	60.00	10.00
Group 6/7	0.00	16.67	33.33	0.00	50.00
Total	14.29	11.11	28.57	23.81	22.22

A dendrogram constructed from pair-wise squared distances between groups of plants, obtained through canonical discriminant analysis, shows plants in groups 4 and 6/7 to be the most morphologically distant from plants in the other groups (Figure 28). A Mantel test was performed to compare the distance matrix used to construct the dendrogram above with the one used to construct the dendrogram of genetic distance between the same groups, shown in Figure 26. The test showed a slight, significant ($P = 0.940$), negative correlation ($Rxy = -0.490$) between the two distance matrices.

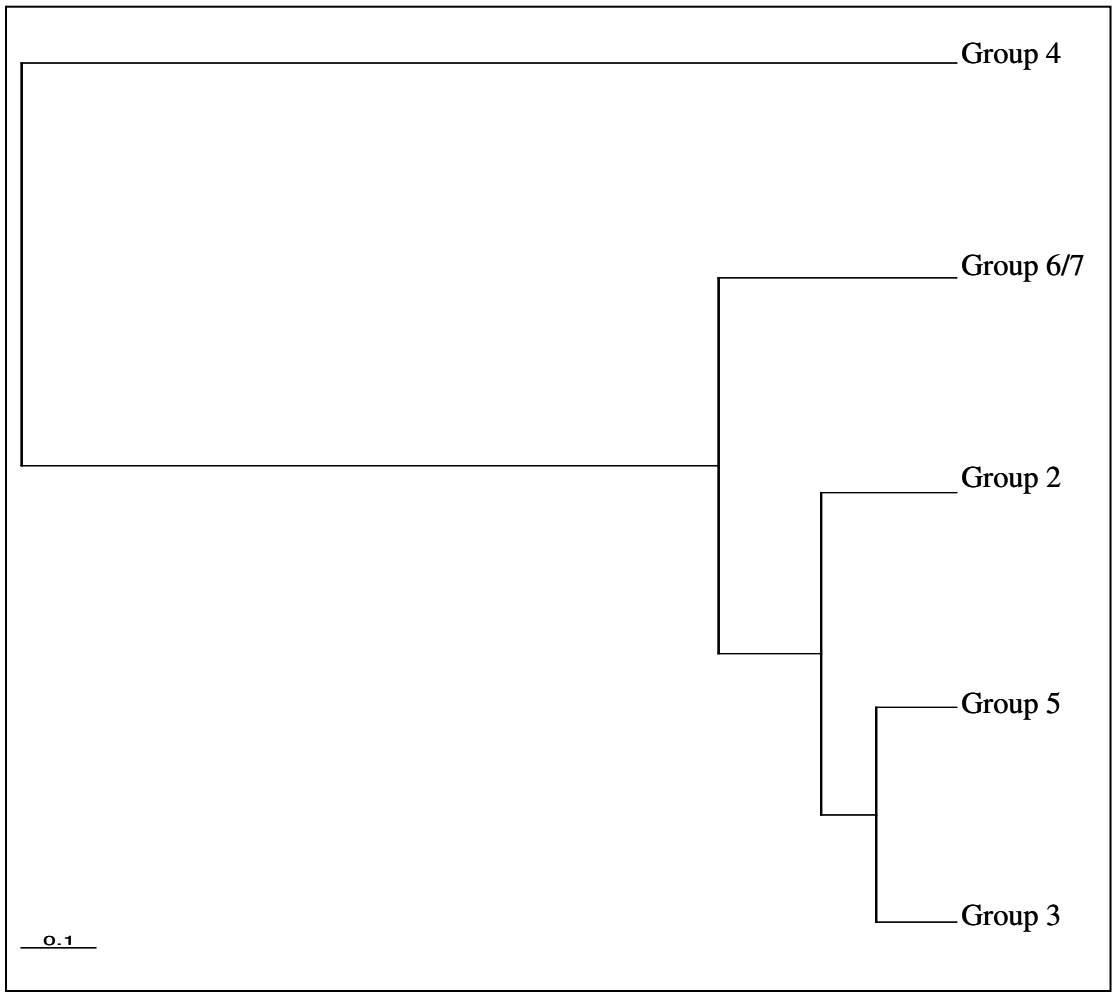


Figure 28: Dendrogram based on pair-wise squared distances of morphological data, obtained through canonical discriminant analysis. The scale bar indicates 0.1 distance units.

The diversity of each variable within the different toxicity groups of plants was determined by calculating coefficients of variation, in order to allow a comparison of morphological variation and genetic variation (Table 42). The most variable plants are generally found in the least toxic areas, while the least variable individuals do not seem to follow a particular trend in toxicity.

Table 42: A comparison of coefficients of variation for each variable in plants from different toxicity groups. The highest coefficient for each variable is shown in bold, with the lowest coefficient shown in italics.

Variable	Group7/6	Group5	Group4	Group3	Group2
Height	*6.69*	9.75	9.67	10.60	**16.87**
Canopy width	*15.45*	47.67	34.09	**57.68**	47.94
Canopy width 90	40.71	52.74	41.25	**56.99**	*35.60*
Leaf length	97.38	100.17	102.01	**138.41**	*82.40*
Leaf width	36.53	28.62	**39.87**	36.50	*26.29*
Canopy area	*54.06*	81.10	59.71	**93.14**	78.48
Canopy volume1	*62.47*	93.19	65.58	99.15	**110.86**
Leaf area	52.48	*33.79*	**64.19**	54.83	46.34
Leaf shape	27.30	24.86	*19.67*	**31.87**	20.07

8. ANALYSIS OF *INDIGOFERA ZEYHERI* ACCORDING TO TOXICITY

Indigofera zeyheri plants were separated into two toxicity groups based on groundwater sulphate levels, measured from boreholes in the area of the slimes dam, which have been shown to be correlated with toxicity (Figure 29). Higher sulphate levels increase the toxicity of the groundwater to plants due to higher levels of osmotic stress. High sulphate levels also indicate a low pH of the groundwater, which leads to a higher concentration of heavy metals in the groundwater through increasing the solubility of these metals (Weiersbye et al., 2002). The depth to groundwater is important as it determines the extent to which the toxicity of the groundwater will impact on plants growing on the surface. A moderate depth to groundwater is evident throughout the area surrounding the New North Complex slimes dam (4-14 mbgl), with slightly shallower levels being evident in higher toxicity zones (Figure 30).

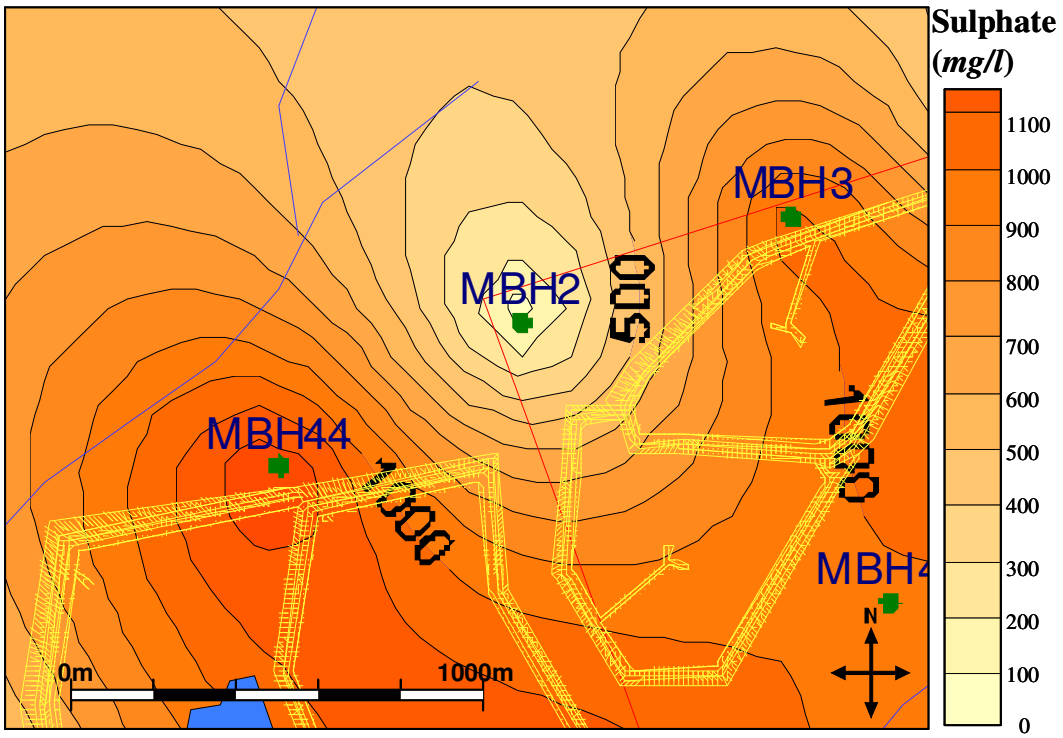


Figure 29: Zones of sulphate (mg/l) surrounding the New North Complex slimes dam. Individuals were separated into two toxicity groups. The low toxicity group contained individuals from zones of 200 – 500 mg sulphate/l, while the high toxicity group contained individuals from zones of 600-800 mg sulphate/l.

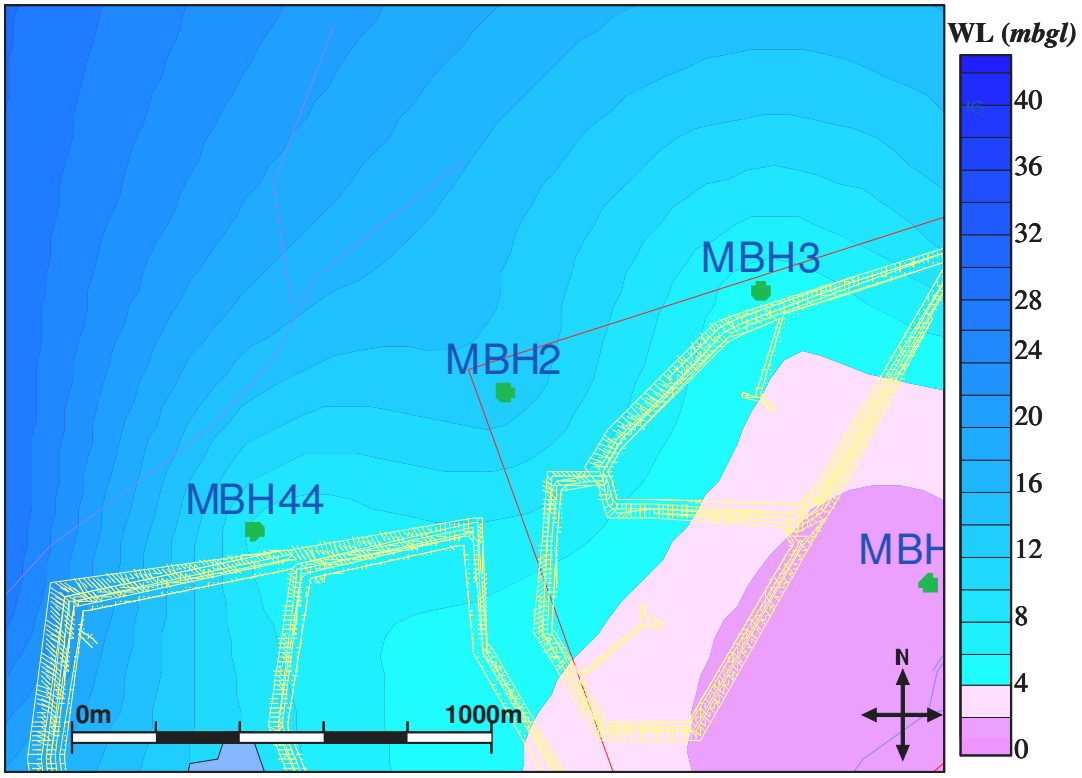


Figure 30: Zones of groundwater level in metres below ground level (*mbgl*) for the two toxicity zones. Individuals sampled were found in zones of 4-14 mbgl.

8.1. Genetic analysis using plants from all areas

Initial analysis of *Indigofera zeyheri* according to toxicity levels involved separating plants into two toxicity groups as described in Figure 29. Toepaddock and off site samples were included in this analysis, in order to determine if a significant difference exists between the two groups regardless of local growth conditions caused by proximity to the slimes dam. Genetic diversity and the percentage of loci polymorphic are higher in plants from the higher toxicity group (Table 43). Total genetic diversity is mostly due to diversity within toxicity groups, indicating that differentiation between plants from different toxicity groups is low. Estimated gene flow levels are high (Table 44).

Table 43: Genetic structure of plants from the two toxicity groups in terms of sample size, percentage of loci polymorphic, Shannon's diversity index (*I*) (mean ± standard deviation), and Nei's (1973) gene diversity (*h*) (mean ± standard deviation).

Area	Sample number	Percentage of loci polymorphic	*h*	*I*
High toxicity	27	79.41	0.2614 ± 0.1961	0.3918 ± 0.2701
Low toxicity	15	67.65	0.2159 ± 0.1953	0.3272 ± 0.2772

Table 44: Nei's gene diversity statistics (mean ± standard deviation) for subdivided populations, showing the genetic structure of the overall population. An estimate of gene flow (*Nm*) is also shown.

Statistic	Total genetic diversity (H_T)	Diversity within groups (H_S)	Gene flow between groups (*Nm*)
Value	0.2553 ± 0.0278	0.2386 ± 0.0265	7.1662

The F_{ST} value is 0.0249 ± 0.00242 (mean ± standard error). A permutation test for genetic differentiation among toxicity groups showed this F_{ST} value to not be significant. The null hypothesis is therefore not rejected, indicating that the groups are not more genetically diverse than a random assemblage of the individuals.

8.2. Morphological analysis using plants from all areas

Initial morphological analysis involved comparing morphology of off site and toepaddock plants that were separated into two toxicity groups. No variable came close to being significantly different between the areas at the $P<0.05$ level (Table 45). These groups of plants are therefore not significantly differentiated from each other. Therefore further morphological analysis was not performed on these groups.

Table 45: T-test showing which variables contribute significantly to differences between the toxicity groups (DF = 40). P values indicate which variables are significantly different between the areas.

Variable	T value	Pr > t
Plant Height	1.39	0.1731
Canopy Width	0.70	0.4854
Canopy Width 90°	0.79	0.4370
Leaf Length	0.35	0.7246
Leaf Width	0.25	0.8009

8.3. Genetic analysis using plants from the toepaddock only

Plants from the toepaddock only were separated into two toxicity groups. This was done to remove the effect of local variations in growth conditions caused by proximity to the slimes dam. As was seen in the initial toxicity groups, genetic diversity and the percentage of loci polymorphic are higher in plants from the group with higher toxicity (Table 46). Total genetic diversity is mostly due to diversity within toxicity groups, indicating that diversity between plants from different toxicity groups is low. Estimated gene flow levels are high, although lower than those estimated for the previous toxicity groups containing off site individuals (Table 47).

Table 46: Genetic structure of plants from the two toxicity groups in terms of sample sizes, percentage of loci polymorphic, Shannon's information index (*I*) (mean ± standard deviation), and Nei's (1973) gene diversity (*h*) (mean ± standard deviation).

Area	Sample size	Percentage of loci polymorphic	*h*	*I*
High toxicity	19	73.53	0.2504 ± 0.2045	0.3730 ± 0.2832
Low toxicity	7	50.00	0.1721 ± 0.1985	0.2591 ± 0.2852

Table 47: Nei's gene diversity statistics (mean ± standard deviation) for subdivided populations, showing the genetic structure of the overall population. An estimate of gene flow (*Nm*) is also shown.

Statistic	Total genetic diversity (H_T)	Diversity within groups (H_S)	Gene flow between groups (*Nm*)
Value	0.2343 ± 0.0303	0.2113 ± 0.0267	4.5879

The F_{ST} value for the overall population is 0.0289 ± 0.0142 (mean ± standard error). A permutation test for genetic differentiation showed this F_{ST} value to not be

significant. The null hypothesis was therefore not rejected, indicating that plants from different toxcity groups are not more genetically diverse than a random assemblage of the individuals.

8.4. Morphological analysis using plants from the toepaddock only

Morphological analysis was performed on the groups used in section 8.3. Differences in plant height are statistically significant at the $P<0.05$ level (Table 48). Graphs were created showing the distribution of plant height in the two different toxicity groups (Figure 31). This shows that plants in the high toxicity group have both a higher average height and a larger variation in height.

Table 48: T-test showing which variables contribute significantly to differences between the toxicity groups (DF = 24). P values indicate whether the variable accounts for significant differentiation between the groups.

Variable	T value	Pr > t
Plant Height	2.06	0.0502
Canopy Width	0.64	0.5274
Canopy Width 90°	0.18	0.8611
Leaf Length	0.12	0.9092
Leaf Width	0.00	0.9983
Canopy area	0.60	0.5538
Canopy volume	0.84	0.4102
Leaf area	0.56	0.5780
Leaf shape	0.21	0.8316

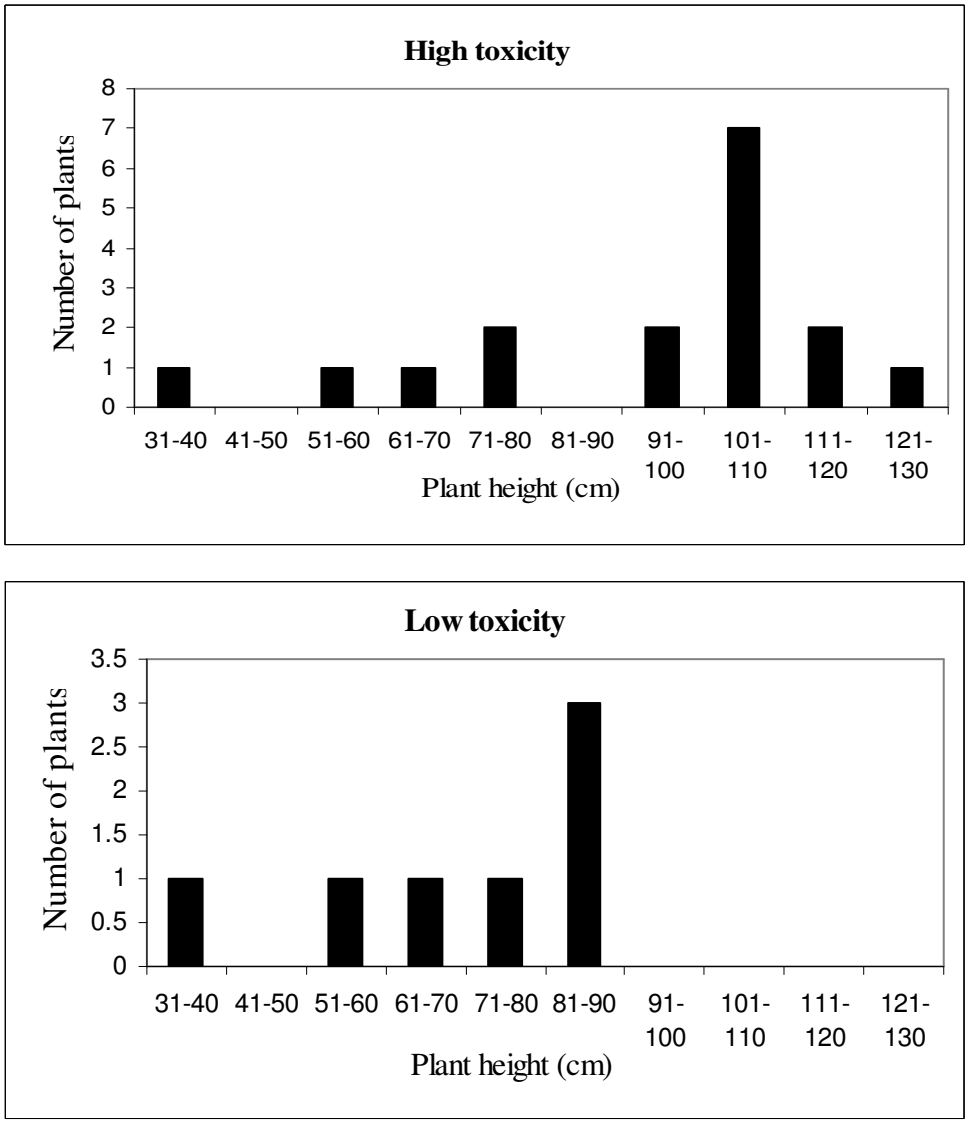


Figure 31: The distribution of plant height in the two toxicity groups. Note the different y-axis scales.

Differences in correlation coefficients are apparent in many pairs of variables. These differences are particularly notable in correlations involving plant height, emphasising the difference in this variable between plants in the different toxicity groups. A marked difference is also seen in the correlation of leaf length and leaf width, with this correlation being significant in plants from the high toxicity group, but not in plants from the low toxicity group.

Table 49: A comparison of correlation coefficients between various variables in plants from the different toxicity groups. The asterix (*) indicates a p value of less than 0.0001, showing the two variables to be highly correlated.

Correlation between	High toxicity	Low toxicity
Height and leaf length	0.518	0.126
Height and leaf width	0.387	-0.576
Height and canopy diameter	0.474	0.918
Height and canopy diameter90	0.390	0.530
Canopy diameter and diameter 90	0.768	0.350
Leaf length and leaf width	0.904*	0.366
Canopy area and leaf area	-0.424	0.091
Canopy volume and leaf area	-0.391	0.205

The diversity of each variable in plants within the different toxicity groups was determined by calculating coefficients of variation, in order to allow a comparison of morphological variation and genetic variation. Higher morphological variation was found in the higher toxicity areas, except in the case of plant height, the only variable shown to account for variation between the areas, and leaf shape, both of which show very similar coefficients of variation in the two groups (Table 50).

Table 50: A comparison of coefficients of variation for each variable in plants from the different toxicity groups. The higher coefficient for each variable is shown in bold.

Variable	High toxicity	Low toxicity
Sample size	19	7
Height	7.22	**7.76**
Canopy Width	**55.48**	45.10
Canopy Width 90	**70.77**	65.38
Leaf Length	**30.41**	16.13
Leaf Width	**22.36**	9.71
Canopy Area	**135.08**	66.00
Canopy Volume	**156.24**	68.60
Leaf area	**40.41**	21.79
Leaf shape	15.76	**15.96**

DISCUSSION AND CONCLUSIONS

1. Genetic differentiation between areas

Relatively lower genetic diversity was observed in plants more closely associated with the slimes dams, on the toepaddock and associated areas of the toepaddock wall and retaining wall, when compared to plants further away from the dam, i.e., off site and far off site areas. This difference was particularly notable when toepaddock and associated areas were compared to off site areas. These groups were sampled in close proximity to each other, and the sizes of the areas sampled were similar. Therefore the only difference between the areas was proximity to the slimes dam, with an increase in slimes-contaminated runoff and other pollution existing in the toepaddock and associated areas. This difference was found in both *Indigofera adenoides* and *Indigofera zeyheri*, although the difference was smaller in *I. zeyheri*. Lower diversity was noted in all measures of genetic diversity, namely the percentage of loci polymorphic, Shannon's information index (*I*), and Nei's (1973) gene diversity statistic (*h*).

It cannot be determined whether this difference in diversity is due to colonisation, persistence, or natural selection. A lower genetic diversity in the toepaddock and associated areas, together with the presence of some bands in the far off site but not toepaddock samples, provides support for colonisation of the dams occurring by a small number of individuals from the surrounding areas. Colonisation of toxic areas results in several members of the founding population dying shortly after colonisation, which would produce a loss of genetic variation in the colonising population, and the presence of unique alleles off the colonised area (Bush and Barrett, 1993).

In addition, changes in diversity could have involved persistence of plants from before the initial disturbance occurred, with individuals pre-adapted to the slimes-contaminated environment surviving, while other genotypes died out over time. It is unlikely that all plants sampled were persisters from before construction of the slimes dams, as many of them appeared very small and were not significantly

wooded, indicating they could not be over 25 years old. Retaining and toepaddock wall plants were not likely to be persisters, as these walls were created when the slimes dams were constructed and the height of the plants makes it unlikely they grew through from below the wall. These plants may, however, have resprouted from root stocks that were included intact when the retaining wall and toepaddock wall were created from soil from the surrounding areas.

The slimes dams studied are both over 25 years old. Both *I. adenoides* and *I. zeyheri* are perennial plants that presumably produce seeds once a year. The generation time is the time taken for newly established seeds to become reproductive adults, and it is not known how long this would take. However, it is likely that there had been several generations of plants between establishment of the dams and the time of the study. Gene flow levels were estimated to be moderate to high between areas. Therefore with these levels of gene flow occurring over so many years, why did differences in genetic variation still exist between areas? And why were unique alleles present in far off site populations that were not found on site? It could be that seedling mortality was so high that only a few plants from each seed production event survived, or natural selection could have played a role, resulting in only particular genotypes being able to survive on the toepaddock and associated areas.

Alternatively, genetic differences may have been maintained due to the observed difference in flowering time between the areas. Flowering occurred earlier in the season on the toepaddock and associated areas, presumably due to increased available water in these areas, due to continued deposition of moist slimes on the top of the dam. Increased amounts of available water could lead to more rapid growth and maturation of plants in toepaddock areas, restricting pollination between the areas and causing an isolation of toepaddock populations. Gene flow between areas would therefore have been due mostly to the movement of seeds rather than the movement of pollen. Lower genetic diversity in the toepaddock and associated areas therefore originated and was maintained through either a founder effect following colonisation, natural selection, flowering time differences, or a combination of these factors.

In *I. adenoides*, the initial grouping of plants (toepaddock, toepaddock wall/retaining wall, off site, and far off site A and B) had an overall genetic diversity (H_T) only marginally larger than the diversity within groups (H_S). This indicated a very low genetic differentiation between groups, indicating that significant gene flow occurred between the areas. This was supported by the measures of F_{ST} and Nm, which indicated high levels of differentiation within compared to between groups, and a large amount of estimated gene flow, respectively. Significant levels of gene flow between slimes dam-associated and off site groups were expected, as these areas are in extremely close proximity to each other and the studied species were shown to be not entirely self-fertilising following seedling analyses. Overall F_{ST} values indicated that the groups of plants were more genetically differentiated than a random assemblage of the individuals, but this appeared to be mostly due to the far off site group, which in pairwise F_{ST} analysis was shown to be significantly differentiated from the other groups. This is supported by the dendrogram based on measurements of Nei's genetic distance, which showed far off site plants to be distant from plants from other areas.

Following subdivision of *I. adenoides* groups (retaining wall, toepaddock wall, toepaddock, off site, far off site A, and far off site B), a more defined population structure became apparent. Toepaddock wall plants were significantly genetically differentiated from retaining wall plants, despite the morphological similarities of the members of these groups, and the fact that they grow in what appears to be very similar substrates on the dam. H_S and Nm values were reduced with this analysis, indicating less gene flow between areas than was seen with the initial set of groups. These groups of plants therefore showed a fair degree of differentiation from each other. This is supported by pairwise F_{ST} analysis, which showed the two far off site groups to be differentiated from all other groups, and from each other. Toepaddock plants seemed to be most similar to toepaddock wall and off site plants.

The sample sizes in the retaining and toepaddock wall groups were relatively small. This may have led to inaccurate indications of diversity in these groups. Plants from these areas had a lower genetic diversity than those from the toepaddock, even though the retaining wall and toepaddock wall would be expected to have less

contamination by slimes due to the raised ground and the presence of grassland soil in these areas. The degree of contamination would be even lower in those regions of the toepaddock wall that were further away from the slimes dam. Genetic diversity was low in plants from far off site A, probably due to the small area that this group was sampled from, when compared to the entire circumference of the dam used for sampling of toepaddock and off site groups, as shown on the sampling map (Figure 6). Far off site B plants were sampled from an even smaller area than those from far off site A, and yet plants from this area showed a high genetic diversity. This is interesting as far off site B was the only group that can be considered relatively unaffected by slimes-associated contamination.

In the overall population analysis of *I. zeyheri*, overall genetic diversity (H_T) was marginally higher than the diversity within groups of plants from each area (H_S). Estimated gene flow between areas was very high. These values indicate very little genetic differentiation between groups of plants due to a high degree of gene flow between areas. This is supported by F_{ST} analysis, which indicated that plants from the different areas were not more genetically differentiated than a random assemblage of the individuals.

The smaller difference in genetic diversity between plants from the two areas observed with this species may therefore be due to high levels of gene flow which reduced any effect colonisation and natural selection may have had. Alternatively, any plants on the toepaddock that had persisted from before the dam was established may not have been exposed to the disturbance for a long enough time to result in marked genetic changes. Younger plants that germinated in the slimes may be more genetically differentiated. The New North Complex dam is older (34 years old at the time of the study) than the South Complex dam (26 years old). This may have led to a smaller difference in diversity between the two areas at the New North Complex dam, as the plants at this site had more generations in which gene flow could lead to an elimination of the low diversity caused by colonisation. It would be necessary to perform a second study several years in the future to determine if this were the case.

2. Differences in morphology between areas

Morphological analysis of both species showed a large degree of differentiation between the areas. Morphological differences may be environmentally caused or may be genetically inherited and a fundamental part of the population (Antonovics et al, 1971). Morphological analysis may therefore indicate genetic changes in the selected genome – the physiological manifestation of traits that have been selected for. Alternatively, it could indicate phenotypic plasticity without a genetic basis. It is impossible to know whether the phenotypic differences observed were based on genetic variation between plants from the different areas, or whether they constituted phenotypic plasticity without a genetic basis. It is also impossible to determine whether the plasticity observed conferred fitness advantages on plants in the different areas or not. Some of the traits appear to have the potential to provide an advantage to the plants, as will be discussed, but this is not necessarily the case. Morphological variation may have been due to inherited differences present in a small colonising population that provide no actual benefit to the plant.

Differences in morphology were found between plants growing on different areas of the slimes dams. *Indigofera adenoides* plants growing on the toepaddock grew in a low, spreading fashion when they achieved a certain (adult) size, and were found growing only in sparsely colonised, slimes inundated areas. *Indigofera zeyheri* plants were found growing amongst other plants in highly colonised areas, and did not exhibit the spreading habit of *I. adenoides*. Differences in canopy area were observed between the areas in both species. Plant height differences were also observed in both species, although these differences were not statistically significant in *I. zeyheri*. Leaf width differences were observed in *I. adenoides* only. In contrast to genetic differences between the areas, morphological differences did not generally follow the same pattern in both species.

Plants adapted to local environments have been shown to have a decreased competitive ability (Antonovics et al., 1971). Therefore the absence of *I. adenoides* plants in highly colonised regions of the toepaddock may be an indication that adaptation and a subsequent reduction in competitive ability have occurred. A

spreading growth habit is also a possible indication of adaptation (Antonovics et al., 1971). The growth characteristics of *I. zeyheri* may therefore suggest that this species is less adapted to the toepaddock environment than *I. adenoides*, or that conditions at the New North Complex dam are less detrimental to plant growth than those at the South Complex dam.

In *I. adenoides*, canopy area was found to be relatively constant in the toepaddock wall, retaining wall, toepaddock, and off site plants, while being significantly lower in the far off site plants. This trend was followed with respect to canopy volume. It would be expected that plants would show a greater size on toepaddock and retaining wall areas due to decreased competition allowing increased growth. The noted trend of a more spreading growth on toepaddock soils would also lead to larger measurements of canopy area in these individuals. This does not however explain the larger size in off site areas, which has higher levels of vegetation cover and therefore increased competition.

A possible reason for the observed differences could therefore be that toepaddock, retaining wall, and off site plants were of larger size when compared to far off site plants due to increased water availability as water from newly added slimes would seep into these areas. Toepaddock and retaining wall plants should therefore have been larger than off site plants, due to reduced competition. These plants may, however, have been restricted in their growth due to the toxic component of an increase in slimes-contamination in these areas.

This suggested hypothesis seems to be supported by canopy area and canopy volume measurements for *I. zeyheri*. This species showed a marked reduction in canopy area and canopy volume in toepaddock plants when compared to off site plants. Since plants on both areas appeared to grow in sites that showed the same levels of colonisation, the levels of competition can be assumed to have been fairly equal and can be excluded from the comparison. Water availability levels should also have been similar between the areas. The difference in plant size may therefore have been entirely due to a difference in toxicity between the areas, with toepaddock plants being restricted in their growth.

Plant height data can also be explained using the same hypothesis. In *I. adenoides*, toepaddock area plants were shorter than plants in the retaining wall and off site areas, which are presumed to have lower toxicity due to the greater proportion of grassland soil present in these areas. Toepaddock plants were however taller than far off site plants, presumably due to less competition and increased levels of water. Far off site areas have more competition due to increased colonisation, and may have decreased levels of available water. In *I. zeyheri*, plant height was lower in off site plants, although not significantly. This may indicate that soils at the New North Complex slimes dam are less toxic than those at the South Complex slimes dam. It may also be an indication of the preferred growth conditions of the two species. Areas of the toepaddock are either inundated with slimes that has spilled out of the dam, or not inundated. *Indigofera adenoides* appeared to prefer slimes-indundated areas of the dam, while *I. zeyheri* appeared to prefer non-inundated areas. Therefore the local environment favoured by *I. adenoides* may be more toxic than that favoured by *I. zeyheri*.

Leaves of many species are known to be highly plastic in response to environmental conditions (Witkowski and Lamont, 1991). In *I. adenoides*, significant differences were found in leaf width between areas, with width tending to be larger in areas with a decrease in slimes-associated contamination. Leaf size has been shown to decrease with increasing altitude, decreasing mean annual temperature, decreasing mean annual rainfall, and lower soil fertility (Givnish, 1987; McDonald et al., 2003). Few studies have been done on leaf width and what variables may affect it. Within habitat variation in leaf size or shape may indicate adaptation to variations in ecological conditions within that habitat (Givnish, 1987).

In *I. adenoides*, leaves were shown to be significantly narrower in retaining wall and toepaddock plants. Altitude and rainfall are no different between the areas. Temperature may be slightly higher on the slimes dam than off due to less vegetation and therefore increased levels of radiation reaching the plants and reflecting off the slimes (white in colour) back at the plants. Plants growing on slimes dams may therefore have had narrower leaves in order to prevent water loss within this increased temperature regime. This hypothesis is supported by leaf

width measurements in *I. zeyheri*, which were not siginificantly different in plants from different areas. The New North Complex slimes dam has more vegetation cover, and appears to have higher rainfall levels and lower average temperatures. Temperature and radiation on and off the dam should therefore be similar.

Dendrograms, ANOVA, and Tukey analyses showed far off site plants, especially far off site B samples, to be morphologically differentiated from the plants in the other areas. This indicates that morphology of plants growing in the relative absence of slimes-contaminated soil is very different from that of plants growing in the presence of slimes-contaminated soil. The differentiation of far off site B plants was not surprising as these individuals were sampled from a site seven kilometers away from the other individuals. It is unlikely that gene flow occurs frequently between the two sites. Similar classification patterns were found with *I. zeyheri* individuals, with toepaddock classifying to itself, but off site classifying equally to toepaddock and off site. Classification analysis suggests that toepaddock plants consist of a subset of off site plants. This is expected since colonisation of slimes-contaminated soil was assumed to have occurred by plants from the surrounding areas.

3. Genetic and morphological differentiation

Genetic and morphological data did not follow similar patterns in most instances. In *I. adenoides*, dendrograms for genetic data showed plants from far off site A and B to be the most differentiated from plants in the other groups, while dendrograms for morphological data showed plants from far off site B to be highly differentiated from plants in the other groups, while far off site A plants were not. Morphological differentiation patterns probably indicate the differences in micro-climate and biotic habitat conditions between the greater slimes dam area, and the nature reserve that was the site of sampling for far off site B.

Morphological variation was highest in plants from the toepaddock for most of the single measurements, and highest in plants from far off site A for most of the composite variables. This is in contrast to genetic variation, which was lowest in the

toepaddock and associated areas, and in far off site A. This pattern was repeated in *I. zeyheri*, with toepaddock plants showing the highest morphological variation and the lowest genetic variation. High morphological variation is likely to be an indication of adaptation to different conditions within the toepaddock, for example, exposed or not, and slimes-inundated or not.

Indigofera adenoides classification analyses gave consistent results with genetic and morphological data. Classification based on morphological data gave the highest percentage of correct classifications in toepaddock and far off site B plants, while genetic classification gave the highest percentage of correct classifications in toepaddock and far off site A plants. Far off site A plants were therefore genetically differentiated from plants in the other groups, but not morphologically differentiated, presumably due to the similar climate in far off site A and the slimes dam areas. Toepaddock plants were shown to be most similar to themselves. This indicates the morphological and genetic differentiation of these plants from those in the other areas, due to the unique environmental conditions in the toepaddock.

It would seem that *I. adenoides* has adapted to toepaddock conditions more successfully than *I. zeyheri*. This was shown by *I. adenoides* plants growing only in sparsely colonised areas of the toepaddock, indicating a reduced competitive ability which may be indicative of adaptation (Hangelbroek et al, 2003), as well as by the prostrate, spreading growth habit seen on the toepaddock, which has been suggested to be an adaptation to low nutrient availability and exposed conditions (Antonovics et al., 1971). It cannot be determined, however, whether these differences are heritable differences, phenotypic plasticity, or a combination of the two.

In summary, high degrees of phenotypic plasticity existed between the different areas. It does not seem likely that all of this variation can be accounted for by genetic diversity, given the high levels of gene flow between areas and the comparatively low differences in genetic diversity. There is therefore a degree of non-genetic phenotypic plasticity, which may or may not confer an advantage to growth on slimes-contaminated soil. Morphological and genetic analyses indicated less differentiation between areas, and higher levels of gene flow, in *I. zeyheri*. This

may indicate less toxic conditions at the New North Complex slimes dam, or it may indicate a reduced ability to grow on toxic soils by this species when compared to *I. adenoides*.

4. Analysis according to groundwater toxicity

Analysis according to groundwater toxicity indicated little effect of groundwater toxicity on genetic or morphological diversity. Toxicity group analysis was performed based on data on the contamination of groundwater in the areas studied. *I. adenoides* plants were divided into six groups, with group 2 having the lowest groundwater toxicity, and group 7 having the highest. Initial analysis was performed containing all individuals, i.e., those from the toepaddock, retaining wall, off site and toepaddock wall. This was shown to result in no or very small morphological differences between the sites.

This was thought to be due to differences in growth conditions between the various areas. For example, the toepaddock has more available water than the other areas, due to seepage from the slimes dams, as well as less competition due to sparser vegetation cover. In contrast, off site areas have higher vegetation cover and therefore a higher organic matter content in the soil than the toepaddock and associated areas (Witkowski and Weiersbye, 1998). These area-specific differences would result in area-specific growth patterns, as was seen in morphological analysis of the area groups discussed above. These differences would result in a high within-group variation in morphological traits, which would result in any toxicity-related differences in morphology being undetectable.

Toxicity analysis was therefore performed with toepaddock plants only, in order to determine if a detectable difference could be noted between toxicity groups once the masking effect of proximity to the slimes dam was removed. In morphological analysis of *I. adenoides*, groups 6 and 7 were grouped together, due to the low sample sizes of the individual groups. Plants in group 4, from a moderate toxicity area, were found to have over twice the canopy area and canopy volume of plants from the other groups. Plants from this group classified accurately with the highest

percentage (75%), and were shown to be morphologically distant from the other groups on a dendrogram. Groups 2 and 3 were found to contain the most morphologically variable plants.

These results are confusing and seem to conflict with the results obtained from grouping plants by area. In area classification, toepaddock plants, which are presumed to grow on the highest toxicity, showed the highest morphological variability. This trend was followed in toxicity group analysis of *I. zeyheri*, where plants in the higher toxicity groups were found to have higher variation of most of the variables, especially canopy area and canopy volume, which were twice as variable in the higher toxicity group. *I. zeyheri* individuals were also found to be taller, and have increased variability in height, in the higher toxicity areas. This pattern was found in the area groups in this species.

A hypothesis has been developed to explain the results found in *I. zeyheri.* Evapotranspiration can cause contaminated groundwater to be drawn to the surface if the groundwater depth is shallow. Groundwater levels in the New North Complex area were, however, no higher than 4 metres below ground level. It is unlikely that the root systems of these plants would reach these levels, as they appear to be relatively shallow-rooted. Plants dug up were mature and obviously several years old, and had root systems only reaching approximately 60 cm deep. Seepage from the dams would also reduce evapotranspiration of the ground water due to it increasing the water level in the soil.

Indigofera zeyheri higher toxicity plants were found growing mostly in between two slimes dams, as was shown in the sulphate zone diagram (Figure 29). This area could have higher available water due to seepage from dams on either side, and also may be slightly protected from wind. This would result in both taller plants, and in a larger population size, which would result in higher genetic diversity. This is supported by genetic data, which showed a marked increase in genetic variation in the higher toxicity group, which could be due to a larger population size. The influence of the contamination level in the groundwater may therefore be

insignificant when compared to direct contamination by slimes runoff from the dams.

This hypothesis is not as easy to apply to results for *I. adenoides*. Samples were taken from all aspects of the South Complex dam, and ground water toxicity zones span the dam completely, making it unlikely that aspect would be responsible for differences in morphology. The groundwater levels in this area are very deep – over 14 metres below ground level. The contamination levels of the ground water are therefore unlikely to have influenced the plants growing on the toepaddock.

It can be postulated that the differences in sample size influenced variation in *I. adenoides*. It was noted that fewer plants were obtained in the higher toxicity groups, indicating the presence of fewer plants in these areas. This could reduce the variation of the plants in these areas due to a decreased gene pool within which gene flow can occur. This hypothesis is supported by genetic data, which showed low genetic variation in group 6/7. Genetic variation followed a pattern of decreasing slightly with increasing ground water toxicity up to group 4, then increasing dramatically in group 5, or group 5/6/7, and then decreasing dramatically to group 6/7, if measured. Low diversity in group 6/7 could simply be due to the smaller sample size of this group, while high diversity in group 5/6/7 could be due to the large area this group was sampled from. Gene flow probably occurred from the surrounding areas, so plants taken from a large area would have had a larger population to obtain genes from. This would increase the diversity of these groups.

F_{ST} values were significant for both populations, indicating that although genetic differentiation between groups was low, it is still statistically significant. Groups 6/7 and 5/6/7 were shown to be significantly different from the other groups, which was emphasised by dendrograms. It is possible that more gene flow from outside sources occurred in these areas. Fewer plants were found in these areas, indicating the population size may be small, which would result in the plants receiving more genes from outside areas.

Aerial photographs showed another slimes dam a short distance to the east of the South Complex slimes dam, close to the higher groundwater toxicity zones (groups 6 and 7). This dam could influence the surface toxicity of the soil in these areas due to slimes runoff and slimes dust blown over the area, which could account for the smaller population sizes noted. Plants in group 4 could therefore have been the largest in terms of canopy area because of increased water availability due to seepage from this dam, and showed a higher morphological diversity due to higher toxicity in this area, as was noted in the area groups. The lack of genetic diversity and sample size in groups 5, 6, and 7 may be because toxicity reached a critical level in these areas due to the presence of two slimes dams, so that sufficient growth and population size may not have been maintained. Plants were the shortest in this area, which may indicate stunting due to high toxicity and exposed growing conditions due to a lack of colonisation.

Any differences in morphology and genetics due to levels of toxicity in groundwater therefore seem to have been masked by differences in surface toxicity due to pollution from slimes dams, or other climatic, biotic, or edaphic factors. It would be necessary to repeat the study using data on surface soil toxicity in order to determine the effect local changes in toxicity have on morphological and genetic variation in these species.

5. Applications and further study

Slimes-contaminated soils had both a morphological and genetic effect on the *Indigofera* species studied. This appears to indicate some degree of adaptation to slimes-contaminated soil, either through phenotypic plasticity or adaptive genetic differences. The species studied therefore seem suitable as candidates for vegetation of slimes dams as well as the revegetation of the surrounding polluted soils.

The lower diversity observed in the toepaddock and associated areas should not have a detrimental effect on populations used for vegetating, as diversity was still comparatively high in these groups when compared to other plant populations studied (Jacquemyn et al., 2004; Solé et al., 2004). It would be important to

maintain the diversity that exists, however. This can be done by using a large population size to vegetate dams, in order to avoid further reductions in diversity through genetic drift due to small population sizes. Vegetation should be performed by taking plants that have naturally colonised the toepaddock and associated areas and using them, and their seeds, as starting populations for vegetation. This would ensure that any specialised genotypes that may exist in the toepaddock-adapted populations were included in initial vegetation populations.

Further studies that would be useful in determining the suitability of these species as candidates for vegetation would include the germination of seeds obtained from toepaddock plants and the analysis of subsequent progeny under controlled, non-toxic conditions to determine if the morphological differences observed are due to plasticity or heritable differences. Further long-term genetic diversity studies could also be performed to determine if diversity of individuals on the toepaddock increases or decreases further with time. Complete breeding system studies could also be performed.

The lack of *I. zeyheri* individuals far away from the dam could indicate this species only grows in the sheltered, higher water availability areas around the dams. Alternatively, individuals of this species may exist far away from the dams, but could not be located. *Indigofera zeyheri* needs to be studied further in order to gain a more in depth picture of this species' response to slimes-associated contamination. This analysis should include comparing slimes dam populations to a far off site population if one can be located.

Further study would be necessary to determine the effect of local changes in soil toxicity on these species. If a loss of genetic diversity occurs and persists with increasing toxicity, the overall adaptive ability of the population would be reduced. Soil toxicity data are needed in order to perform a more accurate study of morphological and genetic variation in response to soil toxicity.

6. Conclusions

1. Lower genetic diversity was observed in those areas around the dams with higher levels of slimes-associated contamination
2. Reduced morphological variation was observed in those areas around the dams with higher levels of slimes-associated contamination
3. Morphological differences were observed between the areas, some of which appear to provide some benefit to the plants
4. The above differences were all observed in both species
5. Some degree of adaptation to slimes-contaminated soil therefore seems to have occurred, with this being more pronounced in *I. adenoides*, although it cannot be determined whether this is purely phenotypic, or a combination of phenotypic and genetic
6. These species therefore seem suitable as candidates for vegetation of slimes dams, although further work must be done to determine the effect of surface toxicity on these species

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APPENDIX A - Raw morphological data

1. *Indigofera adenoides*

	Ht	CW	CW90	LL1	LW1	LL2	LW2	LL3	LW3	LL4	LW4	LL5	LW5
RW1	74	38	65	1.8	0.2	1.3	0.2	1.5	0.3	1.5	0.4	1.2	0.2
RW2	53	32	27	1.2	0.4	1.5	0.4	1.8	0.4	1	0.5	1	0.5
RW3	51	74	38	1.5	0.3	1.4	0.4	1	0.4	0.7	0.2	1.5	0.2
RW4	48	86	83	0.8	0.2	1	0.4	0.8	0.4	0.7	0.3	1.2	0.5
RW5	27	28	17	1.3	0.6	1.2	0.6	0.8	0.3	1	0.5	0.9	0.2
RW6	30	21	28	1.9	0.8	1.3	0.5	1.4	0.7	1	0.5	0.8	0.2
RW7	54	79	48	1.8	0.7	1.3	0.5	1.3	0.6	1.4	0.7	1.8	0.5
RW8	41	69	38	1.8	0.8	3	1.5	2.6	1.1	1.9	0.6	2.1	0.6
RW9	40	71	69	1.4	0.5	1.8	0.6	2.2	0.6	1.3	0.4	1.3	0.5
TPW1	46	44	19	1.5	0.5	1.4	0.6	1.5	0.4	1.2	0.5	1.3	0.5
TPW2	38	41	23	1.5	0.6	1.4	0.7	1.6	0.4	0.9	0.6	1.6	0.4
TPW3	47	23	20	1.5	0.7	1.6	0.5	1	0.5	1.3	0.4	1.5	0.4
TPW4	57	32	13	1.5	0.4	1.3	0.5	1.4	0.5	1	0.3	1.2	0.8
TPW5	42	45	18	1.8	0.5	1.8	0.6	1.8	0.8	1.5	0.8	1	0.6
TPW6	73	39	36	1.6	0.6	1.5	0.5	1	0.4	1	0.3	1	0.3
TPW7	45	69	48	1.4	0.3	1.6	0.4	1.3	0.3	0.8	0.5	1.4	0.4
TPW8	56	60	42	1.7	0.5	2.1	0.6	1.3	0.4	1.9	0.6	1.4	0.4
TPW9	53	50	25	1.7	0.4	1.5	0.4	1.3	0.5	1.6	0.4	1.5	0.4
TPW1(	32	38	26	1.3	0.5	0.9	0.2	1.1	0.4	1.2	0.4	1.1	0.5
TPW1	49	43	37	1.2	0.5	1.7	0.5	1.8	0.6	1.6	0.5	2.4	1
TPW1:	59	69	64	1.6	0.5	1.9	0.6	1.3	0.6	1.9	0.7	1.8	0.5
TPW1:	46	55	56	1.8	0.6	2.3	0.7	2.4	0.8	2.4	0.9	1.6	0.6
TPW1-	26	47	43	1.8	0.6	1.7	0.6	1.9	0.9	1.8	0.4	1.8	0.4
TP1	44	61	45	2	0.6	1.8	0.6	1.8	0.6	1.5	0.5	1.8	0.6
TP2	23	65	52	1.4	0.6	1.5	0.4	1.2	0.4	1.6	0.4	1	0.5
TP3	66	53	48	1.5	0.5	1.5	0.4	1.4	0.4	1.3	0.3	1.3	0.6
TP4	51	13	20	1.2	0.4	1.3	0.6	1.4	0.6	1.3	0.4	1.1	0.4
TP5	34	20	15	1.1	0.6	1.4	0.4	1.5	0.5	1.2	0.5	1.4	0.5
TP6	42	52	26	1.9	0.8	1.5	0.7	1.1	0.4	2	0.6	1.2	0.4
TP7	27	34	23	0.9	0.3	1	0.4	1.1	0.3	0.9	0.4	1.2	0.5
TP8	32	100	87	1.6	0.4	1.6	0.6	1.4	0.5	1.1	0.3	1.3	0.4
TP9	57	66	49	1.8	0.5	1.4	0.4	1.1	0.5	1	0.6	1.3	0.5
TP10	45	98	84	1.9	0.7	1.7	0.4	1.9	0.5	1.8	0.4	2.1	0.6
TP11	34	84	66	1.3	0.5	1.4	0.5	1.4	0.5	1.6	0.5	1.9	0.6
TP12	40	27	21	1.6	0.4	1.8	0.4	1.8	0.4	1.6	0.3	1.6	0.3
TP13	48	86	66	1.3	0.4	1.5	0.5	1	0.5	1	0.3	1.1	0.3
TP14	24	63	45	1.1	0.3	0.9	0.3	1	0.3	1.3	0.4	1.1	0.5
TP15	31	88	51	1.2	0.5	1.4	0.5	1.8	0.6	1.4	0.6	1.1	0.5
TP16	23	16	9	1.1	0.6	1.2	0.5	1.7	0.4	1.6	0.5	1.4	0.7
TP17	38	42	29	1.8	0.9	2.1	0.7	1.8	0.6	1.8	0.5	1.8	0.5
TP18	21	10	12	1	0.5	1.3	0.7	0.7	0.3	0.8	0.6	0.7	0.3
TP19	28	52	26	0.7	0.3	1	0.4	1	0.3	0.9	0.4	1	0.3

TP20	24	12	8	1.3	0.5	1	0.6	1.1	0.6	0.9	0.3	0.9	0.6
TP21	32	103	69	1	0.3	0.9	0.3	0.6	0.3	0.8	0.2	0.8	0.2
TP22	21	10	8	0.8	0.4	0.7	0.4	0.8	0.4	0.7	0.4	0.6	0.3
TP23	29	47	26	1	0.3	1	0.3	0.6	0.2	1	0.3	0.9	0.3
TP24	26	42	29	0.9	0.3	1.2	0.4	1	0.4	1.1	0.4	1	0.3
TP25	38	67	36	1.2	0.4	1.8	0.6	1.8	0.6	1.1	0.4	1	0.2
TP26	23	47	35	0.7	0.4	0.7	0.5	1.2	0.5	1	0.3	0.7	0.3
TP27	55	107	49	1.2	0.4	1.2	0.3	1.3	0.5	1.3	0.4	0.9	0.4
TP28	31	55	45	1.5	0.6	2.1	0.8	1.9	0.5	1.2	0.4	1.3	0.5
TP29	31	20	26	1.4	0.5	1.6	0.5	1	0.3	1.2	0.4	1.1	0.4
TP30	36	32	15	1.8	0.5	1.6	0.5	1.1	0.3	1.8	0.3	1.8	0.3
TP31	35	53	38	1.5	0.3	1.4	0.3	1.5	0.4	1.2	0.2	1.5	0.4
TP32	43	50	26	1.2	1	2.1	0.7	2.4	0.6	2	0.5	1.6	0.5
TP33	4	18	42	1.2	1	2.1	0.7	2.4	0.6	2	0.5	1.6	0.5
TP34	33	32	27	1.2	0.3	1.4	0.4	1.2	0.4	1.4	0.4	1.3	0.4
TP35	44	45	27	1.1	0.3	1.2	0.3	1.3	0.3	1.2	0.2	1	0.4
TP36	31	30	19	1.1	0.2	1.5	0.4	1.5	0.4	1.5	0.4	1.5	0.4
TP37	49	59	64	1.2	0.4	1.8	0.6	1.5	0.4	1.8	0.5	1.2	0.4
TP38	32	37	38	1.1	0.4	1.1	0.5	1.2	0.4	1	0.6	1.1	0.4
TP39	52	45	26	1.6	0.3	1.8	0.5	1.9	0.4	1.1	0.4	1.3	0.4
TP40	32	20	28	1.1	0.3	0.9	0.3	0.9	0.4	0.8	0.3	0.7	0.3
TP41	49	58	40	1.3	0.5	1.5	0.5	1.4	0.4	1.2	0.4	1.3	0.3
TP42	24	55	47	1.1	0.5	1.8	1	2.5	1.2	1.6	0.9	1.5	0.6
TP43	28	63	42	1.7	0.5	1.5	0.5	1.4	0.4	0.8	0.4	1	0.4
TP44	24	62	41	1.8	0.6	1.4	0.5	1.4	0.5	1.8	0.5	1.5	0.4
TP45	24	79	84	0.7	0.4	1	0.4	0.8	0.4	0.8	0.5	0.9	0.4
TP46	18	73	45	0.9	0.2	1.3	0.4	1.1	0.2	1	0.4	1.1	0.2
TP47	50	62	60	1	0.4	0.9	0.2	1	0.2	1	0.2	1.1	0.3
TP48	28	23	10	1.1	0.6	0.9	0.5	1.1	0.4	0.9	0.4	1.5	0.5
TP49	24	46	38	0.9	0.4	0.9	0.6	0.9	0.5	0.8	0.4	0.9	0.5
TP50	35	61	58	1.4	0.4	1.3	0.5	1	0.5	1.1	0.4	1.3	0.4
TP51	24	78	50	1.1	0.3	1.4	0.3	1.4	0.3	1.4	0.4	1.1	0.2
TP52	25	47	18	1.4	0.3	1	0.2	1.1	0.2	0.9	0.2	1.3	0.2
TP53	30	47	20	1.6	0.5	1.2	0.5	1.3	0.5	1.1	0.2	0.8	0.3
TP54	30	46	29	1	0.4	0.8	0.3	1	0.4	0.9	0.4	0.8	0.2
TP55	33	58	29	40	0.5	1.7	0.5	2	0.5	1.7	0.5	2.1	0.4
OS1	27	25	18	2.1	0.5	2.1	0.5	1.6	0.7	1.6	0.6	1.8	0.6
OS2	31	38	36	1.7	0.6	1.9	0.6	2	0.5	2	0.6	1.2	0.5
OS3	30	17	16	0.8	0.3	1.1	0.3	1.1	0.3	0.9	0.4	1	0.4
OS4	45	48	38	1.3	0.4	1.6	0.4	1.6	0.4	2.1	0.8	1.8	0.6
OS5	26	39	20	1.4	0.4	1.4	0.7	1.4	0.6	1.2	0.5	1.2	0.4
OS6	28	61	46	1.9	0.9	2.2	1	2.5	1.1	2.7	1.1	2.2	1
OS7	38	37	30	1.7	0.5	1.5	0.5	1.3	0.3	0.6	0.3	1.1	0.3
OS8	29	23	19	1.6	0.4	1.4	0.5	1.1	0.3	1.4	0.4	1.1	0.5
OS9	28	42	28	1.3	0.5	1.7	0.4	1.4	0.4	1.1	0.4	1.3	0.5
OS10	42	36	28	1.4	0.5	1.5	0.5	1.5	0.5	1.2	0.5	1	0.6
OS11	36	107	83	1.2	1	3.5	0.6	2.7	0.9	2.1	0.8	2	0.6
OS12	22	60	43	1.9	0.5	1.7	0.4	1.7	0.5	1.3	0.2	1.7	0.4
OS13	35	47	34	1.3	0.2	1.8	0.3	1.1	0.4	0.8	0.2	1.6	0.3

OS14	63	15	48	2.4	0.5	2.4	0.5	2.4	0.4	2.4	0.4	2.1	0.3
OS15	35	50	56	2.4	0.4	1.6	0.4	1.6	0.4	1.7	0.4	1.9	0.3
OS16	55	55	49	1.8	0.5	2.4	0.3	1.8	0.3	1.4	0.4	2.2	0.5
OS17	27	76	79	1.5	0.8	1.6	0.8	1.2	0.5	1.4	0.4	1.4	0.5
OS18	35	52	21	1.8	0.6	2.3	1	1.9	0.6	1.9	0.6	1.9	0.7
OS19	53	93	31	1.9	0.7	2.8	1.4	2.8	0.8	1.9	0.8	1.8	0.9
OS20	45	37	36	2	1.3	1.9	0.9	2.2	0.8	2.2	0.9	1.5	1
OS21	41	45	26	1.5	0.5	1.3	0.5	1.2	0.6	1.6	0.5	1.3	0.6
OS22	45	55	59	2.1	0.9	2.2	0.8	2.2	0.8	1.9	0.6	1.9	0.6
OS23	67	55	28	1.3	0.3	1.3	0.4	1.2	0.5	1.2	0.2	1	0.5
OS24	60	43	47	1.4	0.3	1.4	0.3	1.3	0.3	1.3	0.3	1.2	0.2
FOSA1	27	29	24	1	0.4	1.1	0.6	1.2	0.5	1.9	0.4	0.9	0.5
FOSA2	36	42	32	1.8	0.6	1.6	0.6	1.6	0.8	1.1	0.6	1.9	0.6
FOSA3	35	52	38	1.1	0.3	2.5	0.6	2.2	0.6	1.8	0.4	1.5	0.3
FOSA4	31	28	23	1.8	0.6	1.6	0.5	1.7	0.6	1.6	0.3	1.9	0.4
FOSA5	32	34	14	1.4	0.7	1.6	0.5	1.5	0.6	1.6	0.5	1.7	0.4
FOSA6	33	40	34	1.4	0.4	1.5	0.3	1.4	0.4	0.9	0.3	1.3	0.4
FOSA7	31	33	15	1.5	0.6	1.9	0.6	1.6	0.5	1.7	0.6	2	0.6
FOSA8	36	34	31	1.2	0.5	2	0.9	2	0.8	1.8	0.5	1.9	0.6
FOSA9	36	68	56	1.3	0.6	1.1	0.5	1.2	0.5	0.8	0.4	0.8	0.5
FOSA10	35	34	26	2.8	0.7	2.4	1	2.6	0.6	2.9	0.8	2.6	0.7
FOSA11	17	6	3	1.7	0.5	1.2	0.6	1.8	0.5	1.7	0.5	0.8	0.5
FOSA12	34	73	48	1.4	0.6	1.7	0.7	1.7	0.9	1.6	0.6	1.1	0.7
FOSA13	23	41	28	1	0.5	1.1	0.5	1.2	0.5	1.4	0.4	0.9	0.4
FOSA14	21	32	15	1.5	0.6	1.4	0.7	1.4	0.8	1.3	0.7	1.2	0.5
FOSA15	24	40	31	0.9	0.5	0.8	0.4	0.9	0.5	1.3	0.8	0.9	0.4
FOSA16	24	41	36	1.9	0.8	1.4	0.5	1.7	0.4	1.9	0.9	2.1	0.8
FOSA17	25	42	35	2.3	0.6	2.1	1.1	2.2	1	1.8	1	2.3	1
FOSA18	31	33	30	0.9	0.4	1.1	0.5	1.2	0.6	1.3	0.4	1.2	0.4
FOSA19	13	24	16	1.6	0.6	1.6	0.6	1.2	0.5	1.9	0.5	1.4	0.5
FOSA20	21	34	19	1.8	0.6	2.6	0.9	2.9	0.9	3	1.1	1.2	0.4
FOSA21	21	26	16	1.4	0.6	1	0.4	1.2	0.4	1.2	0.4	1.5	0.9
FOSA22	29	28	26	1.1	0.5	0.9	0.3	1.1	0.5	0.9	0.5	1.2	0.4
FOSA23	35	47	45	3.1	1	1.6	0.5	1.9	0.5	2.1	0.7	1.7	0.6
FOSA24	36	66	45	2.2	0.8	2.5	0.8	2.2	0.5	2.2	0.6	2.1	0.6
FOSB1	25	29	14	2	0.8	1.5	0.5	1.9	0.6	1.9	0.6	1.6	0.5
FOSB2	16	17	13	1.6	0.7	1.8	0.5	1.6	0.5	2.1	0.6	1.7	0.4
FOSB3	19	29	14	1.7	0.8	1.7	0.6	1.4	0.4	1.8	0.9	1.9	0.6
FOSB4	45	34	17	1.8	1.3	1.5	1.2	1.2	1	1.3	1	1	0.6
FOSB5	23	26	18	1.3	0.6	1.5	0.5	1.2	0.5	1.3	0.5	1	0.5
FOSB6	28	39	25	2.2	1.1	1.8	0.6	1.4	0.3	1.6	0.4	1.2	0.4
FOSB7	23	29	12	1.3	0.5	1.4	0.7	1.5	0.9	1.7	0.8	1.4	0.6
FOSB8	47	35	28	1.8	0.8	1.3	0.6	1.5	0.6	1.3	0.8	1.5	0.6
FOSB9	26	29	28	1.5	0.6	1	0.4	1.4	0.5	1.1	0.4	1	0.4
FOSB10	24	20	19	1.8	0.8	1.5	0.6	1.3	0.5	1.4	0.3	1.6	0.6
FOSB11	28	32	19	1.5	0.7	1.6	0.4	1.8	0.5	1.9	0.5	1.6	0.5
FOSB12	30	33	22	1.7	0.5	1.8	0.6	1.5	0.4	1.4	0.5	1.3	0.4
FOSB13	27	39	21	1.1	0.4	1.4	0.4	1.2	0.5	1.1	0.4	1.2	0.5
FOSB14	25	24	16	1.4	0.6	1.3	0.4	1.9	0.5	1.4	0.3	1.4	0.4
FOSB15	31	23	18	1.5	0.5	1.2	0.4	1.6	0.3	1.5	0.5	1.5	0.5
FOSB16	24	30	23	1.5	0.4	1.6	0.6	1.6	0.5	1.5	0.4	1.8	0.4
FOSB17	31	40	23	2.5	0.8	2	0.8	1.8	0.6	1.3	0.4	1.2	0.4
FOSB18	18	26	23	1.5	0.6	1.6	0.8	1.4	0.6	1.2	0.6	1.5	0.6
FOSB19	29	11	11	1	0.4	1.8	0.6	1.2	0.3	1.5	0.5	1.6	0.5

2. *Indigofera zeyheri*

	Ht	CW	CW90	LL1	LW1	LL2	LW2	LL3	LW3	LL4	LW4	LL5	LW5
TP1	67	37	50	2.5	2.1	1.8	1.6	3.2	3	2.2	2.4	2	1.9
TP2	79	55	32	2.8	2.1	2.7	2.1	2.5	3	2.2	2.7	2.3	2.7
TP3	84	47	31	3.1	2.8	2.1	2	3.2	2.8	1.8	1.5	2.1	2
TP4	89	55	24	3.2	2.8	2.4	2.1	2.8	2.5	2.5	2.5	2.8	2.7
TP5	88	48	19	3.1	2	3.3	3	2.6	2	2.4	2.1	2.2	1.7
TP6	57	25	15	2.1	2.8	2.7	2.7	2.4	2.5	1.9	1.9	2.3	2.5
TP7	85	37	59	3.8	2.5	3.5	2.5	4	2.7	3.1	2.3	3.9	2.8
TP8	36	8	6	3.1	3	3.3	3	2.3	2.3	3.1	3	2.9	2.7
TP9	96	56	37	2.4	2.1	2.7	2	3.3	3	2.5	2.3	3.5	3.4
TP10	100	59	45	3	3	2	2	3.7	3.6	3.2	3.2	3	3.1
TP11	50	14	11	1.7	1.7	1.9	1.5	1.9	1.9	2	2	2.3	1.8
TP12	75	16	12	3	1.9	2.9	2.5	3	2	2.8	2.2	2.8	2.3
TP13	104	12	32	3.5	2.5	3.5	2.4	3.7	2.9	3.1	2.7	2.8	2.4
TP14	64	18	25	2.1	2	2.4	2.3	2	1.5	2.7	2.7	2.5	2.5
TP15	76	73	23	2.4	2.4	2.4	2.2	2.5	2.3	2.6	2.5	2.3	1.9
TP16	128	102	102	2.2	2.2	2.2	2	1.9	2.1	2	1.8	1.1	1.3
TP17	105	69	55	2.3	2.4	2.6	2.6	1.2	1.4	1.3	1.1	1.9	2
TP18	111	67	59	2.9	3	2.8	2.8	2.5	2.1	2.3	2.4	2.6	2.5
TP19	104	65	28	1.8	1.8	1.7	1.7	1.7	1.8	1.8	1.7	1.7	2
TP20	79	41	25	2.5	3	2.4	2.3	2.5	2.6	2.6	2.6	2	2.1
TP21	118	36	18	3.9	3	3.4	2.6	2.7	2.7	3.8	3.6	3.2	2.9
TP22	34	15	12	1.8	2.2	1.4	1.2	1.5	1.8	1.1	1.3	1	1.2
TP23	56	11	13	3.1	2.9	3.3	3.1	2.6	2.5	2.6	2.8	3	3.2
TP24	110	32	17	3.5	1.9	1	1.8	4.5	2.9	4.5	2.7	4	2.6
TP25	89	49	45	3.1	2.8	3.3	3.2	2.6	2.7	2.6	2.6	3	3
TP26	48	2	4	2	1.9	2.4	1.9	2.4	1.6	2.4	1.8	1.9	1.7
TP27	106	29	20	1.9	2.2	3.1	2.5	2.5	3.3	2.9	3.4	2.6	3.3
TP28	112	24	15	2.9	2.5	3.1	2.4	2.8	2.4	2.8	2.1	3	2.8
TP29	59	10	9	3.1	3.1	2.8	2.6	3.1	3	2.9	3.2	3.2	2.9
TP30	110	18	12	3.5	2.9	3.5	2.5	3.3	2.3	2.8	2.3	2.7	1.7
TP31	87	58	33	2.9	2.9	2.8	2.6	1.7	1.8	2.9	2.8	2.3	2.4
TP32	110	18	17	3.4	3.2	4.1	3.2	3.6	3	3.9	2.8	4.4	2.8
TP33	103	48	20	3.1	3.2	4.1	3	3.8	3.6	3.9	2.5	3.9	4
TP34	94	31	17	3.6	2.4	3.7	2.9	3.5	3.1	3.5	2.9	3.7	2.6
TP35	123	41	14	4	3.5	4.2	3.6	3.6	2.7	3.8	2	3.5	2.6
TP36	120	49	29	3.6	2.2	3.9	2.3	4.2	3	2.8	2.5	4	2.4
TP37	107	38	20	3.5	2	3.3	2.6	3.7	2.4	3.8	2.8	3.5	3
TP38	92	41	37	2.5	1.9	2.1	2	2.4	2.1	2.5	2.3	2.3	2.1
TP39	112	82	55	1.9	2.2	2.2	2.1	2.5	2.3	1.9	1.8	1.2	1
TP40	132	66	63	2.3	1.9	2.7	2	2	1.8	1.5	1.4	3.2	2.6
TP41	62	8	5	3.1	2.8	3.1	2.8	3.2	3	3.1	3.1	3.2	3.2
TP42	132	102	99	3	2.5	2.1	1.9	1.8	1.6	1.3	1.2	1.8	1.9
TP43	105	35	29	3.6	3.5	3.5	3.2	3.7	3.8	1.7	1.8	1.5	1.8
TP44	93	42	26	3.2	3	2.1	1.7	2	2	1.8	1.7	2	1.9
TP45	62	8	5	1.5	1.7	2.3	2	2.8	2.4	3	2.8	1.7	2.1
TP46	86	79	49	2.5	2	2.5	2.1	1.8	1.8	3.1	2.3	3.5	2.8
TP47	96	58	54	3.5	3.3	2	2	1.7	2	1.7	2	1.9	2.2
TP48	71	40	35	2.6	2	2.6	2.2	1.8	1.4	3.4	2.6	3.3	3
TP49	108	68	59	3.7	4.1	3.4	3.3	2.5	3.2	3.5	3.8	2.8	2.7
TP50	72	43	55	3.2	2.9	4	3.5	2.6	2.6	3.8	3.1	3.5	3.1
TP51	131	67	72	2.2	2.4	2	1.6	2	2.3	2.5	2.8	3.2	3.2

TP52	53	30	23	2.8	2.2	2.6	2.4	2.2	2.6	2.9	2.6	2.4	2.2
TP53	94	43	20	1.8	2	2.4	2.3	2.8	3.1	2.6	2.9	3.2	3.5
TP54	55	41	20	2.8	2.5	3.2	3	2.9	3.4	2.5	2.7	3.8	3.4
TP55	91	33	16	3	2.4	2.8	2.2	2.6	2.6	3.2	2.8	2.9	2.9
TP56	59	39	28	3.5	2.8	3.7	3	2.6	2.3	3.7	3.3	3.6	3.4
TP57	86	44	36	1.4	1.6	3	2.9	1.5	1.4	2.6	2.5	3.2	3.6
TP58	115	119	90	3.6	2.4	2.5	2.1	2	2	2	2	3.6	3.2
TP59	117	54	62	2.1	2.2	2.1	2.1	2.2	2.2	2.8	2.4	2.4	2.8
TP60	179	21	18	3.3	3.5	3.5	3	3.2	3.4	3.2	3	1.5	1.4
TP61	33	17	17	2.2	2.5	2.8	2.7	3	3.3	3.4	3.1	2.6	3.1
TP62	97	53	49	3.3	2.7	2.7	2.6	3	2.7	3.2	2.8	3.4	2.8
TP63	60	30	24	3	1.8	3.5	3.4	3.5	2.8	1.4	2.3	3	2.5
OS1	76.5	37	19.5	3.1	3.5	2.3	2.9	3.2	3.3	2.7	2.7	2.3	2.4
OS2	109	54	30	2.6	2.2	2.1	2	3.5	3.6	2.1	2.4	2.7	2.6
OS3	125	110	100	1.9	1.8	1.8	1.3	1.8	1.5	1.9	1.4	1.5	1.4
OS4	72	46	20	2.8	2.8	3.2	3.1	1.8	2.2	3.4	3.1	3.1	3
OS5	81	49	29	3.7	3.2	2.5	2.3	3.6	4.2	2.4	2.4	3.3	3.7
OS6	86	84	45	3.6	3.3	2.1	2.4	3.1	3.2	3.2	2.7	1.7	1.9
OS7	124	37	20	2.1	2.2	2.1	2.5	2.1	1.7	3.6	3	3.8	3.3
OS8	64	16	10	3.2	3.1	3.2	2.9	3.2	2.8	2.7	3	2.3	2.4
OS9	98	46	55	3	2.8	2	1.8	1.8	1.5	1.7	1.5	1.7	1.7
OS10	84	75	51	2.9	3.4	3.5	2.9	3.4	3.6	2.7	2.6	1.7	2
OS11	93	60	28	2.7	3.4	3.1	3.3	2.9	3.7	1.7	2.1	1.6	2.1
OS12	90	160	120	3.1	3.5	3.6	3.5	3.1	3.6	3.4	3.8	3.3	3.4
OS13	58	68	32	2	2.9	2.1	2.8	2.4	3.2	2.6	3.1	2.3	2.8
OS14	135	110	45	2.6	2.8	2.3	2.6	2.3	2.8	2.2	2.6	1.8	2
OS15	122	110	103	2.3	2.4	2.7	2.8	2.3	2.4	1.8	1.8	2.9	2.6
OS16	50	56	48	2.9	3.2	2.8	3.1	3	3.1	3.3	3.8	3.6	3.8
OS17	85	65	63	2.2	2.3	2.2	2.3	2.2	2.4	3.1	3.4	3.3	3.6
OS18	91	65	38	2.8	3.2	2.5	2	3	3.1	2.9	3.2	2.7	2.9
OS19	117	64	43	2.8	2.3	3.3	2.3	3.8	3.6	2.6	2.6	3.9	3.2
OS20	85	71	55	2.1	2	1.9	1.8	2	1.8	2.5	2.2	1.8	1.8
OS21	83	62	68	2	1.8	1.9	2.3	2.4	2.7	2.3	2.3	1.8	2.2
OS22	85	54	52	3.5	2.4	1.8	1.8	2	1.6	1.8	1.6	2	1.8
OS23	84	78	56	1.5	1.6	2.8	3.2	2.6	2.7	2.5	2.3	3	2.9
OS24	92	73	60	2.1	1.9	2.6	2.1	2.2	1.8	2.4	1.7	2	2.1
OS25	82	38	33	3.7	2.5	3.1	2.5	4	2.9	1.6	1.9	1.8	2
OS26	92	30	46	2.9	2.5	3.3	2.6	3.2	3.4	3.4	2.8	3.1	3.5
OS27	114	113	107	1.6	1.8	1.3	1.9	1.5	1.4	1.5	2	2.6	2.5
OS28	90	28	26	1.6	1.4	1.4	1.4	2.6	2.1	1.5	1.6	2.4	2.3
OS29	127	84	110	2.1	2.7	1.3	1.5	2.9	2.8	2	2.6	2.4	2.3
OS30	122	93	105	4.1	3.2	4.1	2.3	2.7	2.3	3.1	2.3	2.8	2.3
OS31	123	77	36	2.6	2.5	2.7	2.8	2.3	2.5	2.7	2.4	2.6	2.6
OS32	91	57	60	2.6	2.2	3.2	2.7	3.1	2.6	2.1	2.2	3	2.2
OS33	123	76	45	1.6	1.5	1.3	1.2	2.1	1.8	2.6	2.6	2.3	2
OS34	111	52	49	3.2	2.4	2.7	2.5	3.4	2.6	3	2.4	2.8	2.4
OS35	118	67	42	2.9	2.8	3.7	3.2	3.6	3	2.5	2.6	2.3	2

APPENDIX B - Raw genetic data

1. *Indigofera adenoides*

RW1 0 0 1 0 0 1 1 1 0 1 1 1 1 1 1 1 0 1 1 1 0 0 1 0 0 1 1 1 1 1 1 0 1 1 0 1 0 1 1 0 1 1 0 1 1
RW2 0 0 1 0 0 0 1 1 0 0 0 1 1 1 1 1 1 0 1 1 0 1 0 0 0 1 0 0 1 1 1 0 1 1 0 1 0 1 1 0 1 1 0 1 1
RW3 1 0 1 0 0 0 1 1 0 1 1 1 1 1 1 1 1 0 1 1 0 0 0 0 0 0 1 1 0 1 1 1 1 1 1 1 0 1 0 1 1 0 1 1 0 1 1
RW4 0 0 1 0 0 0 1 1 0 1 1 1 1 1 1 1 1 0 1 1 1 1 1 0 1 0 1 1 1 1 1 0 1 1 1 0 1 0 1 1 0 1 1 0 1 1
RW5 0 0 1 0 0 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 0 1 0 1 0 0 1 0 1 1 0 0 1 1 0 1 0 1 1 0 1 1 0 1 1
RW6 0 0 1 1 0 1 1 1 0 0 0 1 1 1 1 1 1 1 1 1 0 1 0 0 1 0 0 0 1 1 0 1 1 1 0 1 0 1 1 0 1 1 0 1 1
RW7 0 0 1 0 0 0 1 1 0 1 1 1 1 1 1 1 1 1 1 1 0 1 0 1 1 0 0 0 1 1 0 1 1 1 0 1 0 1 1 0 1 1 0 1 1
RW8 1 0 1 0 0 0 1 1 0 0 0 1 1 1 1 0 1 1 1 1 1 1 0 1 0 0 1 1 1 1 1 1 1 1 1 0 0 0 1 1 0 1 1 0 1 1
RW9 1 0 1 1 0 0 1 0 0 0 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 0 1 1 0 1 0 1 0 1 0 1 1 1 1 1 1 0 1 1

TPW1 1 0 1 0 1 0 1 1 0 1 1 1 1 1 1 1 1 1 1 1 0 1 0 1 0 0 0 0 1 1 1 1 1 1 1 0 1 0 1 1 1 1 1 0 1 1
TPW2 1 0 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 0 0 1 1 1 1 1 0 1 1 0 1 0 1 1 0 1 1 0 1 1
TPW3 1 0 1 1 1 0 1 1 0 0 1 1 1 0 1 1 1 1 1 1 0 1 0 0 1 0 1 1 1 1 1 1 1 1 1 1 0 1 0 1 1 0 1 1 0 1 1
TPW4 1 0 1 0 1 0 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 0 1 1 0 1 1
TPW5 0 0 1 1 1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 0 0 1 1 1 1 1 1 1 1 1 1 0 1 1 0 1 1 0 1 1 0 1 1
TPW6 0 0 1 0 0 0 1 0 1 1 0 1 1 1 1 0 1 1 1 0 1 0 0 0 1 1 0 1 1 1 1 1 1 1 0 1 0 1 0 0 1 1 0 1 1
TPW7 0 0 1 0 1 0 1 1 0 1 1 1 1 1 1 1 0 1 1 1 0 0 1 0 0 1 0 0 1 1 1 1 1 1 1 0 1 0 1 1 0 1 1 0 1 1
TPW8 1 0 1 1 1 0 1 0 0 0 1 1 1 1 1 1 0 1 1 1 0 0 0 1 1 0 1 1 1 1 1 0 1 1 1 0 1 0 1 1 0 1 1 0 1 1
TPW9 1 0 1 1 1 0 1 0 0 0 1 1 1 1 1 1 0 1 1 1 0 1 0 0 1 1 1 1 1 1 1 1 1 0 1 1 0 1 0 1 1 1 1 1 1 0 1 1
TPW10 1 0 1 1 0 0 1 0 1 0 1 1 1 1 1 1 1 0 1 1 1 1 1 1 0 0 1 1 0 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 0 1 1
TPW11 1 0 1 0 1 0 1 0 0 1 1 1 1 1 1 1 1 1 0 1 1 1 1 0 0 1 1 1 0 1 1 0 0 1 1 0 1 0 1 1 1 1 1 1 0 1 1
TPW12 0 0 1 0 1 0 1 0 0 1 0 1 1 1 1 1 1 1 1 1 1 1 0 1 0 1 1 1 1 1 1 0 0 1 1 0 1 0 1 1 1 1 1 1 0 1 1
TPW13 0 0 1 0 1 0 1 0 0 1 0 1 1 1 1 1 1 1 1 1 1 1 0 1 0 0 0 1 1 1 1 1 1 1 1 0 1 1 0 1 0 1 1 0 1 1 0 1 1
TPW14 1 0 1 1 1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 0 0 0 1 1 1 1 1 1 0 0 1 1 0 1 0 1 1 0 1 1 0 1 1
TPW15 0 0 1 0 1 0 1 0 0 1 0 1 1 1 1 0 1 1 1 1 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 0 1 1 0 1 1

TP1 0 0 1 0 0 0 1 1 0 0 0 1 1 1 1 1 0 1 1 1 0 0 1 1 0 1 0 1 1 1 0 1 1 0 1 0 1 1 0 1 1 0 1 1
TP2 0 0 1 0 0 0 1 1 0 1 0 1 1 0 1 1 0 1 1 0 1 0 0 0 0 0 0 1 1 1 1 1 1 1 0 1 0 1 1 0 1 1 0 1 1
TP3 0 0 1 1 0 0 1 1 0 1 0 1 1 1 1 1 1 0 1 1 0 1 1 0 0 1 1 0 1 1 1 0 1 1 0 1 0 1 1 0 1 1 0 1 1
TP4 0 0 1 0 0 0 1 1 0 0 0 1 1 1 1 1 1 1 1 1 1 1 0 1 1 0 1 0 1 1 1 0 1 1 0 1 0 1 1 0 1 1 0 1 1
TP5 1 0 1 1 0 0 1 0 0 0 0 1 1 1 1 1 0 0 1 0 1 0 0 0 1 1 0 1 1 1 1 1 1 1 0 1 0 1 1 0 1 1 0 1 1
TP6 0 0 1 1 0 0 1 1 0 0 0 1 1 1 1 1 0 1 1 0 0 0 0 0 0 0 0 1 1 1 1 1 0 1 1 0 1 0 1 1 0 1 1 0 1 1
TP7 0 0 1 0 0 0 1 1 0 0 0 1 1 1 1 1 0 1 1 0 1 0 0 0 0 0 0 1 1 1 0 0 1 1 0 1 0 1 1 0 1 1 0 1 1
TP8 1 0 1 1 0 1 1 1 0 0 0 1 1 1 1 1 0 1 1 0 0 0 1 1 0 0 1 1 1 0 1 1 1 0 1 0 1 1 0 1 1 0 1 1
TP9 0 0 1 1 0 1 1 1 0 1 1 1 0 0 1 1 0 1 1 1 0 0 0 0 0 0 1 1 1 1 0 1 1 1 0 1 0 1 1 1 1 1 1 0 1 1
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TP12 1 0 1 1 0 0 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 0 0 0 1 1 0 1 1 1 1 1 1 1 1 0 1 1 0 1 1 0 1 1 0 1 1
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TP16 1 0 1 1 0 0 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 0 1 0 1 1 1 1 1 0 1 1
TP17 1 0 1 1 1 0 1 1 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 0 1 1
TP18 1 0 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 0 1 0 1 1 0 1 0 1 1 1 0 1 1 1 1 0 1 1 0 1 1 0 1 1
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TP21 0 0 1 1 1 0 1 0 0 1 1 1 0 1 1 0 1 1 1 1 0 1 0 0 1 0 1 0 1 1 0 1 1 1 0 1 0 1 1 0 1 1 0 1 1
TP22 1 0 1 1 1 0 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 0 0 0 1 1 1 1 1 1 1 0 1 1 0 1 0 1 1 0 1 1 0 1 1
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TP25 0 0 1 1 0 0 1 1 0 1 1 1 1 1 1 1 1 1 1 0 1 0 1 0 0 0 1 0 0 1 1 1 0 1 1 0 1 0 1 1 1 1 1 1 0 1 1

TP26 1 0 1 1 1 0 1 0 1 1 0 1 1 1 1 1 1 1 1 0 1 0 1 1 0 1 0 1 1 1 1 0 1 0 1 0 1 1 0 1 1 0 1 1
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TP29 0 0 1 0 1 0 1 0 0 0 1 1 1 1 1 0 1 1 1 0 0 0 0 0 0 1 1 1 1 1 1 1 1 0 1 0 0 0 1 1 0 1 1 0 1 1
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TP31 0 0 1 0 0 0 1 1 0 0 1 1 1 1 1 1 1 1 0 1 1 0 0 0 0 1 1 1 1 1 1 1 1 0 1 1 1 1 0 1 1 1 1 1 0 1 1
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TP33 0 0 1 0 1 0 1 1 0 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 0 1 1 0 1 1 0 1 1
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TP38 0 0 1 0 1 0 1 1 1 0 0 1 1 1 1 0 1 0 1 1 1 0 1 1 1 1 1 1 1 1 1 0 1 1 0 1 0 1 1 1 1 1 1 0 1 1
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2. *Indigofera zeyheri*

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