Cycad forensics:

Tracing the origin of poached cycads using stable isotopes, trace element concentrations and radiocarbon dating techniques

by
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Abstract

In South Africa, the greatest threat to cycad populations is the illegal removal of wild plants for landscaping purposes and collector's gardens, resulting in declining populations that are already threatened with extinction. Radiocarbon dating in conjunction with stable isotopes and trace element concentrations could provide the essential evidence needed in court when prosecuting poachers and collectors. The aim of this study was to determine the feasibility of using radiocarbon dating, stable isotopes and trace element concentrations to identify specimens removed from the wild. To test this method, we sampled two individuals of *Encephalartos lebomboensis* and *E. arenarius*, with known wild origins and relocation histories (1946 and 1992 respectively), and compared these to individuals from the same wild location that had not been relocated (controls). Vascular rings were sampled using an increment corer from the upper, middle and lower sections of the cycads, which were cut into sections so that the inner, middle and outer rings for each core could be sampled. These samples were reduced to cellulose for $\delta^{18}O$ and $\delta^{13}C$ isotope analysis. Leaf bases were sampled from the upper, middle and lower sections of the cycad stem using a hammer and chisel, and petioles were cut from the cycad to represent the most recent growth. These samples were analysed for the following stable isotopes $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, $^{87}Sr/^{86}Sr$, $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{207}Pb$, and the concentration of trace elements were analysed for the leaf bases. The means and variance were compared between the relocated and control specimens and a principal component analysis was done. The radiocarbon ages of *E. lebomboensis* tissues were more recent than expected, dating from 2006 to 1979, suggesting that this method would only be appropriate for specimens relocated within the past 6 to 30 years. Consistent with this, our $\delta^{18}O$ and $\delta^{13}C$ in cellulose, as well as the $\delta^{34}S$, $^{87}Sr/^{86}Sr$ ratio and principal component analysis indicated that the relocated *E. lebomboensis* specimen (relocated 66 years ago) was significantly different from the control, implying that the stem of the relocated specimen did not grow in the wild. In contrast, the relocated *E.arenarius* specimen (relocated 20 years ago) was isotopically indistiguishable from the control in older tissues indicating a common origin, but was significantly different for the upper leaf bases, as well as a shift in $\delta^{18}O$ cellulose for the upper vascular rings, indicating that the plant has been relocated. Thus, the use of radiocarbon dating and stable isotopes showed great potential as a forensic technique for cycads and suggestions for future development of this technique are given.

Chapter 1:

Status of cycads and background to developing a forensic technique

This chapter will discuss the current threats and conservation strategies for South African cycads and will provide the motivation behind why we should care about stopping the illegal cycad trade. We will provide the theory needed to develop a forensic technique that will help combat poaching by tracing wild cycads back to their origin. The plausibility for using various stable isotopes, trace element concentrations and radiocarbon dating to develop a method for tracing the origin of poached cycads will be summarized. Reviewing cycad anatomy, life history, and tissues age will allow us to develop an appropriate sampling method. Thereafter the second chapter will test if stable isotopes, trace element concentrations and radiocarbon dating can trace poached cycads from the wild. Development of this method will potentially stop illegal trade by targeting the end user, i.e. cycad buyers and collectors.

Why are cycads threatened?

According to the fossil record, cycads have been on Earth since the Early Permian period, 280 million years ago (MYA; Donaldson, 2003). Extant cycads are often referred to as "living fossils" and over the past few decades there has been an increased interest in cycads due to their ancient history and rarity (Norstog and Nicholls, 1997). Recent phylogenies, however, show that most extant cycads have recently diversified, as evidenced by the small genetic distances between species (Treutlein and Wink, 2002). Therefore, extant cycads are not as ancient as previously thought, but an evolving group of gymnosperms (Treutlein and Wink, 2002). For example, species within the genus *Encephalartos* (Zamiaceae) have spread through Africa and diversified only 5 to 1.6 MYA (Treutlein and Wink, 2002).

There are over 3 000 species within 11 genera in the Order Cycadales, which are distributed within the tropical, subtropical and warm temperate regions of the Americas, Southeast Asia, Australia and Africa (Hermsen et al., 2006). Although cycads have a large geographic distribution, species have naturally small and scattered populations, which limit their ability to reproduce (Donaldson, 2003). Cycads are long lived species with varying life-spans, growth-forms and reproductive biologies and have adapted to persist in isolated populations (Raimondo and Donaldson, 2003). Long generation times make cycads vulnerable to rapid changes in their environment, such as anthropogenic disturbances, because they are unable to adapt within the rapid time frame of change, increasing their risk of extinction (Giddy, 1995; Norstog and Nicholls, 1997; Brooks et al., 2008).

According to the global conservation assessment in 2010, cycads are the most threatened group of plants in the world (Raimondo et al., 2009). Of the 261 species with known population trends 79% of them are declining, and it is so severe in South African populations that is has been referred to as the "cycad extinction crisis" (M. Pfab pers. comm., 2011; Cousins et al., 2012). Within the African genus *Encephalartos,* four species are already extinct in the wild, 18 are Critically Endangered and 10 are classified as Endangered (IUCN, 2012). South Africa is a centre of cycad diversity with 37 of the 66 *Encephalartos* species and the single *Stangeria* species (Donaldson, 2008). Unfortunately, 78% of South African cycads are classified as threatened (Raimondo *et al*., 2009) and listed under the Convention on International Trade in Endangered Species (CITES) Appendix I, meaning they are threatened with extinction or will become threatened unless trade is restricted (Raimondo et al., 2009; CITES, 2012; Cousins et al., 2012).

The greatest threat to cycads is the illegal harvesting of wild plants for ornamental purposes in private gardens and landscaping, affecting all but two of South Africa's 38 cycad species (IUCN, 2012). Although mature plants are easily propagated, cycads grow slowly and take decades to reach a desirable or reproductive size, thereby promoting the removal of already established plants from the wild. However, exploitation for landscaping purposes is not enough to drive cycads into extinction; it is the exaggerated value of cycads because they are rare that results in them becoming even more desirable to collectors (Courchamp et al., 2006). This is called the anthropogenic allee effect, where humans place inflated value on species as they become rarer and the cost of finding species increases (Courchamp et al., 2006). Private collectors are prepared to pay up to R100 000 (US $15 000 – $20 000) per meter of stem height (Whitelock, 2002; Da Silva, 2005). For example, a large specimen of the Albany cycad (*E. latifrons*) was sold for $20 000 in the United States to collectors (Arendse, 2004), and an individual of *E. woodii,* which is extinct in the wild, was sold for R425 000 at a legal auction in Durban (Wray, 2004). Current prices of cycads can range from R8 to R5 000 per centimetre of stem depending on the sex and rarity of the specimen. The more common variety of *E. arenarius* would sell between R8 to R100 per centimetre of stem compared to the rarer, blue variety of *E. arenarius*, which would sell between R150 to R500 per centimetre of stem (R. Kunite pers. comm., 2013). The large monetary value placed by collectors on rare cycads gives incentives for people to poach wild cycads despite the risk involved.

Local communities utilize the starch rich stems and cones as a food resource, especially during famine, and sometimes the leaves are taken to make straps and baskets (Norstog and Nicholls, 1997; Donaldson, 2003). Some communities depend on traditional healers who harvest wild cycads for traditional medicines (Cousins et al., 2011). During 2009, the Warwick market in Durban sold an estimated 9 metric tons of bark strips and/or whole stems as traditional medicine (Cousins et al., 2011). Twenty-five *Encephalartos* species are known to be sold at Durban and Johannesburg markets for medicinal purposes, and although these practices have been ongoing for generations, increasing human populations has resulted in harvesting rates that are unsustainable compared to historical use (Cousins et al., 2011).

Habitat loss through the construction of roads and dams, clearing of vegetation for agriculture and timber, and encroachment of alien invasive species all contribute to the decline of cycads (Cousins et al., 2011; Whitelock, 1995). With the multiple factors threatening cycads over the past two decades, and the nature of cycad life history, it seems unlikely that future generations will be able to observe these "living fossils" within their natural habitat.

2. Importance of cycads

Why should we be concerned with conserving cycads in the wild? From an ecological perspective, is losing a few cycad species problematic to ecosystem functioning? Cycads have co-evolved with a number of non-symbiotic and host specific invertebrates, and their existence is dependent on cycad survival, therefore with the decline in wild cycad populations we are currently losing a number of species dependent on cycad survival (Norstog and Nicholls, 1997; Donaldson, 2003). Some cycad genera even have their own genus of curculionid (weevil) responsible for their pollination (Schneider et al., 2002). These weevils can even be species specific, indicating a long history of co-evolution (Schneider et al., 2002). Cycads play an important role in the life history of other species, due to their symbiotic relationships with nitrogen fixing cyanobacteria (arbuscular mycorrhizae), insect pollinators, bird and mammal dispersal agents, and obligate cycad feeders, all of which contribute to ecosystem functioning (Norstog and Nicholls, 1997; Donaldson, 2003).

The loss of cycads in the wild has social impacts for traditional healers who use them for medicinal practices, as well as communities who supplement their diet with the starch rich stems and seeds (Cousins et al., 2011). Already, traditional healers have to travel further to find the cycad material they need, for example, traditional healers collecting *Stangeria*

eriopus in 1988 had to travel an estimated 45% further in 1996 to collect materials in some parts of South Africa (Donaldson, 2003).

Furthermore, cycads can be considered umbrella species in certain areas that are associated with high biodiversity or threatened habitats, therefore the conservation and protection of these charismatic plants can protect unique habitats and species simultaneously (Donaldson, 2003). Not all cycads are associated with high biodiversity and threatened habitats. Therefore listing cycads as flagship species protects small cycad populations found in transformed habitat, as well as pockets of indigenous and threatened habitat that would otherwise be overlooked (Donaldson, 2003).

3. Current conservation strategies

The recent amendment of the National Environmental Management Biodiversity Act 2004, Section 57 (2) (NEMBA) in May 2012, is expected to help achieve the aims of Biodiversity Management Plans (BMP) through more stringent regulation of the cycad trade. Currently, removal and trade of any part of *Encephalartos* specimens in the wild that is listed as threatened or protected is illegal in South Africa, unless required for conservation or enforcement purposes (DEAT, 2012). Artificially propagated *Encephalartos* specimens listed as critically endangered or endangered with a stem diameter of more than 15 cm or in the case of subterranean stems more than 7 cm may not be traded within South Africa for the next five years or until conservation targets are achieved (DEAT, 2012). Threatened or protected *Encephalartos* specimens within this same size class may not be exported from South Africa (DEAT, 2012). Notwithstanding these strict protection measures, enforcement is hampered by human resource and budgetary constraints facing conservation authorities, subsequently resulting in unsustainable removal of wild cycads.

One of the action plans developed by the International Union for the Conservation of Nature (IUCN) cycad specialist group is to artificially propagate cycads using seeds collected from the wild to help restore wild populations (Donaldson, 2003). Nurseries run by communities in the vicinity of wild populations would sell rare species propagated from wild populations to satisfy the collector's and landscaper's market, thereby preventing illegal trade (Donaldson, 2003). This action plan also creates incentives to protect the wild populations providing the seeds as well as their habitat (Donaldson, 2003). Successful projects have been set up in Mexico with local communities, but the effectiveness of this plan is limited by adequate

marketing systems and developing reliable trading partners to ensure selling of the plants for the project to be self-sustaining (Donaldson, 2003). In South Africa, the BMP aims to implement this strategy on *E. latifrons*, a Critically Endangered cycad with less than 60 individuals left in the wild (Da Silva et al. 2012), with the aim to secure and restore existing populations (DEAT, 2010). This BMP allows landowners or communities to trade with propagated seedlings from wild cycads on condition that they have the correct permits and a certain percentage of the seedlings are used for restoration purposes (DEAT, 2010).

Actively restoring individuals into the wild from cycads propagated in botanical gardens and nurseries is another conservation strategy in South Africa (Eloff, 1995; Da Silva et al., 2012). Researchers are validating such initiatives to determine how ecologically valid these approaches are, because many of the populations have been in isolation for long periods of time and adding new specimens to the wild may disrupt the genetic diversity in these isolated populations. So far, Da Silva et al., (2012) have shown that re-introducing propagated *E. latifrons* cycads from Kirstenbosch National Botanical Garden into wild populations would not disrupt genetic diversity and is a valid approach. Such research as well as other information on life history traits, for instance pollination, are important for the effective restoration of other *Encephalartos* populations and is currently lacking for many species.

It would be optimal to conserve cycads within their natural environment; however, when species are critically endangered in the wild, *ex situ* conservation must be considered (Eloff, 1995). Ensuring a good genetic seed bank in botanical gardens and nurseries will allow for the re-establishment of wild populations from propagations in the future. The Lowveld National Botanical Garden has an excellent propagation program and genetic seed bank to ensure the *ex situ* survival of many threatened species, and allow for the future introduction and augmentation of wild populations. Conservation of cycads outside the wild will only ensure species existence in the short term future and resorting to this type of conservation should not be used as an excuse for allowing illegal collection of plants from the wild to continue (Eloff, 1995). Unfortunately, cycads are not even safe in botanical gardens, for example, the Lowveld National Botanical Garden and Kirstenbosch National Botanical Garden have had cycads stolen out of the garden despite the tight security measures in place, emphasizing the severity of the current situation (W. Froneman pers. comm., 2012).

Many of the threatened cycad species in southern Africa occur outside official protected areas and are found mostly on privately owned land (Cousins et al., 2008). This makes cycads

vulnerable to land transformation for agriculture and targets for poachers. Over the past decade, there has been an increase in the number of private conservancies, game reserves and ranches (Cousins et al., 2008). For example, endemic cycad species in Limpopo are found primarily in private game reserves (Cousins et al., 2008). Current official protected areas lack the resources to monitor cycad poaching, especially with other rife problems, such as rhinoceros horn and elephant ivory poaching. Conserving cycads on private land provides the opportunity for the costs of conservation to be carried out by the private sector; therefore, private game reserves and conservancies play a vital role in the successful conservation of wild cycad populations.

There have been attempts to use micro-chips to identify individual cycads removed from the wild (Da Silva, 2005), which is crucial for successful law enforcement. Micro-chipping has had limited success, because poachers can detect and remove the micro-chips using X-ray equipment or the micro-chip is rejected by the plant (Da Silva, 2005). DNA fingerprinting of cycads is another potential tool; however, this technique requires DNA references of all cycad subpopulations in order to relate poached cycads to the subpopulation they originated from (Da Silva, 2005; Da Silva et al., 2012). Furthermore, if there is no genetic diversity between subpopulations then individual plants must have their DNA barcode processed prior to poaching. There are limited facilities and experts available for the extensive field and laboratory work needed to successfully implement this approach; therefore, DNA fingerprinting is impractical.

There is no single solution to stopping the illegal cycad trade and the combination of community propagation programs, conservation on private and national protected areas, tracking techniques, *ex situ* conservation and propagation in botanical gardens and nurseries is needed to conserve cycads successfully. With the continual improvement of technology, more options become available to help combat illegal trade. For example, stable isotopes are being used more frequently in forensic sciences and could provide another useful tool to combat illegal trade in cycads.

4. Stable isotopes in forensic science

Stable isotopes are an effective natural recorder of ecological pattern and process (West et al., 2006). Increasingly, stable isotopes are being used in forensic science to help stop criminal activities by sourcing the origin of illegally traded items, thereby contributing to combating

the illegal trade in species. Stable isotopes have been used to trace the origins of confiscated heroin and cocaine (Ehleringer et al., 1999), to identify the source of poached ivory in southern Africa for directing anti-poaching measures at the correct locations (Vogel et al., 1990), and to determine the authenticity of food origin, such as beef (Rossmann, 2001; Schmidt et al., 2005). Although, the potential for using stable isotopes is vast it is still novel to many research and conservation arenas. Hence before using stable isotopes as a forensic tool for cycads, we need to know what influences stable isotopes, and more importantly, what is a stable isotope?

4.1 What are stable isotopes?

Stable isotopes are elements that occur in more than one stable form with each form having the same number of protons, but different number of neutrons (i.e. the same atomic number but with different atomic mass). For example, oxygen occurs naturally in the atmosphere as ^{16}O, ^{17}O and ^{18}O, where ^{16}O is the lightest isotope with 16 neutrons as opposed to ^{18}O with 18 neutrons (Barbour, 2007). The ratio of the stable isotopes found within a substance is compared relative to an internationally agreed upon isotopic standard: where Pee Dee Belemnite (PDB) is used for carbon ($\delta^{13}C$), atmospheric air for nitrogen ($\delta^{15}N$), Canyon Diablo meteorite for sulphur ($\delta^{34}S$), and Vienna Standard Mean Ocean Water for oxygen ($\delta^{18}O$) and hydrogen ($\delta^{2}H$). These comparisons are calculated using the delta notation below, which is expressed in parts per thousand (‰):

$$\delta\ ^{X}E = (R_{sample}/\ R_{standard}\ -1)1000$$

Where E, is the element of interest, X is the atomic mass of the heavier isotope, R_{sample} is the ratio of the heavier to the lighter isotope within the sample, $R_{standard}$ is the ratio of the international standard (Baugh et al., 2004). Samples that contain more of the heavier isotopes are referred to as “enriched” compared to samples containing less of the heavier isotopes are referred to as “depleted” (Dawson et al., 2002). By definition, isotope standards have a delta value of 0‰, therefore a positive delta value for a sample indicates that the sample is more enriched in the heavier isotope than the standard and a negative delta value indicates that the sample is more depleted in the heavier isotope relative to the standard (Dawson et al,. 2002).

The ratio of the heavy to light isotope within plants and animals varies according to the initial ratio of the isotopes found in the environment and the fractionation of those isotopes by metabolic processes within the organism (Ehleringer et al., 1999). Fractionation is the change

in heavy to light isotopic ratio when the element transfers from one phase to another (e.g. liquid to vapour), causing the final phase to have a different isotopic ratio compared to original phase, because the rate of change is proportional to the mass of the element (Dawson et al., 2002). Fractionation can also occur through a one-way kinetic reaction resulting in one of the isotopes (usually the heavier isotope) being discriminated against, subsequently changing the isotopic ratio compared to before the reaction (Dawson et al., 2002). Changes in an organism's environmental conditions alter the degree of fractionation, which results in a change in isotopic signature (Dawson et al., 2002).

The following subsections briefly describe how environmental change and metabolic processes influence different isotopes, to give an overview of why isotopes would be an effective forensic tool to trace the movement of poached cycads.

4.2 Carbon

$\delta^{13}C$ is sensitive to changes in environmental factors that influence the rate of photosynthesis and the conductance of CO_2 into the leaf (Warren et al., 2001). The rate of photosynthesis as well as the ratio of CO_2 partial pressure found inside compared to outside the leaf, influences the rate at which CO_2 enters the leaf through diffusion, influencing the $^{13}C/^{12}C$ ratio of CO_2 inside the leaf, available for photosynthesis (Warren et al., 2001). Different types of photosynthetic pathways can be distinguished from one another through different rates of fractionation of the carbon isotope. Typically, $\delta^{13}C$ for plants using C_4 photosynthetic pathways, such as grasses, vary between -14 ‰ to -9 ‰ compared to C_3 plants, which vary from -35 ‰ to -20 ‰ (Decker and De Wit, 2006). Plants that use crassulacean acid metabolism (CAM) cycling have $\delta^{13}C$ that lie between – 35 ‰ and -9 ‰ (Decker and De Wit, 2006).

The combination of a number of environmental variables determine $\delta^{13}C$, such as availability of soil water, temperature, humidity, precipitation, nitrogen availability, vapour pressure deficit, and irradiance, because they determine the rate of CO_2 assimilation, transpiration and the kinetic activity of enzymes (Warren et al., 2001; Dawson et al., 2002). For example, high humidity and soil water availability compared to low humidity and water availability can cause plants to have a 4 ‰ to 6 ‰ differences in $\delta^{13}C$ values (Ehleringer et al., 1999). Altitude also shows a strong relationship with $\delta^{13}C$ with a consistent decrease in fractionation with increasing altitude (Warren et al., 2001). This decrease in discrimination is mostly due to decreasing partial pressure with increasing elevation, as well as the plant's morphological

and physiological adaptations to survive at higher altitudes (Warren et al., 2001). Decreasing $\delta^{13}C$ with increasing altitude may also be related to an increase in rainfall and decrease in temperature with higher altitudes.

The availability of soil water, transpiration rates and humidity are strongly related to $\delta^{13}C$, because water availability and water use is a key part of CO_2 assimilation (Warren et al., 2001). Water use efficiency (WUE) is the ratio of CO_2 assimilation rate to the rate of transpiration, and is an integrated measure used to determine plant water stress or water use (Roden et al., 2005).

Changes in climate can be determined through the sampling of cellulose in tree rings, because cellulose is immobile after it is formed reflecting the climatic conditions at that time. Past climatic conditions can be inferred based on isotopic changes in carbon and oxygen isotopes, because they are an integrated measure of water use efficiency, vapour pressure deficit, and availability of water within the environment that the plant grew in (Roden et al., 2005).

In cycads, stable carbon analysis may be a useful tool to determine if the plant was relocated from a water rich to a water stressed environment. This is because cycads switch from C_3 photosynthetic pathway to CAM (Crassulacean Acid Metabolism)–cycling (Vovides et al., 2002) when water stressed resulting in higher $\delta^{13}C$ values in water stressed conditions. $\delta^{13}C$ alone is probably not an effective tracer, but would be more useful when viewed simultaneously with $\delta^{18}O$ isotopes, which reflects more about the plant water source and status.

4.3 Oxygen

Stable oxygen isotopes within rainfall vary spatially across the globe (Bowen and Wilkinson, 2002). Thus, $\delta^{18}O$ provides an excellent geographic tracer (Bowen et al., 2005) sensitive to changes relating to the water cycle and can trace changes in precipitation, humidity, and soil water within a plant or across a landscape (Barbour, 2007). Plant cellulose is generally used to measure $\delta^{18}O$ isotopes, because cellulose is immobile once formed, thereby capturing the oxygen signature of the climate that the plant occurs in. The primary factors that influence $\delta^{18}O$ in plant cellulose are humidity, air temperature and moisture source, while the secondary variables that respond to changes in the primary climatic variables, are leaf temperature, stomatal conductance and transpiration (Kahmen et al., 2011).

Generally, $\delta^{18}O$ values become depleted as temperature decreases with higher altitudes and latitudes (Barbour, 2007; Kahmen et al., 2011). Regardless of temperature effects, $\delta^{18}O$ values in rainfall tend to decrease with distance inland due to "rain-out effects", whereby the first water from precipitation events is more enriched with the heavier oxygen isotope, and progressively becomes depleted as the precipitation event moves across the landscape (Barbour, 2007). The effect that humidity and air temperature have on the $\delta^{18}O$ value in cellulose is determined through an integrative measure called vapour pressure deficit (VPD) between the surrounding air and inside the leaf (Kahmen et al., 2011). Increasing VPD results in an increase of $\delta^{18}O$ cellulose values, this is because the water within the leaf becomes more enriched due to the greater evaporation rate of $H_2{}^{16}O$ through the stomata, increasing the $H_2{}^{18}O$ within the leaf (Kahmen et al., 2011).

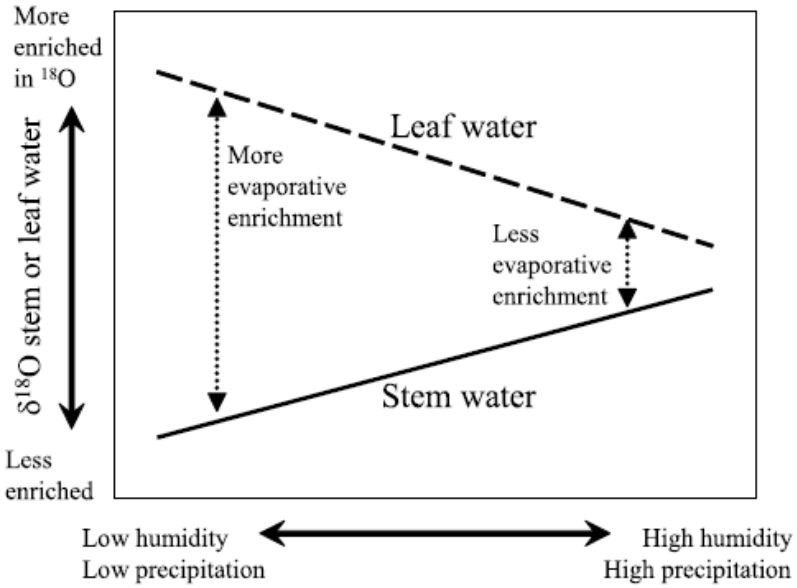

Figure 1. The potential effects of environmental variables across a precipitation transect on stem and leaf water $\delta^{18}O$, where precipitation is generally more depleted in ^{18}O with increasing altitude. Diagram taken from Roden et al., (2005).

No significant fractionation of $\delta^{18}O$ occurs during water uptake by plants, making it a potentially effective tool to observe changes in climate over time, or trace the origin of poached plants, such as cycads, based on differences in water source (Dawson et al., 2002; Barbour, 2007). For example, differences of more than 15 ‰ in $\delta^{18}O$ can be seen between plants grown in different temperate regions, which could be informative to trace poached cycads (Ehleringer et al., 1999). There is a strong negative relationship of source water $\delta^{18}O$ values with increasing elevation (Bowling et al., 2003; Kahmen et al., 2011). However, the

evaporative enrichment of $\delta^{18}O$ values may cause $\delta^{18}O$ in plant cellulose to be much higher than expected in source water at high elevations (Figure 1; Roden et al., 2005; Kahmen et al., 2011). This is due to the influence of evaporative enrichment (see Figure 1) causing opposing $\delta^{18}O$ values in the cellulose than what would be expected from the source (or stem) water (Roden et al., 2005; Kahmen et al., 2011). Therefore, understanding which environmental and biological variables are driving the $\delta^{18}O$ values in cellulose is important for forensic studies, especially when tracing plants or animals back to their origin. The use of $\delta^{18}O$ in conjunction with $\delta^{13}C$ and other stable isotopes tracers that vary consistently across landscapes is necessary to trace the movement of cycads (Bowen et al., 2005).

Although multiple drivers influence $\delta^{18}O$, it is one of the most spatially resolved isotope tracers for plant material (West et al., 2010). For the purposes of this study, $\delta^{18}O$ may be a perfect tracer for cycads relocated from a dry to a humid environment, resulting in a change in VPD, or between regions with different precipitation seasonality, causing $\delta^{18}O$ values to be different within the source water.

4.4 Nitrogen

Understanding the assimilation and fractionation of stable nitrogen isotopes is not as straight forward as oxygen and carbon isotopes, because differences in $\delta^{15}N$ values are often not directly linked to soil and surrounding climatic conditions and can vary up to 10 ‰ between co-occurring plants (Evans, 2001; Dawson et al., 2002). $\delta^{15}N$ values generally varies between 2 ‰ to 3 ‰ depending on which tissue is sampled; however extreme differences of up to 7 ‰ have been recorded in desert plants (Evans, 2001; Dawson et al., 2002).

Nitrogen can be absorbed into the plant as three different forms (NH_4^+, NO_3^- and amino acids), which can vary in isotopic value and are easily altered by anthropogenic activity, such as pollutants in water, soil and the atmosphere (Evans, 2001). Variations seen in $\delta^{15}N$ values are primarily due to differences in soil compositions, plant physiology (i.e. differences in pathways of nitrogen assimilation, uptake mechanisms and nitrogen recycling within the plant), and plant morphology (i.e. deep- roots versus shallow-roots, or variation in leaf form) (Evans, 2001; Baugh, 2004). Many plants also interact with nitrogen fixing mycorrhizae resulting in different $\delta^{15}N$ values within the plant compared to the soil (Evans, 2001). Nitrogen isotopes, when analysed in combination with carbon and oxygen isotopes, has the potential to detect changes in physiological processes and responses within cycads as well as their source of nitrogen (Dawson et al., 2002). Therefore, $\delta^{15}N$ may have forensic potential

through detecting changes in nitrogen availability in the soil, either through a change in substrate or through the use of fertilizer, which may be reflected within a relocated cycad.

4.5 Sulphur

Sulphur occurs in a few different chemical forms (SO_2, H_2S and SO_4) in the atmosphere, which are reduced to sulphate through oxidation in the atmosphere (Faure, 1986). Sulphate compounds are removed from the atmosphere through precipitation (i.e. acid rain), and can potentially cause acidification of soils, streams and lakes (Faure, 1986). Sulphur inputs into the atmosphere occurs naturally through volcanic activity, seawater spray, and sulphate-reducing bacteria, or anthropogenically, through industrial and urban pollution (Faure, 1986).

Sulphur isotopes have been underutilized in plant studies and are mostly used to detect sources of pollution through certain bio-indicator plants (Trust and Fry, 1992). Although sulphur is essential for a number of biochemical processes, it occurs in very small amounts in plants, because even moderate concentrations of sulphur are toxic to most plants (Trust and Fry, 1992). The most common source of plant sulphur is from inorganic sulphates in the soil and incorporation of SO_2 directly from the atmosphere (Trust and Fry, 1992). $\delta^{34}S$ values in plants are usually 1.5 ‰ less enriched than $\delta^{34}S$ values found in the surrounding environment (Trust and Fry, 1992). This is because little fractionation of $\delta^{34}S$ occurs during the assimilation of sulphur to cysteine and acetate, which are needed to produce other sulphur containing compounds for plant functioning (Trust and Fry, 1992).

In cases where plants are exposed to high concentrations of SO_2, they release H_2S into the atmosphere to prevent toxification, resulting in higher $\delta^{34}S$ values, particularly in new leaf growth. Thus high $\delta^{34}S$ values in vegetation are thought to be an indicator of sulphur stress (i.e. presence of anthropogenic pollution; Trust and Fry, 1992). Therefore, $\delta^{34}S$ may have forensic potential, through detecting changes in exposure to atmospheric pollution or sulphur availability in the soil, which may be reflected within a relocated cycad.

4.6 Strontium

Strontium is released into the soil through the weathering of rock and the ratio of strontium isotopes ($^{87}Sr/^{86}Sr$) within plants is usually a reflection of the underlying geology (Vogel et al., 1990). The ^{87}Sr isotope is formed as a result of the slow radioactive decay of rubidium (^{87}Rb; Vogel et al., 1990). The initial quantity of ^{87}Rb present, as well as the decay of ^{87}Rb to form ^{87}Sr determines the age of the underlying geology (Vogel et al., 1990). A greater

amount of ^{87}Sr results in a higher $^{87}Sr/^{86}Sr$ ratio, which is indicative of an older geology (Vogel et al., 1990).

There is no significant amount of isotopic fractionation when strontium is absorbed into the plant, allowing strontium to be a useful indicator of the surrounding geology (Rummel et al., 2010). Strontium isotopes have been previously used to track the source of materials such as bone, ivory or rhino horn back to their original location, through characteristics of the geology in an area (Vogel et al., 1990). However, strontium isotopes are not informative in highly variable soils with mixed geology, because the strontium isotope can vary greatly within a small spatial scale. Also, strontium isotopic values in plants is not necessarily a true reflection of the geology and it is the available strontium in the soil for up take by the plant which is correlated to the strontium found within the plant tissues (Price et al., 2002). Factors that can significantly affect the strontium values in vegetation are aerosols, dust, sea spray and fertilization (Rummel et al., 2010). These factors can potentially mask the expected ^{87}Sr signal that would be expected from the underlying geology. In cycads, the use of strontium ratios might be useful for detecting a shift in soil type or proximity to the ocean if a cycad has been relocated.

4.7 Lead

Four different lead isotopes occur in nature; ^{208}Pb, ^{207}Pb and ^{206}Pb and are decay products of uranium (U) and thorium (Th) which increase in abundance as U and Th decays over time, whereas ^{204}Pb is non-radiogenic with a constant abundance over time (Faure, 1986; Komárek et al., 2008). Like strontium, lead isotopes reflect the underlying geology in which a plant grows; however, anthropogenic addition of lead through the combustion of gasoline and coal has resulted in an increase of lead into the environment (Faure, 1977). The mixing of natural and pollutant lead isotopes results in generally lower lead ratios ($^{206}Pb/^{207}Pb$) than what would naturally be expected in most modern sediments (Renberg et al., 2002). The source of pollution can be recognised through distinct or overlapping isotopic ranges and can trace different sources of pollutants through different lead isotope ratios (Komárek et al., 2008). Anthropogenic pollution is not always the main driver of lead isotopic composition in soils and the decay of bedrock can cause the ratios of the radiogenic lead isotopes to be naturally higher than what would be expected from pollution lead (Komárek et al., 2008).

There is no significant fractionation of the lead isotope when it is incorporated into the plant and in general, physico-chemical fractionation processes do not affect lead isotopes

(Komárek et al., 2008). Some studies have used tree rings to trace the increase in lead pollution over time. However, the incorporation of lead pollution through the wet and dry deposition in bark pockets of trees is thought to be a better indication of atmospheric pollution than lead in tree rings, which provide better records for lead isotopes and concentrations in the soil (Komárek et al., 2008). In cycads, the relocation of poached specimens to cities may be reflected through lead isotope ratios that are indicative of pollution, thereby being a potential tracer of cycad movement.

5. Trace element concentrations

Trace elements usually refer to elements that are found in low abundances in the soil and are present at concentrations of less than 0.1% (Adriano, 1986). These elements can be essential to the nutrition of organisms, or can have no known physiological function (Adriano, 1986). The concentration of trace elements in the soil as well as their mobility and availability for absorption depends on the geochemical, climatic, and biological origin of those elements (Kabata-Pendias, 2004). Usually, acidic and well-aerated soils promote the mobility of a number of metal trace elements, especially cadmium (Cd) and zinc (Zn), compared to poorly aerated alkaline soils which tend to have less mobile metal trace elements (Kabata-Pendias, 2004).

Determining the concentration of trace elements in plants, can give an indication of which elements are present and most abundant within the soil, providing evidence of the underlying geology. Plants have adapted multiple ways of obtaining certain essential trace elements, which may give concentrations that are much greater in the plant compared to the soil it is growing in (Kabata-Pendias, 2004). Furthermore, if certain elements are in excess in the soil, especially heavy metals, they can be detrimental to plants in high concentrations, and plants can adapt ways of selecting against those elements (Kabata-Pendias, 2004). The ability of plants to be selective in which trace elements they take-up, may give distorted concentrations of elemental concentrations compared to those in the soil. This is only problematic when certain essential micro-nutrients are limiting and strongly selected for, or if there are high concentrations of metal elements, due to anthropogenic pollution (Kabata-Pendias, 2004).

In poached cycads, determining which trace elements are present and their concentrations may if indicate a shift in soil type occurred. The use of trace elements to identify information

regarding the source of nutrients into the environment can provide an additional method in conjunction with the stable isotopes to trace the origin of poached cycads.

6. Principles for using isotopes as a tracer

To successfully use isotopes across the landscape one needs to have adequate knowledge of the climate, geology, ecology and as well as any other important information specific to the region in which the organism occurs, so that the results can be ground truthed (Hobson et al., 2010). This is necessary to know what is expected from your results and to ground truth isotopic findings (Hobson et al., 2010). In addition, factors and physiological processes that influence the discrimination of elements must be understood within the tissue being studied, especially across trophic levels and changes associated with metabolic rates (Hobson et al., 2010).

The turnover rate of stable isotopes may differ within tissues of an animal or plant (Hobson et al., 2010). Isotopes are usually retained and assimilated in immobile tissues such as bone, feathers, or wood, but are replaced regularly within metabolically active tissues, such as muscle, blood or plant leaves (Hobson et al., 2010). Consequently, metabolically active tissues will provide an isotopic signal of elements recently absorbed into the organism and immobile tissues will provide an assimilation of isotopic signals over time (Hobson et al., 2010). In plants, cellulose in cell walls or wood is fairly immobile and will record isotopic signatures absorbed at the time the cellulose or wood was formed (Hobson et al., 2010). Compared to starch stored in parenchyma cells and leaves, which has new nutrients and carbohydrates regularly added and removed within the plant tissue, thus recording recent isotopic signatures (Hobson et al., 2010). Studies that use stable isotopes from tree rings generally use cellulose extracted from the vascular rings in order to get values that are more accurate within each tree ring (Battipaglia et al., 2008). Bulk tissue has different isotopic ratios compared to the pure cellulose, therefore knowing which material will be most useful to sample for developing a forensic technique is essential.

7. Radiocarbon dating

Radiocarbon (^{14}C) is the radioactive isotope of carbon, and the amount of radiocarbon present in an organism or in the atmosphere decays over time (Faure, 1986). The rate of ^{14}C decay is referred to as the Libby's half-life which is 5 730 years, and is used to determine the age of an organism (Faure, 1986). Once an organism dies the atmospheric ^{14}C absorbed into the

plant starts to decay and a date of when that organism existed can be determined by comparing the amount of decay to what the expected ^{14}C concentrations were in the atmosphere (Faure, 1986).

There are processes that increase the ^{14}C concentrations in the atmosphere, such as the interaction between nitrogen, carbon and oxygen isotopes with cosmic-ray neutrons, which give ^{13}C an extra neutron to form ^{14}C when they react with CO or CO_2 (Faure, 1986). Together with the production and slow decay of ^{14}C, atmospheric concentrations have been relatively consistent with some fluctuation. However, the ^{14}C concentrations were disrupted between the late 1950s and early 1960s due to the testing of the atom bomb, which resulted in a rapid spike of ^{14}C concentrations in the atmosphere (Vogel et al, 2002; Figure 2). Since the testing stopped, the ^{14}C concentrations have decreased in the atmosphere partially due to ^{14}C decay, but mostly due to absorption into the ocean and terrestrial land sinks (Levin and Kromer, 2004; Levin et al., 2008). The effect of increased CO_2 from fossil fuel emissions has also resulted in the dilution of ^{14}C compared to ^{13}C concentrations. This change in ^{14}C concentrations over the past 60 years was measured at different laboratories across the world and their combined records of atmospheric ^{14}C has allowed for the precise dating of samples (Levin and Kromer, 2004; Levin et al., 2008).

Applying radiocarbon dating to illegally traded and endangered cycads can provide a time line of when plant material was grown and linking these dates to the time of the poaching event. The material selected is critical for forensic investigations and knowing the age of the different tissues being analyzed will determine the feasibility of using isotopes and trace element concentrations as a forensic technique. In order to meaningfully sample and interpret the radiocarbon dates, stable isotopes and trace element concentrations, a detailed account of cycad anatomy and growth is necessary.

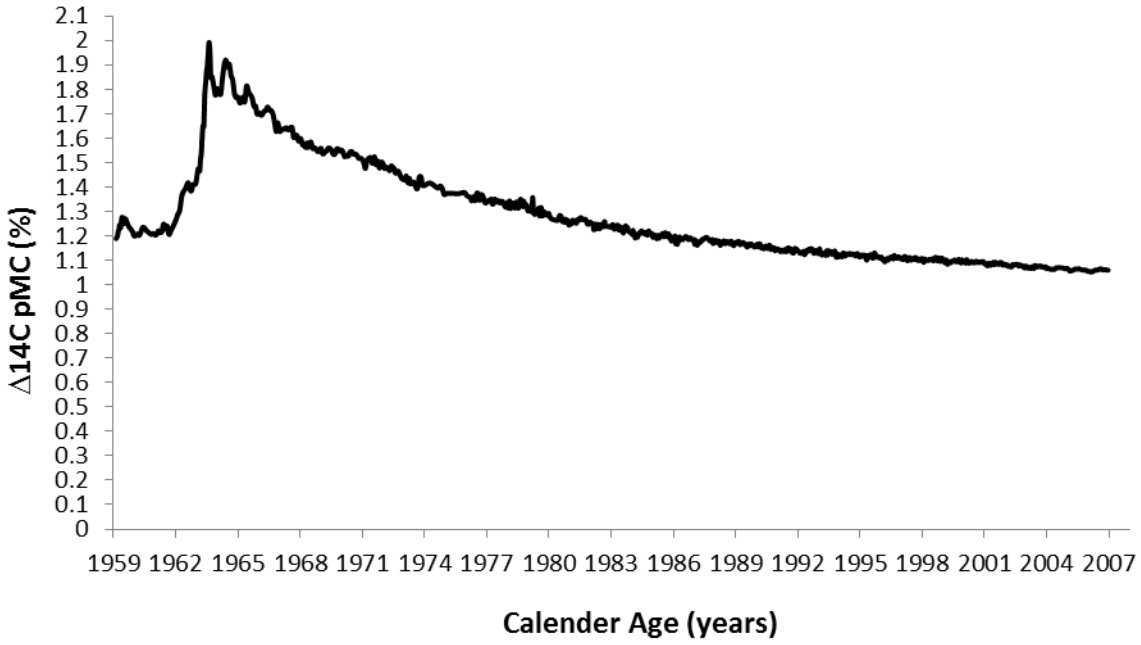

Figure 2. The "bomb-curve" showing the percent modern carbon (^{14}C) in the atmosphere for the Southern Hemisphere from 1959 to 2006, data from Levin and Kromer (2004) and Levin et al., (2008).

8. Cycad life history, anatomy and age of tissues

The initial cycad anatomy work was done by Von Mohl (1832), Mettenius (1861), Worsdell (1896), Miller (1919) and there are also numerous valuable writings by Chamberlain (1909; 1911; 1919). There are detailed descriptions of the genera *Dioon*, *Ceratozamia*, *Zamia* and *Microcycas*, but since Chamberlain's work in the early 1900s there has been limited research on cycad anatomy due to the lack of specimens to work on, because they are slow growing and threatened. Most research since the early 1900s has focussed on cycad pollination, cultivation, taxonomy and paleobiology (Wang et al., 2011). Cycad anatomy has become more popular in the literature over the past decade with findings of fossils and understanding of cycad evolution (Hermsen et al., 2006; Wang et al., 2011). To develop a new conservation tool it is essential to understand the life history traits and anatomy of cycads.

The growth form of cycads is uniform across genera with a large stem that grows from a single apical meristem, initially in breadth and then in length (Norstog and Nicholls, 1997). Stem growth forms can be distinguished between subterranean (below the ground) and arboreal (above the ground) with some species having multiple stems. All cycads are perennial with individual male and female plants and usually have sets of leaves that emerge

10 to 25 leaves at one time, which are long lived for one to three years (Norstog and Nicholls, 1997; Donaldson, 2008).

The African genus *Encephalartos* has a polyxylic stem which consists of a number of concentric vascular rings with the newest vascular ring forming on the outside of the older (inner) rings as the stem increases in diameter with age (Norstog and Nicholls, 1997). The amount of vascular tissue that increases with size is relatively small when compared to the cortex and pith (see Figure 2) which forms the bulk of the stem (Norstog and Nicholls, 1997). The vascular rings are made up of xylem tracheids, which transport water, and phloem tracheids that transport nutrients and other solutes throughout the plant (Norstog and Nicholls, 1997). Tracheids are long pitted tubes with thin cell walls that are connected to bordering tracheids, and are pointed at their ends with sclariform (ladder-like) perforation plates as they are connected longitudinally (Chamberlain, 1911). The pith and cortex both consist predominately of parenchyma tissue with relatively unspecialized cells that are used for storage of starch and have numerous mucilage canals with various viscous polysaccharide substances (Norstog and Nicholls, 1997).

During our anatomical exploratory phase of the genus *Encephalartos*, several concentric rings with increasing age were seen (see Figure 3; Norstog and Nicholls, 1997). Vascular tissue that forms the concentric rings around the soft pith does not represent annual growth rings (Chamberlain, 1911), nor do they correspond to the number of crowns or cones produced, and number of seasons (Chamberlain, 1911). Cycads can undergo resting periods that create indentations sometimes seen along the outer surface of the stem of the cycad. It has been suggested that during resting periods new concentric rings may be formed (Chamberlain, 1911).

Rays are bands of parenchyma cells that are a few cells thick, which connect the pith to the cortex and run transversely through the vascular parts of the stem (Norstog and Nicholls, 1997). Leaf traces are composed of vascular tissue that extends from the vascular ring to the base of the leaf or leaf bases (Figure 4), and the large medullary rays that form horizontally between the vascular rings are leaf traces, which become larger as tissue grows around it (Figure 3; Chamberlain, 1911).

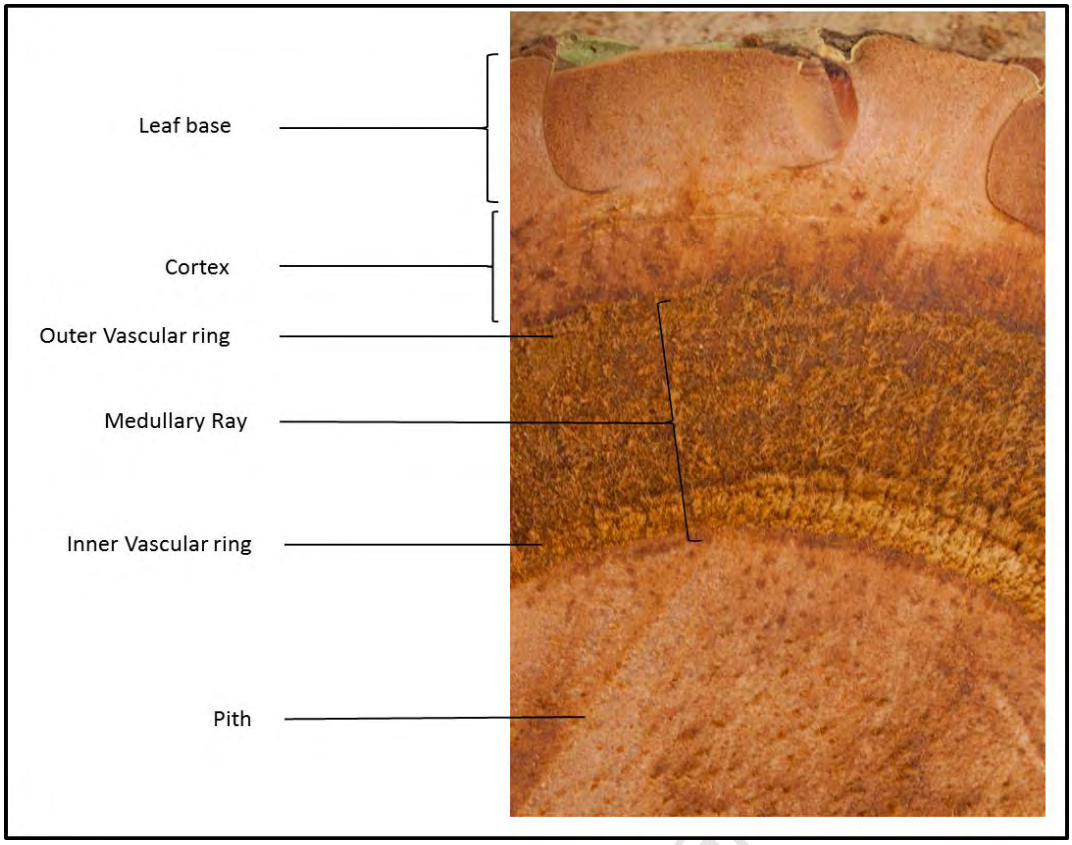

Figure 3. Internal anatomy of *Encephalartos altensteinii* stem showing part of the horizontal slice at the base of the stem (2012).

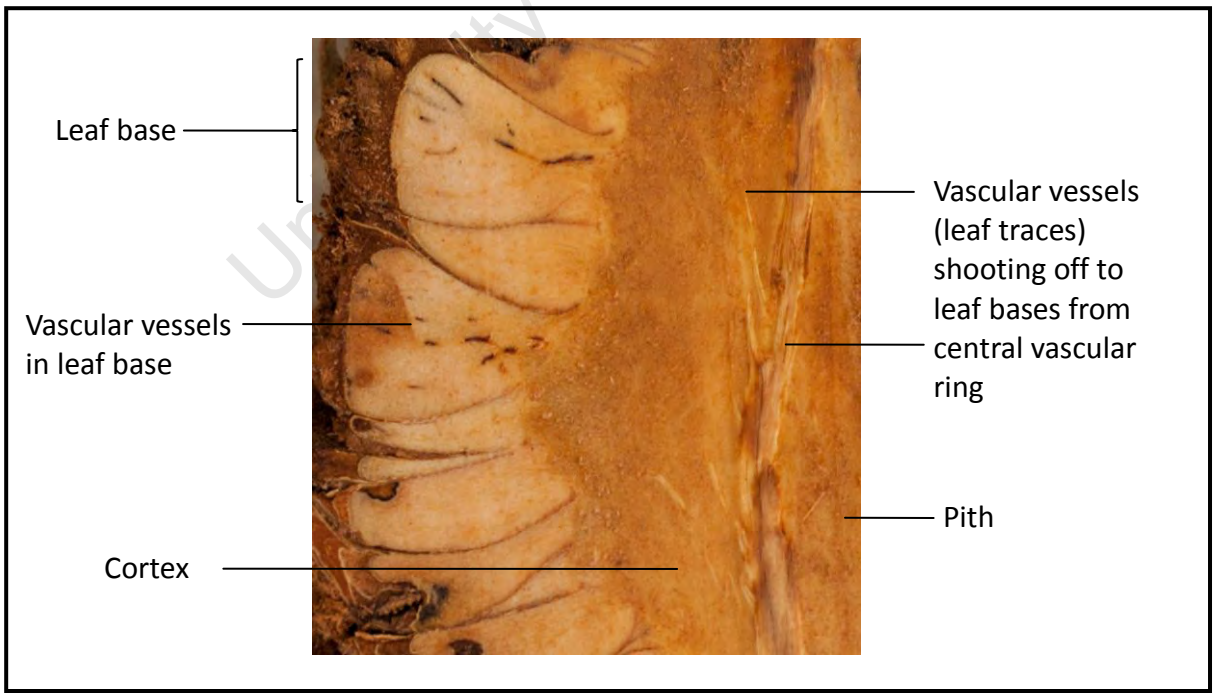

Figure 4. Internal anatomy of *Encephalartos altensteinii* stem showing a section of a longitudinal slice into the stem (2012).

The resprouting of new stems through basal offshoots, called suckers, from the lead stem makes it impossible to truly know the age of an individual plant, because a new stem keeps replacing the old one before dying back. Previous studies have used radiocarbon dating to estimate age and growth rates of cycads, which depends on the species-specific growth strategy and environmental conditions (Vogel and van der Merwe, 1995; Vogel et al., 2002). For example, an *Encephalartos transvenosus* individual from a nature reserve in Limpopo with a 7 meter tall stem was estimated to be about 150 years old and have a growth rate of about 7.5 to 5.5 centimetres per year (Vogel et al., 2002). Compared to an *Encephalartos eugene-maraisii* specimen from a game lodge in Limpopo with a 2.1 meter stem, which was estimated to be 210 years old with a growth rate of 10 millimetres per year (Vogel and van der Merwe, 1995).

The plant tissue that Vogel and van der Merwe (1995) used to radiocarbon date the cycads was taken from the leaf base of the stem (Figure 4). The samples taken decreased in chronological order from the top (younger) to the base (older) of the cycad; however some leaf bases were contaminated with new plant growth. This is because as cycads grow broader or are damaged, new plant tissue forms around the old leaf-base giving mixed radiocarbon dates (Vogel and van der Merwe, 1995). Radiocarbon dating on extracted cellulose is suggested to give improved results of cycad age (Vogel and van der Merwe, 1995). Therefore, to prevent dating recently grown plant material, sampling of the vascular tissue (leaf traces) just beneath the leaf base or vascular rings within the stem can be done and would represent old growth.

There are a number of challenges in determining the appropriate tissue to select for sampling. This is because different tissues grow at various rates and deciding which tissue is of an appropriate age and turn over time is challenging. It is uncertain the time required for a relocated plant to have grown within the new environment before a forensic technique can be applied and this needs further investigation. Old tissue being mixed with new tissue could be problematic and may result in the attenuation of isotopic signal; therefore, it is challenging to determine which tissue was grown from the wild. In addition, comparing between different types of tissue (petiole, leaf bases, or cellulose) is problematic because they have different assimilation rates; therefore retrieving a continuous timeline of tissue age from most recently grown to the oldest tissue may not be possible. Hence, a sampling strategy that includes numerous possible sampling methods is required to determine the appropriate tissue for a forensic method.

Chapter 2:

Applying radiocarbon dating, stable isotopes and trace element concentrations to cycads

1. Introduction

Illegal trade in wildlife and plants is a global issue, resulting in many species being pushed towards the brink of extinction. Currently, South Africa is fighting a losing battle with a number of species that are traded illegally. For example, abalone (*Haliotis midae*) are being harvested out of our oceans at an alarming rate and exported to East Asia where it is considered a delicacy to eat (Warcol et al., 2003). Poaching of rhinoceros horns for its apparent medicinal value is one of the most exposed illegal trades in South Africa, yet the number of rhinoceros being poached is increasing dramatically each year, despite huge financial efforts towards anti-poaching measures (Warcol et al., 2003). Numerous plant species, such as aloes and cycads are threatened, due to their illegal removal for medicinal uses, aesthetic pleasure or as collector's items (Donaldson, 2008).

Cycads are the most threatened group of plants in the world with 63% of the 303 extant species being threatened with extinction (IUCN, 2010; Da Silva et al., 2012). The decline in cycad populations is predominantly due to the illegal removal of adult plants from wild populations for private gardens and landscaping (Donaldson, 2008; IUCN, 2012). Other factors such as slow recruitment, habitat destruction and collection for traditional medicine have also contributed to the extirpation of cycad populations, as discussed in Chapter 1, section 1.1 (Donaldson, 2008). For conservationists to be ahead in the battle of illegally traded species, such as cycads, we need novel ways of thinking and new strategies for conservation.

In South Africa, the use of first world conservation strategies based on strict legislation to protect cycads is ineffective due to the lack of enforcement (Giddy, 1995; Thomas, 2011). Authorities have inadequate capacity to monitor populations in the wild or to prevent the illegal movement of cycads across borders (Thomas, 2011). Furthermore, nurseries and private collectors that have large specimens they claim were legally propagated or obtained and it is nearly impossible to prove that cycads in the possession of collectors originated from the wild to the satisfaction of a court of law (M. Pfab pers. comm., 2012).

The advancement of technology and use of science in conservation can provide alternative methods for regulating trade and preventing illegal removal of wild cycads. The use of micro-chipping to mark cycads in the wild has been successful in apprehending and prosecuting poachers and collectors who have obtained these specimens illegally. One hindrance with

these techniques is that each cycad needs to be marked prior to the poaching event (Da Silva, 2005; Da Silva et al., 2012). Establishing if cycads are removed from the wild using stable isotopes, trace element concentrations and radiocarbon dating techniques could provide the evidence needed to prosecute cycad poachers even if cycads have not been previously marked in the wild. Development of such a forensic technique would be particularly valuable for proving the wild origin of cycads in nurseries and private gardens. This would target enforcement at the end users, who are far removed from the poaching events, and allow for prosecution a number of years after the poaching event.

One limitation of using stable isotopes is that there will be a time lag before tissue from the new location is incorporated into the plant. Furthermore, results from isotopes are inferential and unlikely to conclusively identify a location of origin. Rather, isotopes are used to exclude the possibility of the plant originating from certain locations and can reinforce other supporting information (Ehleringer and Matheson, 2010). Despite these limitations, using stable isotopes as an additional technique to prosecute poachers would apply more pressure on the illegal cycad industry.

The aim of this study is to validate the use of stable isotopes, trace element concentrations and radiocarbon dating as a forensic technique to trace cycads to their wild origin. The key question asked is: Can stable isotopes, radiocarbon dating and trace element concentrations trace the movement of a cycad with a known relocation history? The methods used must be robust enough to stand up in court and simple enough to implement. It is uncertain which plant tissue would be the oldest and retain the best signature from the wild, therefore two sampling methods will be explored. One method will sample vascular rings using a tree corer, and the second method will sample leaf bases along the height of the stem.

Radiocarbon dating will age the material sampled to determine which plant material grew in the wild based on known relocation dates of the cycad. Reducing the vascular tissue to cellulose will increase the precision of $\delta^{18}O$ and $\delta^{13}C$ results, which may show weak differences between vascular rings if analysed as bulk material (Rinne et al., 2005). The bulk tissue of the leaf bases will be analysed for $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, $^{87}Sr/^{86}Sr$, $^{206}Pb/^{207}Pb$, $^{208}Pb/^{207}Pb$, and trace element concentrations. It is hypothesised that the stable isotope value and trace element concentrations for a relocated plant would be different in the old tissue (grown in the wild) compared to the new tissue (grown in a new location), indicating that the cycad has been moved. A wild specimen will serve as a control (null hypothesis) and it is

expected that there will be no significant difference in isotopic signature or trace element concentrations between the old and new tissue, because it has remained in the same place.

2. Methods

2.1 Sampling selection and sites

The following criteria were used to select specimens to sample: 1) an arboreal growth form, threatened status, 2) removed from the wild at least three years ago, 3) grown in an *ex situ* climate that is different from the wild, and 4) there are specimens remaining in the wild to sample as a control. Two species met these criteria, and one relocated and control specimen was selected for each species. The first species *Encephalartos lebomboensis*, commonly known as the Lebombo cycad, naturally occurs in isolated populations in Kwazulu-Natal, Mpumalanga and Swaziland (Norstog and Nicholls, 1997). It grows at high altitudes on rocky slopes or on cliffs and ravines in grasslands or savannas (Norstog and Nicholls, 1997; Donaldson, 2009a). *Encephalartos lebomboensis* is an arborescent species and can have multiple stems that grow to a height of two to three metres (Norstog and Nicholls, 1997). *Encephalartos lebomboensis* is listed as Endangered by the IUCN, because of its narrow distribution range and is often poached from wild populations for collectors, which has resulted in considerable population declines over the past 90 years (Donaldson, 2010a). Today, fewer than 5 000 individuals remain in the wild (Donaldson, 2010a).

One individual of *E. lebomboensis* was sampled at Kirstenbosch National Botanical Garden, in Cape Town. This plant was moved to the gardens in 1946 and originated from a wild population in the northern part of KwaZulu-Natal, approximately 1400 km from Cape Town (Figure 5; Google Earth, 2011). This cycad had two stems, and the lead stem was selected for sampling, which was approximately two meters in height. This cycad could be identified as a female plant, because it was coning at the time of sampling and was in a healthy condition, except for some yellowing of the older leaves. Kirstenbosch National Botanical Garden is situated within the Western Cape which has winter rainfall peaking from May to August with a mean annual precipitation of 1400 mm (Harris et al., 2010) and hot dry summers with mean annual temperatures that range from 7 ˚C to 26 ˚C (Mucina and Rutherford, 2006). The geology is derived from Cape Peninsula Pluton of the Cape Granite Suite and is situated at an elevation of around 150 m above sea level (Mucina and Rutherford, 2006; Google Earth, 2011).

A control specimen of *E. lebomboensis* was sampled from the wild, in KwaZulu-Natal near the origin (within 80 km) of the relocated specimen at Kirstenbosch. The cycad stem was approximately two and a half meters in height, with multiple male cones and had many small and large stems originating from its base. This specimen was growing on the side of a sandstone cliff face in shallow soil at an elevation of 1100 m above sea level (Google Earth, 2011) and appeared to be much older than the relocated *E. lebomboensis* specimen at Cape Town. The wild cycad was healthy in appearance with moss and lichen growing on the outside of the leaf bases and some of the leaves were weather beaten. This area experiences a mean annual precipitation of 1053 mm (Schulze, 2000), which peaks in midsummer and the geology consist of quartzite of the Mozaan Group (Mucina and Rutherford, 2006).

Encephalartos arenarius or the Alexandria cycad was the second species selected for sampling, which can grow up to a meter in stem height and occurs in isolated populations distributed on the coastal sand dunes of the Eastern Cape (Norstog and Nicholls, 1997; Donaldson, 2009b). This species grows in sandy soils usually on sloping hills or dunes, either under tree canopies or is found in open grasslands due to altered habitat for pastures (Donaldson, 2009b). It is listed as Endangered by the IUCN criteria due to its restricted distribution range and the population has decreased by 50 % over the past 60 years (Donaldson, 2010b). Illegal collection and habitat removal are the main reasons for the declines seen in *E. arenarius* populations and there are estimated to only be between 850 to 1 500 mature individuals remaining in the wild (Donaldson, 2009b).

One specimen of *E. arenarius* was sampled at the Lowveld National Botanical Garden, in Nelspruit, which arrived at the garden in 1992 after it was confiscated from poachers. This cycad originated from a wild population in the Eastern Cape, approximately 1020 km from Nelspruit (Figure 5; Google Earth, 2011). The cycad stem was about a meter in length; and half of the stem was partially imbedded in the ground with a small sucker growing at the base of the stem. One side of the cycad was more exposed to the rain and was starting to show signs of decay and had moss growing on the leaf bases. Sampling was done on the side of the cycad that was not exposed to the rain for this reason. This would prevent water from entering the inside of the cycad and avoiding collecting decaying leaf bases which may contaminate results. This specimen had recently grown a new set of leaves and an obvious change in stem growth could be seen from when the cycad had been moved to Nelspruit, therefore the top 20 cm to 30 cm of the stem was known to have grown within the new locality, i.e. since 1992. Nelspruit has mild to hot sub-tropical conditions, with summer

rainfall from September to March and mean annual precipitation ranging between 600 mm and 1 100 mm (Mucina and Rutherford, 2006). The geology within Nelspruit consists of granite and gneiss, and the elevation is around 650 m in the gardens (Mucina and Rutherford, 2006; Google Earth, 2011).

The control specimen for *E. arenarius* was sampled at the same locality that the confiscated cycad at the Lowveld National Botanical Garden originated from, making it the perfect scenario for testing our hypothesis. This cycad was in healthy condition and found growing under a canopy of shade on a steep slope. The cycad stem was between a 1 m and 1.5 m in length, with more than half of the stem buried in the ground. There was a recently deceased stem next to the lead stem and a new sucker started to emerge with no leaves. This species grows along the Albany Coastal Belt, which receives between 450 mm and 900 mm of mean annual precipitation throughout the year with peak rainfall during March and October/November (Mucina and Rutherford, 2006). This species was growing at an elevation of around 100 m and mean annual temperatures range between 5 ˚C and 32 ˚C for the region (Mucina and Rutherford, 2006; Google Earth, 2011). *E. arenarius* is usually found near the coastal sand dunes, which consist of Quaternary sands (Mucina and Rutherford, 2006).

Table 1. Environmental and climatic variables for the four sampling sites.

Variables	**Cape Town**	**KwaZulu-Natal**	**Nelspruit**	**Eastern Cape**	**References**
Mean annual temperature (°C)	16	19	19	18	Schulze, 2000
Mean annual precipitation (mm)	1400	1053	775	755	Schulze, 2000; Harris et al., 2010
Precipitation seasonality	Winter (May to August)	Summer (September to March)	Summer (September to March)	All year (March, October/November)	Mucina and Rutherford, 2006
Mean humidity for January (%)	69	66	71	71	Schulze, 2000
Mean humidity for June (%)	73	58	58	67	Schulze, 2000
Mean elevation (m)	150 m	1100 m	650 m	20 m	Google Earth, 2011
Predicted $\delta^{18}O$ precipitation for January	-2.6	-4.7	-3.9	-3.3	Bowen et al., 2005; Bowen, 2013
Predicted $\delta^{18}O$ precipitation for June	-4.2	-2.3	-1.4	-3.4	Bowen et al., 2005; Bowen, 2013
Predicted $\delta^{18}O$ tap water	-3.4 to -3.0	-0.9 to -0.5	-2.9 to -2.5	-0.4 to 0	West et al., 2011
Annual mean pan evaporation (mm)	1984	1898	1884	1810	Schulze, 2000
Generalised Geology	Cape Peninsula Pluton; Cape Granite Suite	Quartzite; Mozaan Group	Granite and Gneiss	Quaternary sands	Mucina and Rutherford, 2006
Soil fertility: 0 (low) to 9 (high)	1.2	5	3.3	6.8	Schulze, 2000

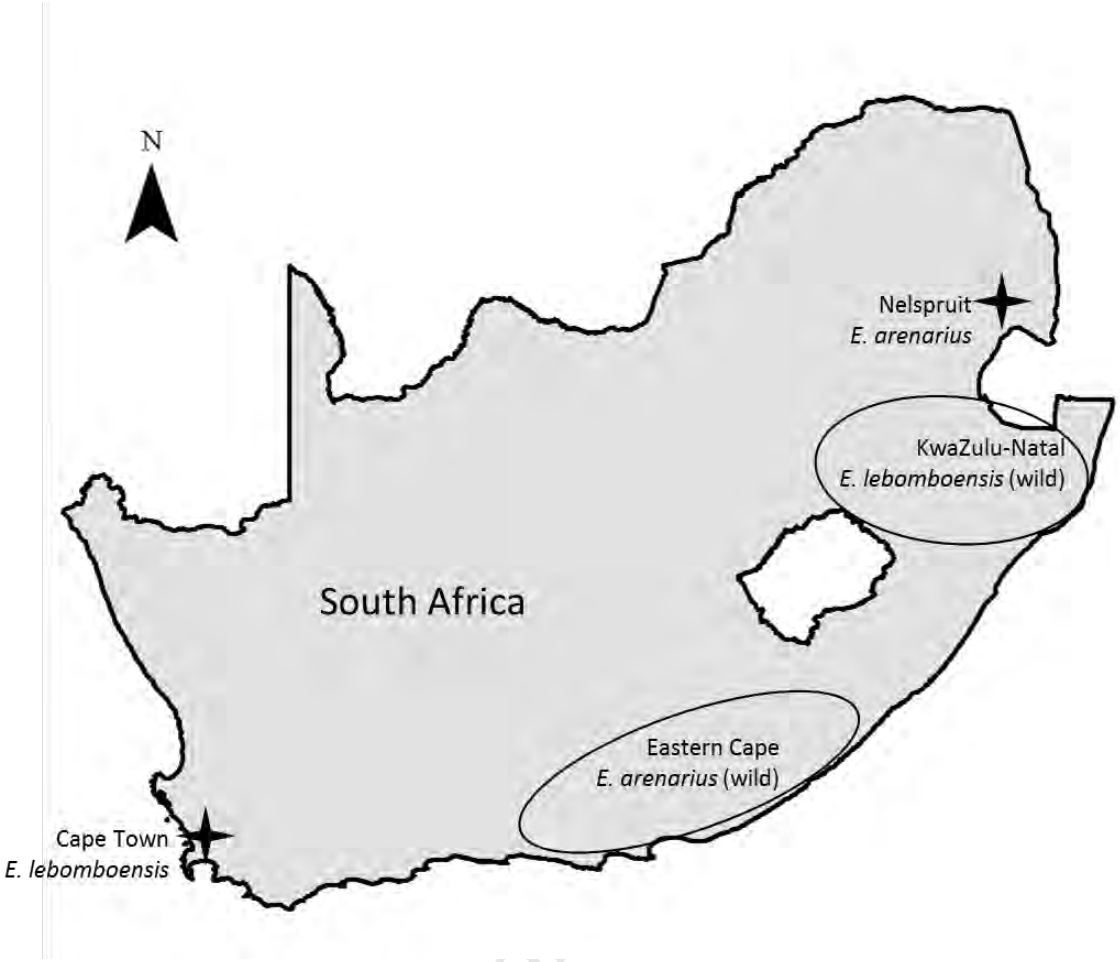

Figure 5. Map of South Africa showing *E. lebomboensis*, which was sampled at Kirstenbosch National Botanical Garden (Cape Town) and in KwaZuluNatal, and *E. arenarius*, which was sampled at Lowveld National Botanical Garden (Nelspruit) and in the Eastern Cape (2012). (To protect the localities of the wild specimens the region they occur in is encircled and not specified)

2.2 Sampling techniques

The sampling technique must be non-destructive to the plant due to the threatened status of cycads. Taking tree cores from the stem using a hand held increment corer is the best way to sample vascular rings from the cycad (Norton, 1998; Fowler and Boswijk, 2003; Mantgem and Stephenson, 2004). Increment coring, a method used globally, extracts a core of plant material from the stem leaving a hole the diameter of a pencil (5-10 mm; Norton, 1998; Fowler and Boswijk, 2003; Mantgem and Stephenson, 2004). Coring has mostly been done on conifers and other hard wood species for reconstructing past climatic changes (Mantgem and Stephenson, 2004). Although, cycads have soft wood and increment coring has not been done on them before, it is thought that they would respond in the same way to the coring as hard wood trees. Cycads are well adapted to survive natural damages from fire and insects

boring into the stem, due to the numerous mucilage ducts that can produce large amounts of sap and resin when the plant is damaged (Norstog and Nicholls, 1997). Micro-chipping of cycads have shown no negative effects, therefore the hole created by the corer is thought to have a non-damaging effect on cycads (W. van Staden pers comm., 2012).

The greatest danger with this technique is potentially spreading diseases; however sterilizing the increment corer between uses avoids this danger. Leaving the hole created by the corer unsealed versus plugged should not make a difference, because sap and resin naturally produced by the plant fills the hole and serves an anti-bacterial function (Norton, 1998). However, plugging of holes is more likely to hinder the process of compartmentalisation, which is a process that excludes damaged cells to prevent decay inside the plant (Fowler and Boswijk, 2003). The plugs can also act as infiltration points for fungi, compared to unsealed holes (Fowler and Boswijk, 2003); therefore, it would be better practice not to plug the holes in the cycad stem after coring.

Figure 6 (a) shows the increment corer that is screwed into the cycad stem with the core being removed out of the corer. Figure 6 (b) is an image of the core once removed from the stem. The size of the holes and the extent of damage done by scraping off the leaf bases with a hammer and chisel are displayed in Figure 7 (c) and (d). Due to the lack of knowledge on the most efficient sampling method, the sampling done on these cycads was more extensive than what will be necessary once the technique has been refined. When returning two weeks later to examine the holes left by the tree corer, we found that sap had clogged most of the holes and was even oozing down the stem.

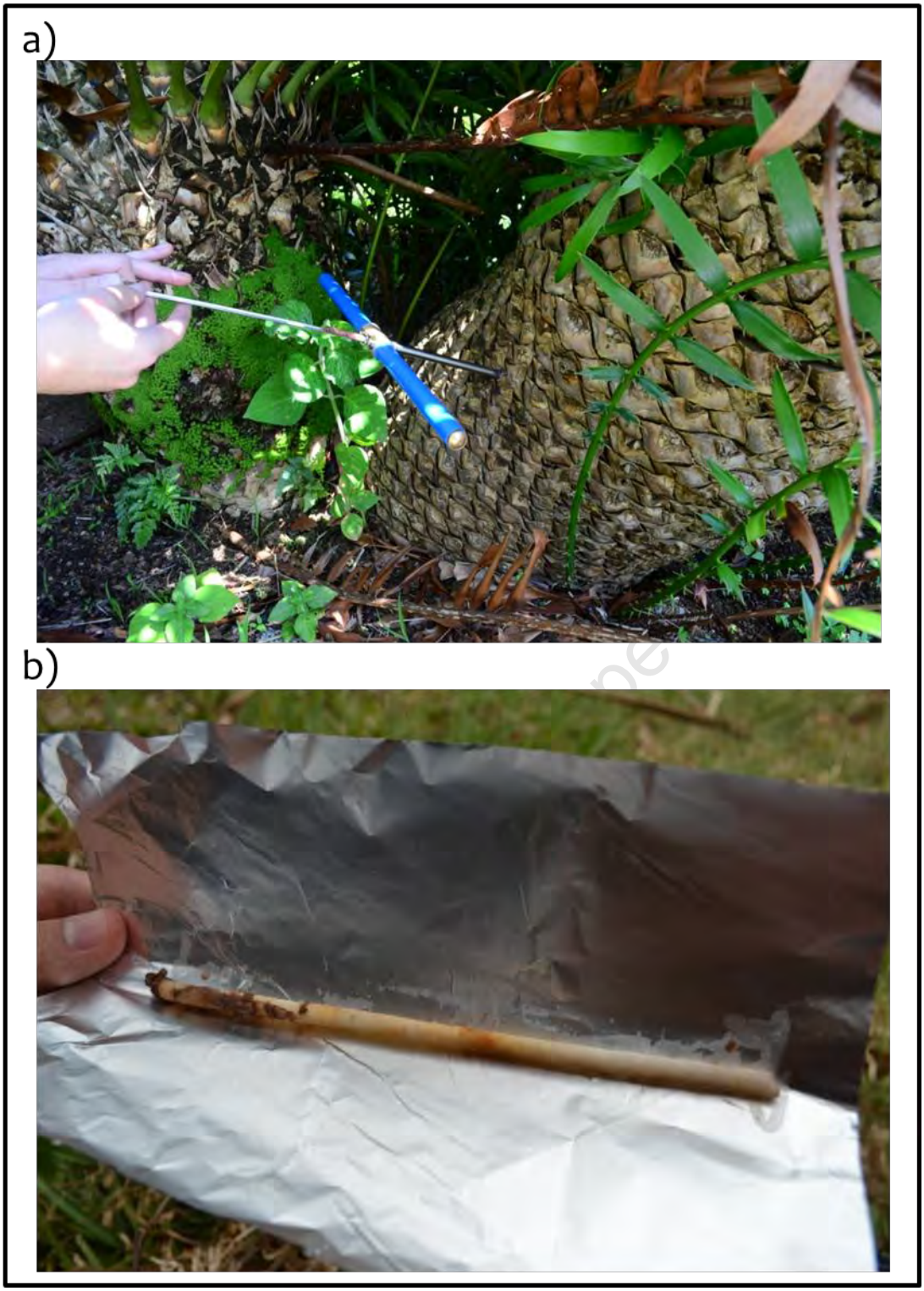

Figure 6. Picture a) shows the hand held increment corer screwed into the cycad stem with the core being removed. Picture b) shows the tree core that was ~15 cm in length and 0.5 cm in diameter.

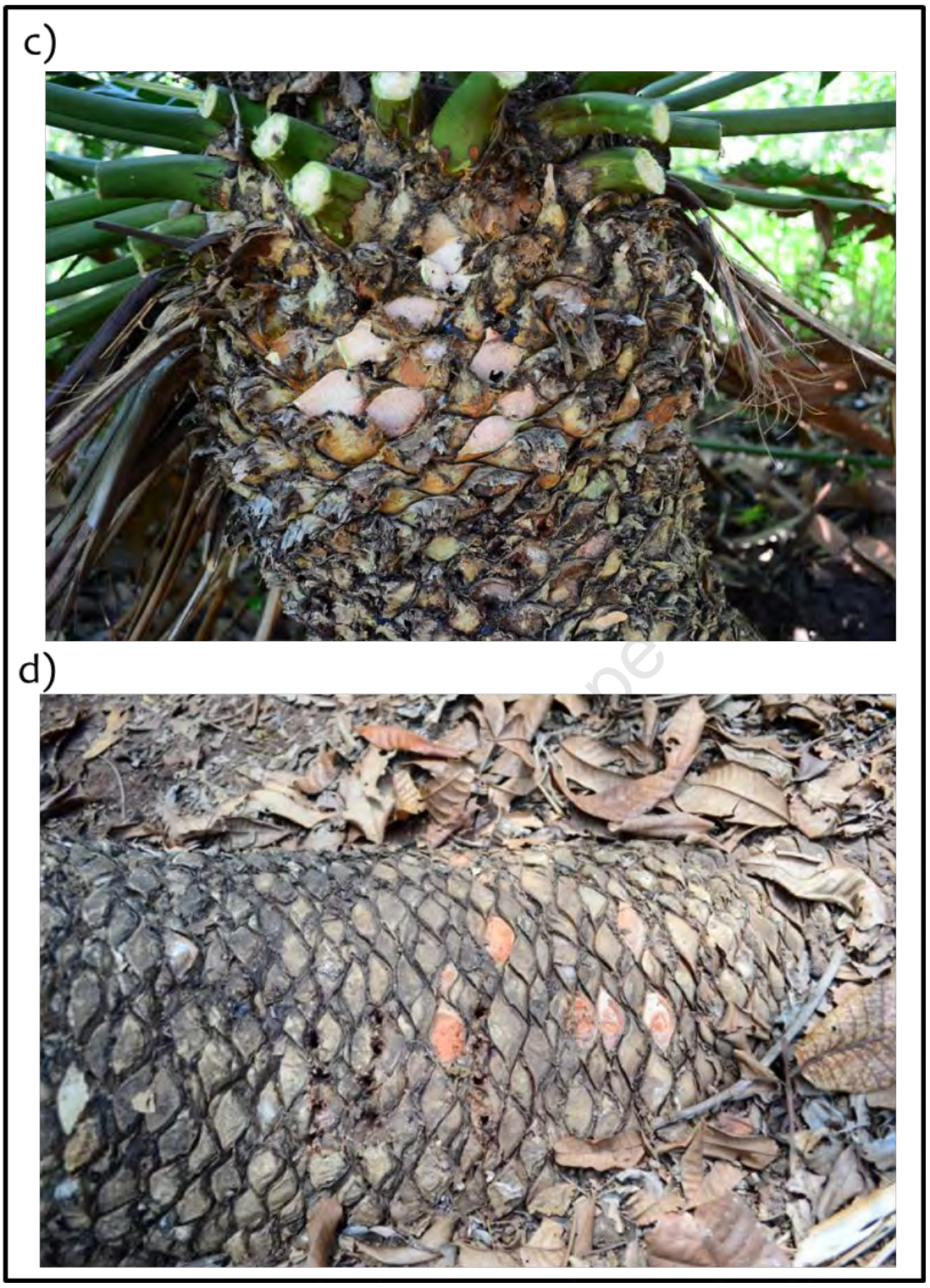

Figure 7. Image c) shows the upper section, d) shows the lower section of the cycad stem after the leaf bases were scraped off, and coring was completed.

The tree coring experimental design in Figure 8 gave the possibility of detecting a shift in stable isotope composition between the inner and outer vascular ring segments at the bottom of the cycad, or between the upper and lower vascular segments along the height of the

cycad. Tree cores were collected from the lower, middle and upper parts of the cycad stem. It is expected that the upper most core would have more recent growth as the cycad grows taller than the lower tree cores. There should be a difference in age between the inner and outer ring segments, with new tissue growing on the outermost vascular ring, causing the oldest vascular tissue to be closest to the pith (Figure 3). The upper most vascular tree ring, the inner and outer vascular tree rings from the middle part of the stem, and the outer, middle and inner vascular tree rings from the lower part of the cycad stem were collected as indicated in the illustration below (Figure 8).

The total number of tree cores taken per plant varied between 33 and 40 cores, depending on the size of the vascular rings in the tree core. This is because some specimens had larger individual ring segments than others and the vascular rings became smaller as they reached the meristem at the top of the cycad. Therefore, more tree cores were required per replicate for the upper part of the stem compared to the lower part of the stem. An estimate of 100 g to 300 g of dry weight wood was required for each sample. The vascular tissue from the tree ring samples were analysed for carbon and oxygen stable isotopes.

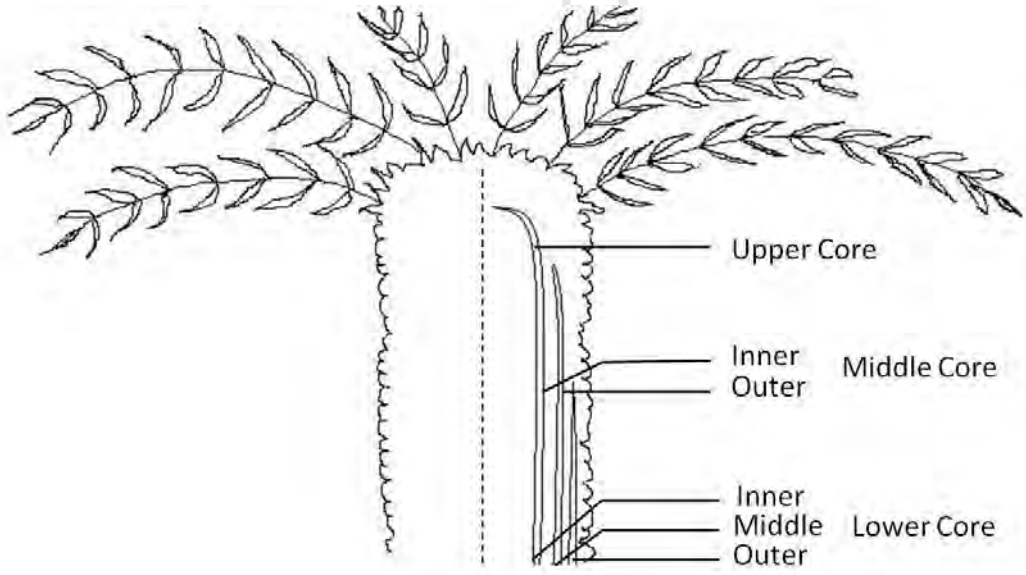

Figure 8. Tree cores were taken from the upper, middle and lower sections of the cycad stem using an increment corer and the vascular rings were cut out of the cores to sample the inner, middle and outer sections from the lower tree core, the inner and outer sections from the middle tree core and one sample from the upper tree core.

Vogel and van der Merwe's (1995) method of taking the leaf bases from the upper, middle and lower regions of the cycad stem were repeated (Figure 9). The amount of plant material needed to do the analyses required two to three leaf bases (approximately one to two grams) taken from the upper, middle and lower parts of the stem as indicated in Figure 8. The most recently grown plant tissue would be the upper part of the stem with the oldest plant tissue at the lowest leaf bases. The leaf bases were sampled by shaving the outer part of the leaf base off with a hammer and chisel (about one to two centimetres into the stem), and the leaves were cut at their base with secateurs to sample the petiole. The leaf bases and petioles were analysed for carbon, nitrogen, and sulphur isotopes, as well as trace element concentrations.

The experimental design was limited to two pairs of cycads, thus there was no replication of individuals for each species. Future work should explore variance between individuals within a species potentially improving our inferential power. A minimum of three replicates for each sample in Figure 8 and Figure 9 were collected in order to statistically test the difference in isotopic signature between the samples. Replicates are important for knowing how much isotopic variability is within the plant, because the surrounding environment has a certain amount of natural variability that is incorporated into the vascular tissue. Therefore, control specimens that have remained in the same location are essential to understand how much variability can be expected within the plant.

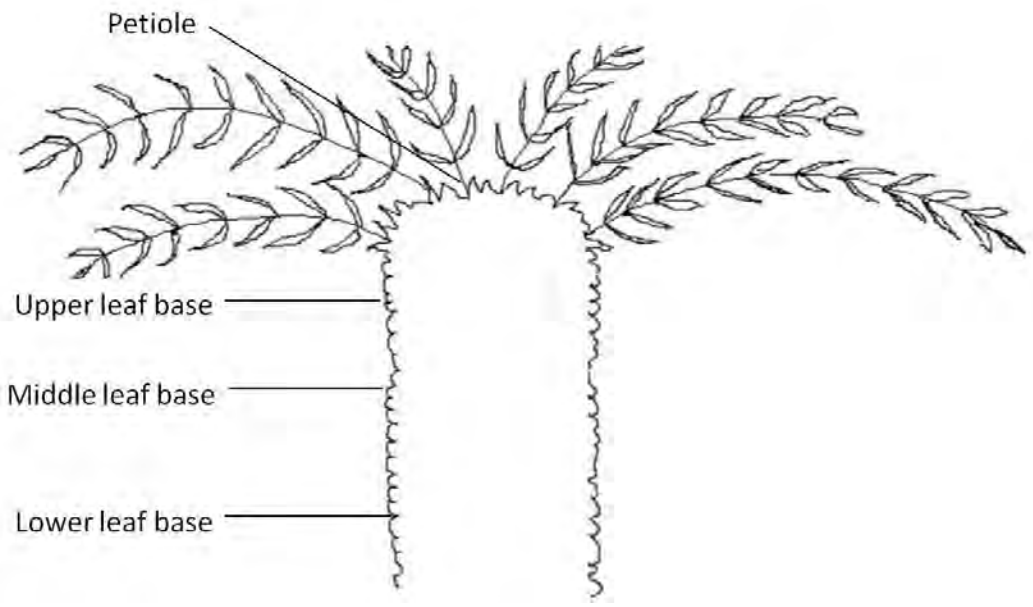

Figure 9. Sampling of the petiole, as well as the upper, middle and lower leaf bases were done using a hammer and chisel.

2.3 Processing samples

The tree cores, leaf bases and petioles were placed in a laboratory oven at 60 ˚C for 48 hours to dry. Tree cores were cut under a dissecting microscope using a razor blade to extract the vascular rings. The vascular rings were cut into sections so that there was ideally an inner, middle and outer sample of the tree core at the lower part of the cycad stem, an inner and outer sample for the middle part of the cycad, and one sample at the upper part of the stem (see Figure 8). Each vascular ring segment was approximately 5 mm in diameter and between 3 mm to 10 mm in length and could easily be distinguished from each other. The diagram in Figure 9 shows how three tree cores were placed next to each other and the outer vascular tissue for each vascular ring was cut out and combined in order to have enough material for one replicate. This was repeated another two times so that there were three replicates for that sample. The same was done for the other samples in Figure 8.

Between 80 mg and 200 mg of material (each ring segment weighed between 20 mg and 50 mg) was needed for each replicate, because through the cellulose extraction process the mass of the replicates are reduced by 90%. Therefore if the mass of three tree cores combined, as shown in Figure 10, was less than 80 mg a fourth or even a fifth tree core was added in order to get enough material for the cellulose extraction.

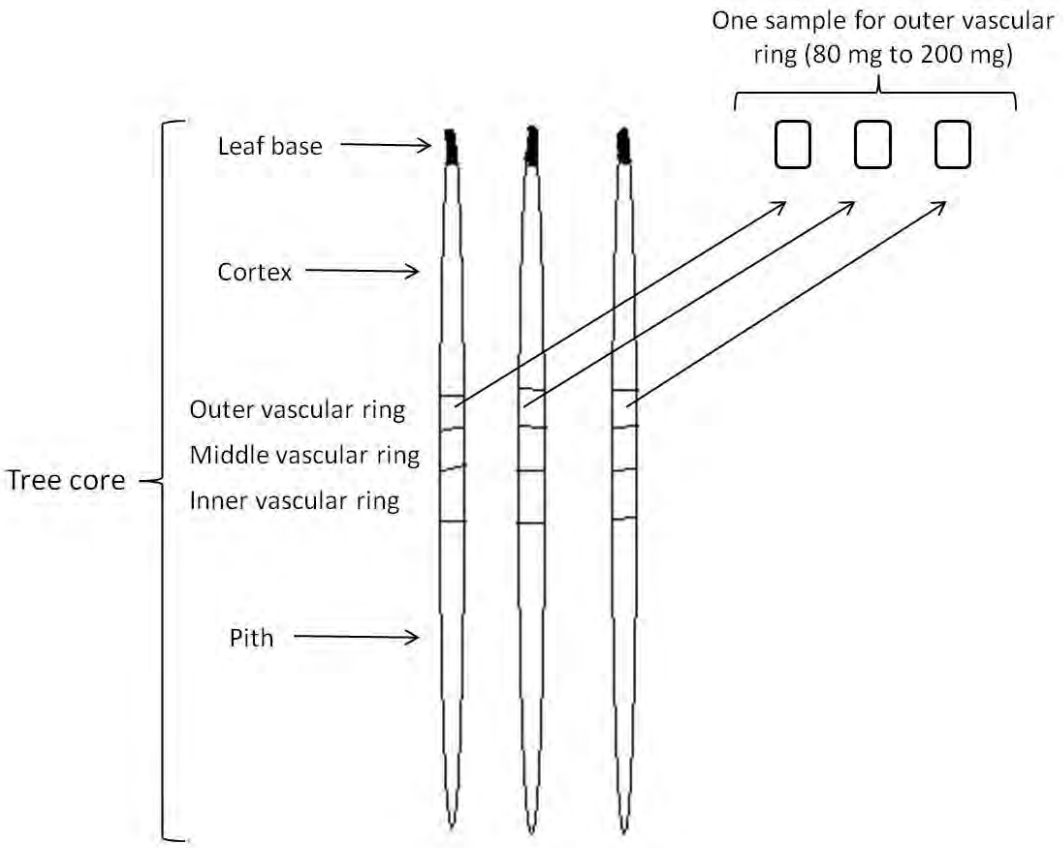

Figure 10. Diagram of three tree cores next to each other and demonstrating how the outer vascular ring was cut out of the tree cores and combined together to get one replicate.

The middle vascular ring from the lower core for the control *E. arenarius* specimen could not be sampled, because there were only two vascular rings present in the tree cores, but all other samples from the middle and upper part of the cycad stem could still be collected. Any lichen, moss and algae growing on the leaf bases were scraped off using a sharp razor blade to prevent contamination of results. The leaf bases and vascular rings from the cores were ground to a fine power for 5 to 10 minutes in a ball mill and the petioles were ground to a fine powder using an electric mill.

2.4 Cellulose extraction

Cellulose is usually preferred to whole tissue samples for analysis of carbon and oxygen stable isotopes, on the basis that cellulose is immobile compared to other wood components that can be recycled into different tissues (Cullen and MacFarlane, 2004). Samples reduced to cellulose also minimises the variability in isotopic signature, because different wood

components assimilate carbon differently (different biosynthetic pathways) resulting in differing isotopic signatures (Cullen and MacFarlane, 2004).

The cellulose extraction followed the method described by Leavitt and Danzer (1993) and will briefly be summarized. The ground tree core samples were put into non-carbon F57 filter bags (ANKOM technology) and were heat sealed. To extract the resins, waxes and oils the samples were placed in a soxhlet extractor and the reservoir flask was filled with 2:1 toluene-ethanol solution, which was run over night (12-16 hours). The next day the toluene-ethanol solution was replaced with 96 % ethanol in the reservoir flask and the whole procedure was repeated. Thereafter, samples were air dried, rinsed in deionised water at 70°C and left to air dry again for four hours each. They were placed into the Ehrlenmyer flask with deionised distilled water, 6.7 g of sodium chlorite and 3 ml of glacial acetic acid for three hours at 70°C, after which the sodium chlorite and glacial acetic acid was added without changing the solution another two times every three hour intervals. This last step was repeated the following day, and the samples were left over night to air dry. Finally the samples were thoroughly rinsed in deionised water and dried overnight at 40°C. The F57 filter bags were cut open and the cellulose was scraped out of the bags and put into eppendorfs.

2.5 Oxygen and sulphur stable isotopes

The oxygen and sulphur stable isotopes were analysed at the Iso-Analytical laboratory in the United Kingdom using a Europa Scientific 20-20 Isotope-Ratio Mass Spectrometer (IRMS) and a modified Sercon Elemental Analyser. The cellulose samples were weighed (1.0 ± 0.1 mg) into silver capsules (8 mm by 5 mm) for the oxygen stable isotope analysis. The capsules with the cellulose samples and standards (IAEA-CH-6 and IAEA-C-3) were put in a micro-titre plate and placed in a laboratory oven to dry at 60°C for seven days. Another standard (IAEA-601) was added to the samples before they were combusted in the IRMS. The known isotopic signatures for the standards IAEA-CH-6 (sucrose, $\delta^{18}O_{V\text{-}SMOW}$ = 36.4 ‰), IAEA-C-3 (cellulose, $\delta^{18}O_{V\text{-}SMOW}$ = 32.2 ‰), and IAEA-601 (benzoic acid, $\delta^{18}O_{V\text{-}SMOW}$ = 23.3 ‰) were used as reference material and served as quality control checks during the analysis. The standards used were inter-laboratory comparison standards which have generally agreed upon $\delta^{18}O$ values and are distributed by the International Atomic Energy Agency.

The ground leaf base and petiole samples were weighed out (6.0 ± 0.1 mg) into tin capsules for the sulphur isotope analysis and then loaded into an automatic sampler with the standards and vanadium pentoxide, which acts as a catalyst (Iso-Analytical Laboratory Report

unpublished). The stable sulphur isotopes were expressed relative to Canyon Diablo Troilite (CDT). The SO^+ ion beam had to be calibrated and corrected for the ^{18}O contribution by using the following standards: IA-R025 (Iso-Analytical working standard barium sulfate, $\delta^{34}S_{V\text{-}CDT}$ = +8.53 ‰), IA-R026 (Iso-Analytical working standard silver sulfide, $\delta^{34}S_{V\text{-}CDT}$ = +3.96 ‰) and IA-R061 (Iso-Analytical working standard barium sulfate, $\delta^{34}S_{CDT}$ = +20.33 ‰). IA-R061 was also the reference material used for analysing ^{34}S isotope. The standards used to monitor the quality of the analysis while the samples were being analysed were IA-R061 and IA-R027 (blue whale baleen, $\delta^{34}S_{CDT}$ = +16.3 ‰).

2.6 Carbon and nitrogen stable isotopes

Using a Sartorius micro balance, 0.4 ± 0.01 mg of the dried cellulose samples and 3 ± 0.1 mg of leaf base and petiole samples were weighed into tin capsules for the stable carbon and nitrogen analysis. The carbon and nitrogen isotopes were analysed at the Department of Archaeology at the University of Cape Town using a Flash 2000 organic elemental analyser which combusts the samples and passes the gases to a Delta V Plus isotope ratio mass spectrometer (IRMS) via a Conflo IV gas control unit (Thermo Scientific, Bremen, Germany). In-house standards were used for the analyses which were calibrated against International Atomic Energy Agency standards. The following reference standards with known isotopic values were analysed with the samples to check the quality of the analysis: Choc (Chocolate-egg mixture $\delta^{13}C/^{12}C$ = -17.75, $\delta^{15}N/^{14}N$= 4.13), MG (Merck Gel $\delta^{13}C/^{12}C$= -20.05), Lentil (dried lentils $\delta^{13}C/^{12}C$= -26.74), Nast (Dried nasturtium leaves $\delta^{13}C/^{12}C$ = -28.68, $\delta^{15}N/^{14}N$= 6.75) and Acacia (*Acacia saligna* leaves $\delta^{13}C/^{12}C$ = -27.75, $\delta^{15}N/^{14}N$= -0.7). The nitrogen isotopic ratio was expressed relative to atmospheric nitrogen and the carbon isotopic ratio was expressed relative to Pee-Dee Belemnite.

2.7 Strontium, lead and elemental concentration analyses

The strontium ($^{87}Sr/^{86}Sr$), lead ($^{206}Pb/^{204}Pb$; $^{207}Pb/^{204}Pb$; $^{208}Pb/^{204}Pb$) isotope analyses and trace element concentrations (^{6}Li, ^{45}Sc, ^{51}V, ^{52}Cr, ^{59}Co, ^{59}Ni, $^{63.5}Cu$, ^{65}Zn, ^{70}Ga, $^{85.5}Rb$, ^{89}Y, ^{91}Zr, ^{93}Nb, ^{137}Ba, ^{139}La, ^{140}Ce, Pr, ^{144}Nd, ^{150}Sm, ^{152}Eu, ^{159}Tb, ^{157}Gd, $^{162.5}Dy$, ^{165}Ho, ^{167}Er, ^{169}Tm, ^{173}Yb, ^{175}Lu, $^{178.5}Hf$, ^{181}Ta, ^{207}Pb, ^{232}Th, ^{238}U) were analyzed in the Department of Geology at the University of Cape Town. Between 200 mg and 500 mg of ground leaf base and petiole was weighed into crucibles. The crucibles were placed into a furnace for ashing, and the temperature was initially set at 300˚C, then increased every hour by 100˚C and left

over night once the temperature reached 800˚C. The crucibles were weighed again after ashing and the difference in weight was used to determine the element concentrations.

The following is a summary of the sample preparation and digestion before the strontium isotopes, lead isotopes and element concentrations were analysed and is based on methods described by Pin *et al*. (1994) and Miková and Denková (2007). Between ten and fifty micrograms of ashed sample was weighed out into 7 ml teflon beakers and digested in HF:HNO_3 acids. The acid digestion involved adding 1 ml of 65% 2B HNO_3 to each sample using a volumetric pipette and the samples were placed on a hot plate at 140˚C for one hour with the beaker's lids closed. The lids of the beakers were then opened and the solution was dried at 130˚C. Another 2 ml of 65% 2B HNO_3 was added to the dried residue in such a way that the sides of the teflon beaker were rinsed to ensure the entire sample was at the base of the beaker. The samples were placed back on the hotplate to dry a second time. Once the solution was dried, the samples were cooled and 1.5 ml of 2.0M 2B HNO_3 was added to the beakers to redissolve the samples. The strontium and lead isotope samples were transferred into centrifuge tubes and spun at 4000 rotations per minute for 20 minutes, whereas the trace element concentration samples were placed in a hot bath for an hour, which was then turned off and left at room temperature over night.

To extract the strontium and lead isotopes a 200 ul Eichrome Sr spec resin bed in a polypropylene column was needed for each sample. A waste beaker was placed underneath each polypropylene column for the residual acid that flows through the column before the strontium and lead can be extracted. The resin bed was first conditioned before use by adding 1 ml of 2.0M 2B HNO_3 into the column, which was added again once the acid had run through the column. The sample solution was added in three intervals of 0.5 ml, 0.5 ml and 0.4 ml, after which six washes of 0.5 ml 2.0M 2B HNO_3 was done. The waste beakers were replaced with new teflon beakers for collecting the strontium within the resin beds. Strontium was collected out of the resin beds by adding 0.02M 2B HNO_3 in three intervals of 0.5 ml, 0.5 ml and 1.0 ml. The beakers with the strontium solution were then replaced with the waste beakers and the resin beds were washed five times with 0.1 ml 3.1M HCl. New teflon beakers replaced the waste beakers in order to collect the lead out of the resin beds. The lead was collected out of the resin beds by adding 6.2M HCl in volumes of 0.5 ml, 0.5 ml and 1.0 ml with intervals in between.

The collected strontium and lead solutions from the resin beds were then dried on a hotplate at 130˚C, and cooled. Two drops of concentrated 2M HNO_3 was added to the lead samples and then they were dried again. Thereafter 1 ml of 2% HNO_3 was added and the lead samples were placed in an ultrasonic bath for 30 minutes to accelerate the dissolution of the samples. The strontium samples were also placed into the ultrasonic bath for 30 minutes, after 2 ml of 0.2% HNO_3 was added to the samples.

The strontium and lead isotopes were analysed in the AEON EarthLAB using a Nu Instruments Nu Plasma HR instrument. Strontium was analysed as 200ppb 0.2% HNO_3 solution using NIST SRM987 as a reference standard following methods outlined by Miková and Denková (2007), which were then normalized against an $^{87}Sr/^{86}Sr$ ratio of 0.710255 (Miková and Denková, 2007). The results were corrected for rubidium isobaric interference on ^{87}Sr by measuring the signal for ^{85}Rb and the natural $^{85}Rb/^{87}Rb$ ratio (Miková and Denková, 2007). A value of 0.1194 and the exponential law was used to correct for the instrumental mass fractionation on the $^{86}Sr/^{88}Sr$ isotope (Miková and Denková, 2007).

The lead isotopes were analysed as 50ppb 2% HNO_3 solutions using Nu Instruments DSN-100 desolvating nebuliser. All of the samples and standards had NIST SRM997Tl standard added to them in the ratio of 10:1 Pb:Tl. The precision of lead isotope ratios used to be limited by variations in instrumental mass fractionation, resulting in standard deviations of around 300 ppm to 500 ppm (Galer and Abouchami, 1998). However, with the introduction of a triple lead spike by adding an aliquot of $^{206}Pb/^{204}Pb$, $^{207}Pb/^{204}Pb$ and $^{208}Pb/^{204}Pb$ to the analysis reduces the standard deviation of the isotope ratios to ~100 ppm (Galer and Abouchami, 1998). The reference standard used was NIST SRM981, which gave normalized values of 36.7219± 0.0044, 15.4963± 0.0016 and 16.9405± 0.0015 for $^{206}Pb/^{204}Pb$, $^{207}Pb/^{204}Pb$ and $^{208}Pb/^{204}Pb$ respectively (Galer and Abouchami, 1998). The standard with the known isotopic values for each lead isotope ratio were compared to the analysed samples, which gave the accuracy of the analysis. The samples were corrected for interference with mercury isotopes (^{204}Hg) by monitoring the presence of ^{202}Hg in the sample (Thirlwall, 2002; Will et al., 2010). The Pb isotopes were also corrected for instrumental mass fractionation using the exponential law and a value of 2.3889 for the $^{205}Tl/^{203}Tl$ standard that was added to the lead samples and standards (Thirlwall, 2002; Will et al., 2010).

For the trace element concentrations, the digested samples were diluted in 5% HNO_3 and analysed using a Perkin Elmer/Sciex Elan 6000 inductively coupled plasma mass

spectrometry (ICP-MS). Only one replicate was analyzed for each sample for the following trace element concentrations d: ^{6}Li, ^{45}Sc, ^{51}V, ^{52}Cr, ^{59}Co, ^{59}Ni, $^{63.5}$Cu, ^{65}Zn, ^{70}Ga, $^{85.5}$Rb, ^{89}Y, ^{91}Zr, ^{93}Nb, ^{137}Ba, ^{139}La, ^{140}Ce, Pr, ^{144}Nd, ^{150}Sm, ^{152}Eu, ^{159}Tb, ^{157}Gd, $^{162.5}$Dy, ^{165}Ho, ^{167}Er, ^{169}Tm, ^{173}Yb, ^{175}Lu, $^{178.5}$Hf, ^{181}Ta, ^{207}Pb, ^{232}Th, ^{238}U. Calibration curves were constructed using multi-element standards with concentrations of 10 ppb (2A- Alkaline Earth Elements), 20 ppb (REE- Rare Earth Elements) and 30 ppb (HFSE- High Field Strength Elements) which was compared to the concentrations found in the sample. A blank with no sample was run to serve as a quality check for the analysis.

2.8 Radiocarbon dating

Four samples from the *E. lebomboensis* specimen at Kirstenbosch National Botanical Garden were sent for radio carbon dating to the Beta-Analytical Laboratory in Miami, Florida, USA. Radiocarbon dating was also done for *E. arenarius*, however the results were not available before completion of this project. One sample each from the inner and outer vascular ring at the lower part of the cycad stem was sent for radiocarbon dating and cellulose was extracted from these samples at the Beta-Analytical Laboratory. Leaf bases were taken at the upper and lower section of the cycad stem. All four samples were pre-treated with acid-alkali-acid (AAA) wash, using HCl-NaOH-HCl (Beta-Analytic, 2012). Before the samples were analysed using accelerator mass spectrometry (AMS), the AAA pre-treated samples were first cryogenically purified to carbon dioxide through the combustion of the sample, and then reduced to solid graphite. The ^{14}C was measured as percent modern carbon (pMC) with one relative standard deviation, because there was more ^{14}C present in the samples than in the modern reference standard (AD 1950). The ^{14}C values were calculated relative to oxalic acid I (NIST SRM-4990B), which was the reference material used to correct for isotopic fractionation. The calendar age for the samples were determined using percent modern carbon from the "bomb spike" curve (Figure 1; Levin and Kromer, 2004; Levin et al., 2008), because the samples were younger than 1950.

2.9 Data Analysis

The δ^{13}C, δ^{18}O, δ^{15}N, and δ^{34}S isotopes were compared between the relocated and control specimens for each species. The isotope samples were checked for normality and tested for equal variances using Levene's test in the program mini-tab (Minitab Inc., 2000). If the data were normally distributed with equal variances a two-sample t-test was done and for non-

parametric data a Mann-Whitney U test was done between the control and the relocated specimens for each species.

Working with small samples sizes is unavoidable when sampling endangered species, therefore a trade off was made at the start of the project. This study focussed on sampling a variety of isotopes and plant material, instead of selecting one method with numerous replicates. When comparing between variables it is important to be aware of Type 1 error (rejecting the null hypothesis falsely), which would result in accepting that there is a significant change in the isotopic signature when there is actually no change. Analysis of variance test (ANOVA) is sensitive to type 1 errors when sample sizes are small and unequal. Therefore a non-parametric Kruskal-Wallice test was done to compare between the different variables, because the data consisted of small (four or less) sample sizes, which would be sensitive to unequal variance.

The $\delta^{13}C$ and $\delta^{18}O$ isotopes were compared between the different vascular rings for each specimen using a Levene's test (for equal variance) and Kruskal-Wallice test to determine whether the vascular rings within the relocated plant were different between the new and older vascular tissues. The sulphur concentration in the E. *arenarius* petioles and the upper leaf bases from the control was too low to analyse the $\delta^{34}S$ isotopes. The petioles were compared between the relocated and control specimens for both species and a Mann-Whitney U test was done for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ isotopes. The effect size for the $^{87}Sr/^{86}Sr$, $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{207}Pb$ isotopes was too small to test for significance between the control and relocated plants petioles. The leaf bases were compared between the relocated and control specimens for both species and a Levene's test (for equal variance) and Mann-Whitney U test was done for $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, $^{87}Sr/^{86}Sr$, $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{207}Pb$ isotopes. A Kruskal-Wallice test was done between the upper, middle and lower leaf bases for the same isotopes.

Principal component analysis (PCA) was done in R-gui version 2.15.2 (R Development Core Team, 2013). The *E. lebomboensis* and *E. arenarius* specimens were analysed separately, because when they were analysed together the first component separated the two species from each other. A PCA analysis was done with trace elements that had concentrations greater than 0.01 ppm and with the following stable isotopes $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, $^{87}Sr/^{86}Sr$, $^{206}Pb/^{207}Pb$, and $^{208}Pb/^{206}Pb$.

3. Results

3.1 Radiocarbon dating

The upper leaf bases for the relocated *E lebomboensis* specimen were calibrated between 2004 and 2006, whereas the lower leaf bases were calibrated to 1979 (Figure 11). Cellulose from the outer vascular ring of the lower tree cores were calibrated to between 1996 and 1997, whereas the inner vascular ring was calibrated to either between 1959 and 1961 or between 1982 and 1984 (Figure 11). Due to the bell shaped curve of the "bomb spike" graph (Figure 2), two of the samples (lower leaf bases and the inner vascular ring from the lower section of the stem) had two possible dates (Table 2). The most parsimonious date would be the most recent dates, due to the slope of the curve being much steeper between 1958 and 1965, therefore the probability of the date falling on that section of the curve is less likely than post 1970. To tell which date is more accurate another sample from the middle of the stem or the middle of the tree core should be analysed.

The leaf bases provide the oldest and more recent material compared to the vascular rings. Assuming that the most parsimonious age is correct, the radiocarbon age for the inner vascular rings at the bottom of the stem was younger (1982-1984) than the lower leaf bases (1979). This indicates that new plant material is included into the old vascular tissue giving it a younger age than the lower leaf bases. The outer vascular tissue from the lower section of stem was older than expected (1996-1997) compared to the upper leaf bases (2004-2006). This indicates that new plant material takes longer to be incorporated into the outer vascular rings than the upper leaf bases.

The chronological increase of leaf base age from the top to the bottom of the cycad stem is consistent with Vogel and van der Merwe's (1995) findings (Figure 11). The decrease in tissue age from the inner to the outer vascular rings was consistent with Chamberlain's (1911) anatomical observations, where new vascular rings growing on the outside of older vascular rings as the cycad increases in girth (Table 2; Figure 11). *E. arenarius* is expected to also conform to the same sequential tissue growth pattern within the plant.

Considering that the *E. lebomboensis* specimen was moved in 1946, the radiocarbon dates indicate that our samples all date from after the plant was relocated to Kirstenbosch. This suggests that there is continous addition of new material or turn over in both leaf bases and internal vasculature within the plant resulting in younger tissue ages than expected. Another

explanation is that the original cycad stem that arrived at Kirstenbosch gardens 66 years ago died and a new lead stem, which never grew in the wild, replaced it between 30 and 40 years ago.

Table 2. Radiocarbon dates for the relocated *Encephalartos lebomboensis* showing the $\delta^{13}C$ values and the conventional radiocarbon age used to calibrate the samples.

Sample name	Conventional ^{14}C age (pMC)	$\delta^{13}C$ (‰)	Calibrated date
Lower tree core			
outer vascular ring	112.3 +/- 0.4	-22.50	1996-1997
inner vascular ring	122.0 +/- 0.6	-22.40	1959-1961 / 1982-1984
Leaf bases			
upper part of stem	106.2 +/- 0.3	-21.30	2004-2006
lower part of stem	129.4 +/- 0.6	-25.00	1979

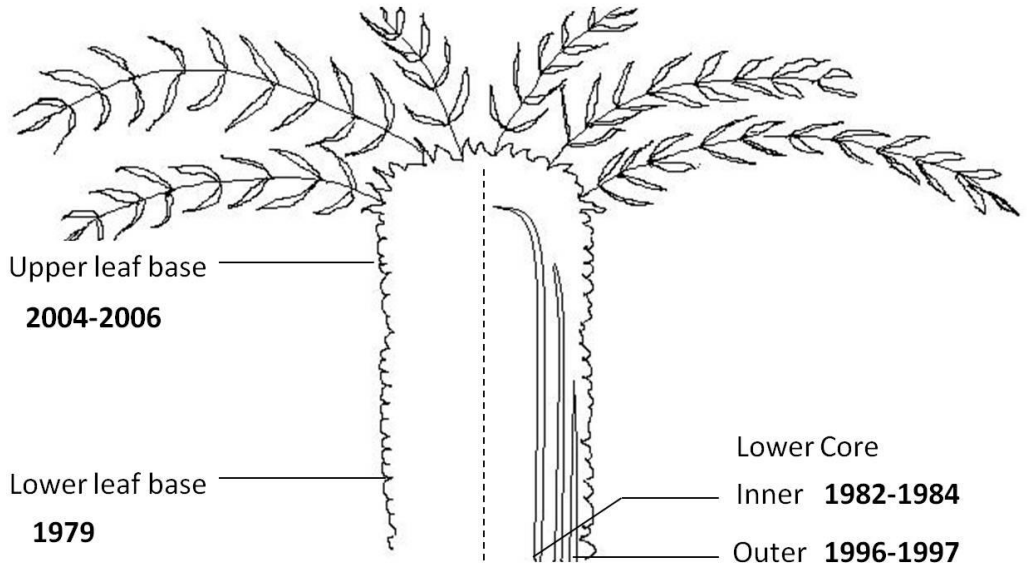

Figure 11. Diagram of *E. lebomboensis* showing calibrated radiocarbon ages for leaf bases and tree cores sampled. Dates in **bold** represents most parsimonious dates based on the shape of the bomb-curve (see results).

3.2 Tree core sampling technique

The *E. lebomboensis* specimen relocated in 1946 is resembling the variation found in a plant that has not been relocated, compared to the more recent relocation of *E. arenarius*, which had significantly more variance than the control. $\delta^{13}C$ ranged betweeen -20 ‰ to -22.5 ‰ for

the *E. lebomboensis* specimens and -22 ‰ to -27 ‰ for the *E. arenarius* specimens (Figure 12 and 13). Testing for equal variances using Levene's test between the relocated and the control specimen found that $\delta^{18}O$ was not significantly different in variance for *E. lebomboensis* (N = 25, 37; F-statistic = 0.201; $p > 0.05$), however the *E. arenarius* specimens were different (N = 19, 15; F-statistic = 11.825; $p < 0.005$). The variance in $\delta^{13}C$ for the relocated and control *E. lebomboensis* specimens were not different from each other (N = 25, 37; F-statistic = 0.001; $p > 0.05$). *Encephalartos arenarius* did have significantly different variances between the relocated and control specimens for $\delta^{13}C$ (N = 19, 15; F-statistic = 5.416; $p < 0.05$).

To test if the sample means for the $\delta^{18}O$ and $\delta^{13}C$ were different between the relocated and control speicmen a two sample t-test was done for *E. lebomboensis* and a Mann-Whitney U-test for *E. arenarius*. Both $\delta^{18}O$ and $\delta^{13}C$ were significantly different between the relocated and control *E. lebomboensis* specimens (Table 3; $\delta^{18}O$: Df = 59, $p < 0.001$, $\delta^{13}C$: Df = 53, $p < 0.001$). This suggests that the vascular rings from the relocated *E. lebomboensis* originated from a different climate compared to the control specimen.For *E. arenarius*, $\delta^{13}C$ was significantly different between the relocated and control specimens, but $\delta^{18}O$ was not (Figure 13; Table 4: $\delta^{18}O$: N = 19, 15, $p > 0.05$, $\delta^{13}C$: N = 19, 15, $p < 0.05$). These isotopic signatures may be reflecting both the wild and new environment, because the relocated plant was recently moved in 1992.

Table 3. Two-sample t-test between the vascular rings from the relocated and control *E. lebomboensis* specimens for the $\delta^{13}C$ and $\delta^{18}O$ isotopes.

Two sample T-Test	**Mean**	**SE Mean**	**N**	**Df**	**T-value**	**p**
$\delta^{18}O$						
E. lebomboensis (relocated)	33.15	0.07	25	59	15.29	**< 0.001**
E. lebomboensis (control)	31.52	0.08	37			
$\delta^{13}C$						
E. lebomboensis (relocated)	-21.76	0.09	25	53	-9.15	**< 0.001**
E. lebomboensis (control)	-20.68	0.08	37			

Table 4. Summary of Mann-Whitney U-test between the relocated and control specimens for $\delta^{13}C$ and $\delta^{18}O$ isotopes from *E. arenarius* vascular tree rings.

Mann-Whitney U test	**Median**	**N**	**U**	**p**
$\delta^{18}O$				
E. arenarius (relocated)	29.8	19	369.5	> 0.05
E. arenarius (control)	29.52	15		
$\delta^{13}C$				
E. arenarius (relocated)	-24.5	19	421.5	**< 0.05**
E. arenarius (control)	-25.53	15		

$\delta^{13}C$ in the *E. lebomboensis* control specimen was lower than the relocated specimen (Figure 12), which may be caused by water stess. The Kruskal-Wallice test (Table 5: N = 37, Df = 6, $p < 0.05$) between the different vascular rings within the control *E. lebomboensis* specimen was significantly different in $\delta^{13}C$, possibly due to environmental variability (Figure 12). The upper vascular rings from the control *E. lebomboensis* were significantly lower in $\delta^{13}C$ compared the vascular rings sampled at the middle and lower sections of the stem (Figure 12), possibly because the plant experienced less water stress during this growth period. $\delta^{18}O$ was different between the vascuar rings within the relocated *E. lebomboensis* specimen, but the large overlap between the various vascular rings is indicative that this difference was caused by environmental variation, and not by the relocation of the plant.

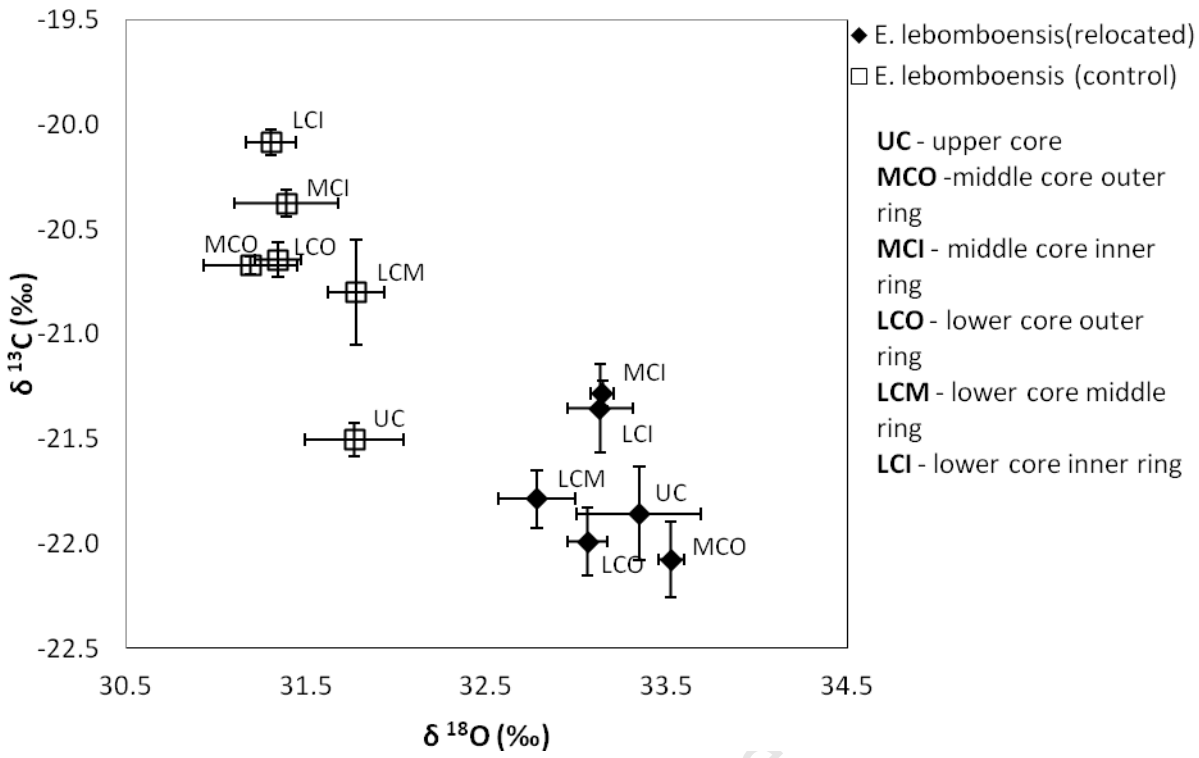

Figure 12. The mean with standard error for $\delta^{13}C$ and $\delta^{18}O$ isotopes from the cellulose extracted vascular rings for the relocated and control *E. lebomboensis* specimens.

Table 5. Summary of Kruskal-Wallice test between the all sampled vascular tree rings for the relocated and control *E. lebomboensis* and *E. arenarius* specimens.

Kruskal-Wallis test	N	Df	H	p
$\delta^{13}C$				
E. lebomboensis (relocated)	25	5	10.14	> 0.05
E. lebomboensis (control)	37	6	25.5	**< 0.05**
E. arenarius (relocated)	19	5	14.46	**< 0.05**
E. arenarius (control)	14	3	12.2	**< 0.05**
$\delta^{18}O$				
E. lebomboensis (relocated)	25	5	11.46	**< 0.05**
E. lebomboensis (control)	37	6	9.96	> 0.05
E. arenarius (relocated)	19	5	12.32	**< 0.05**
E. arenarius (control)	14	3	1.86	> 0.05

The $\delta^{13}C$ from the inner vascular ring of the lower stem was more positive compared to the rest of the vascular rings for the relocated *E. arenarius* specimen (Figure 13; Table 5: N= 19, Df= 5, $p < 0.05$). This could represent tissue formed during a period of water stress. The outer vascular rings from the middle section of the control *E. arenarius* stem was lower in $\delta^{13}C$ compared to the outer and inner vascular rings sampled from the lower section of the stem (Figure 13). This may be indicative of a period with less water stress resulting in lower $\delta^{13}C$.

$\delta^{18}O$ was similar between the different vascular rings sampled for the *E. arenarius* control specimen (Table 5; Figure 13). This was expected, because the plant remained in the same climate and therefore the variability in isotopic value should be minimal. The upper vascular ring from the relocated *E. arenarius* specimen had higher $\delta^{18}O$ compared to the rest of the vascular rings (Figure 13). The upper section of this relocated plant was grown within Nelspruit since 1992, and the increased $\delta^{18}O$ may be due to higher VPD in Nelspruit. Variability in the $\delta^{18}O$ of the source water may explain why the inner vascular ring from the middle section of the relocated *E. arenarius* stem had lower $\delta^{18}O$ compared to the rest of the vascular rings (Figure 13).

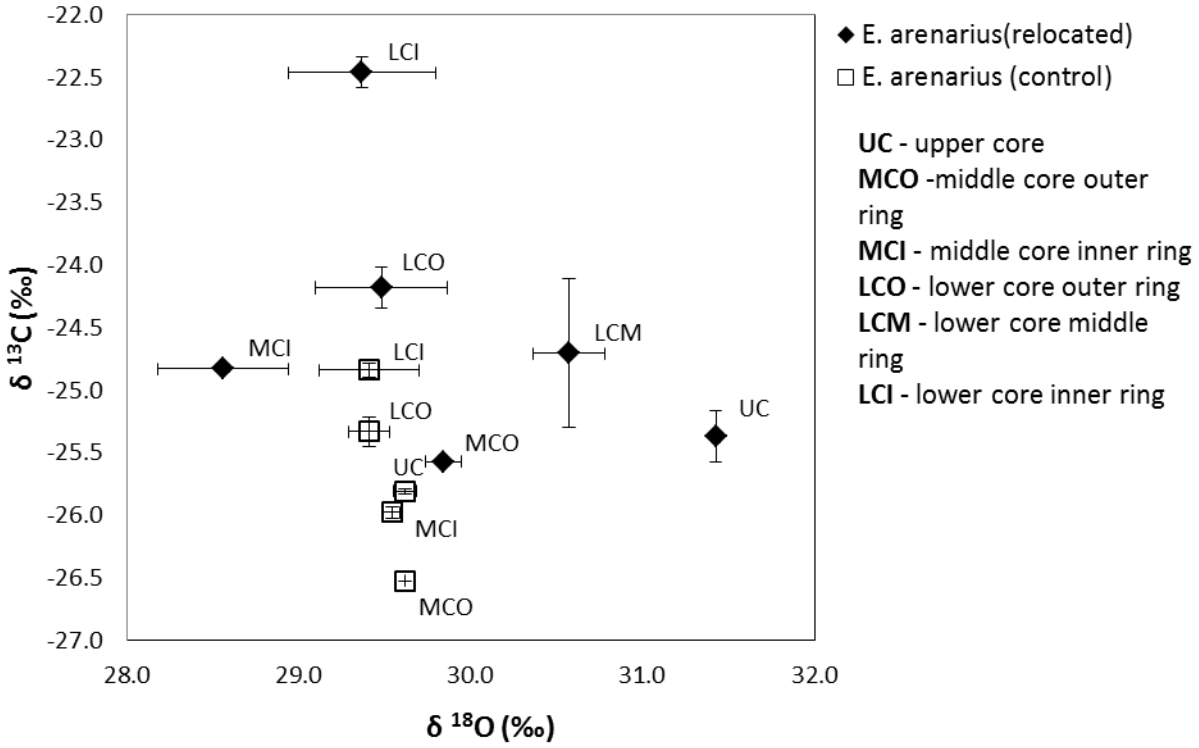

Figure 13. The mean with standard error for $\delta^{13}C$ and $\delta^{18}O$ isotopes from the cellulose extracted vascular rings for the relocated and control *E. arenarius* specimens.

3.3 Petiole sampling technique

Variation in isotopic composition between the different sites is assuring for potentially using petioles as a forensic tracer. $\delta^{13}C$ and $\delta^{34}S$ for *E. lebomboensis* were almost significantly different between the relocated and control specimens, and with more replication the samples may be statistically different (Table 6; Figure 14). $\delta^{13}C$ and $^{87}Sr/^{86}Sr$ ratios appeared to be different in isotopic composition between the control and relocated plants for both species (Figure 14). $\delta^{13}C$, $\delta^{34}S$ and $^{87}Sr/^{86}Sr$ may be the most useful for measuring a shift in location using petiole samples, because they reflect a change in isotopic value for both species. Whereas the $\delta^{15}N$, $^{206}Pb/^{207}Pb$, and $^{208}Pb/^{207}Pb$ isotopes do not show a clear separation between the control and relocated specimens for both species (Figure 14).

Table 6. Summary of Mann-Whitney U-test between the relocated and control specimens for $\delta^{15}N$, $\delta^{13}C$ and $\delta^{34}S$ isotopes from *E. lebomboensis* and *E. arenarius* petiole samples.

Mann-Whitney U test	Median	N	U	P
$\delta^{15}N$				
E. lebomboensis(relocated)	-2.41	4	11.00	> 0.05
E. lebomboensis(control)	-1.82	3		
E. arenarius (relocated)	-2.21	3	12.00	> 0.05
E. arenarius (control)	-2.31	3		
$\delta^{13}C$				
E. lebomboensis(relocated)	-22.995	4	10.00	> 0.05
E. lebomboensis(control)	-22.48	3		
E. arenarius (relocated)	-27.45	3	6.00	> 0.05
E. arenarius (control)	-26.59	3		
$\delta^{34}S$				
E. lebomboensis(relocated)	22.99	4	10.82	> 0.05
E. lebomboensis(control)	11.44	3		

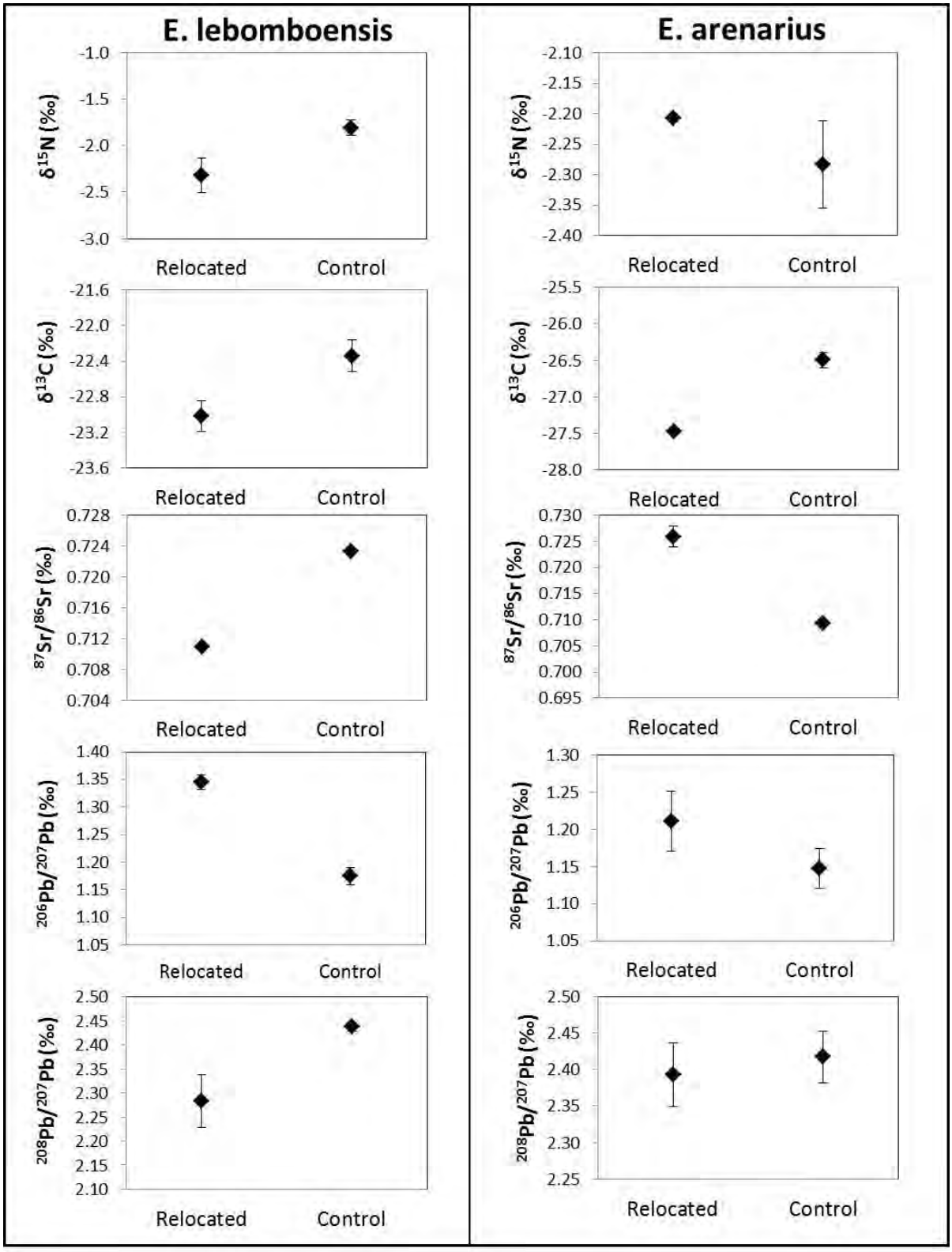

Figure 14. Comparing the means of $\delta^{15}N$, $\delta^{13}C$, $^{87}Sr/^{86}Sr$, $^{206}Pb/^{207}Pb$, and $^{208}Pb/^{207}Pb$ with standard error between the relocated and the control specimens for petiole samples from *E. lebomboensis* and *E. arenarius*.

3.4 Leaf base sampling technique

3.4.1 Nitrogen, Carbon and Sulphur

It was found that $\delta^{13}C$ was more variable for the relocated *E. lebomboensis* specimen than the control and $\delta^{15}N$ was more variable within the relocated than the control specimen for *E. arenarius* (Table 7). This might suggest that the relocated plants have greater variability than control specimens do, because they have isotopic values from different environments. For *E. lebomboensis* there were no differences in variance between the control and relocated plants for $\delta^{15}N$ and $\delta^{34}S$ and no difference in variance for $\delta^{13}C$ and $\delta^{34}S$ between the *E. arenarius* specimens (Table 7). Based on results in Table 7, using variance as a proxy for determining if a cycad has been relocated is not consistent with the relocated specimen having a greater variance than the control specimen for either of the isotopes, therefore this is unlikely a plausible forensic method.

Table 7. Levene's equal variance test for $\delta^{15}N$, $\delta^{13}C$ and $\delta^{34}S$ isotopes between the relocated and control specimens for *E. lebomboensis* and *E. arenarius*.

Levene's test	**N**	**F-statistic**	**p**
E. lebomboensis			
$\delta^{15}N$	9, 12	0.008	> 0.05
$\delta^{13}C$	9, 12	10.307	**< 0.05**
$\delta^{34}S$	9, 12	0.097	> 0.05
E. arenarius			
$\delta^{15}N$	12, 12	5.89	**< 0.05**
$\delta^{13}C$	12, 12	2.798	> 0.05
$\delta^{34}S$	12, 12	3.432	> 0.05

The $\delta^{13}C$ isotopic values ranged between -21.5 ‰ and -24.5 ‰ for the *E. lebomboensis* and between -24 ‰ and -27.5 ‰ for the *E. arenarius* specimens (Figure 15 and 16). The upper and middle leaf bases had higher $\delta^{13}C$ values compared to the lower leaf bases for the relocated *E. lebomboensis* specimen (Figure 15). Although the Kruskal-Wallice test was not significant, the p-value was close to significance (Table 9: $\delta^{13}C$: N = 9, Df = 2, p = 0.061), and the lower leaf base would possibly be different from the upper and middle leaf bases if the sample size was increased. The $\delta^{13}C$ values for the *E. lebomboensis* control specimen were similar for all of the leaf bases along the height of the stem and ranged between -23 ‰

and -24 ‰ (Figure 15). These constant $\delta^{13}C$ values are indicative of an invariable photosynthetic rate and little environmental variablity which was expected as this specimen remained in the same locality. The $\delta^{15}N$ isotopes were similar between (Table 8: N = 9, 12, p > 0.05) and within (Table 9) the relocated and control *E. lebomboensis* specimens and ranged between -0.5 ‰ and -2 ‰ (Figure 15). This suggests that the source or rate of nitrogen assimilation is similar within the relocated and control *E. lebomboensis* specimens.

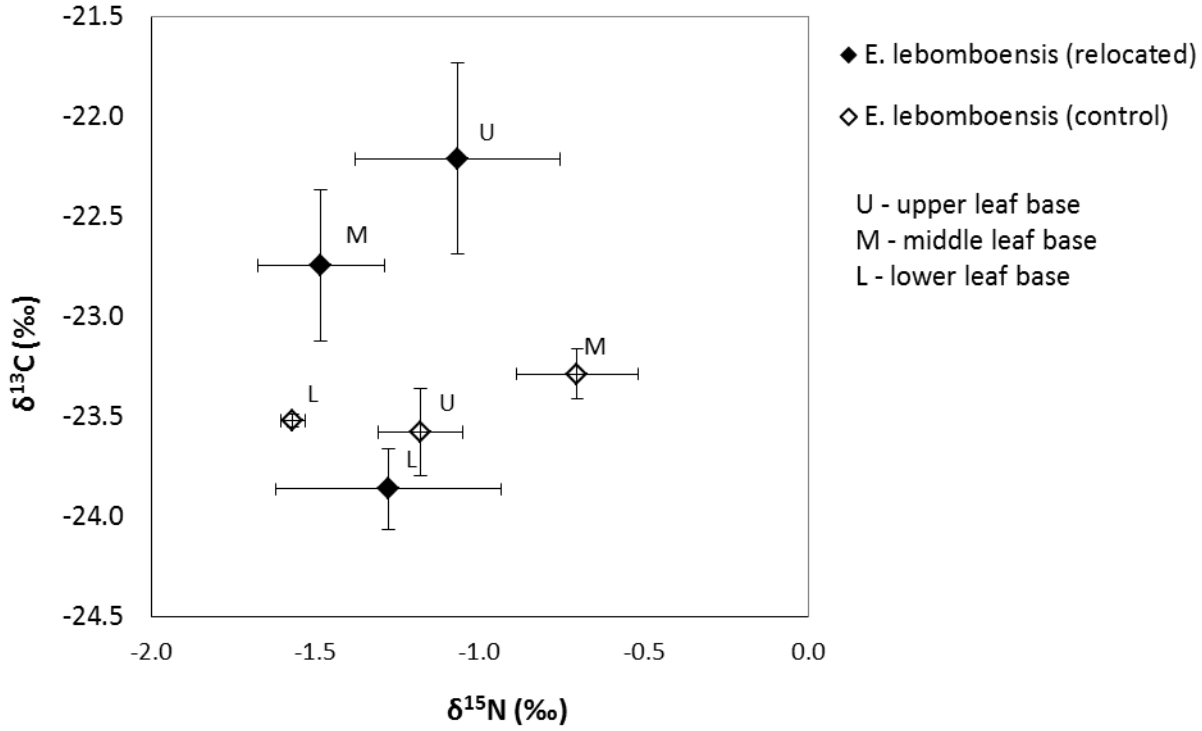

Figure 15. The $\delta^{13}C$ and $\delta^{15}N$ isotope values with standard error for the relocated and control *E. lebomboensis* specimens.

There was no difference between the control and relocated *E. arenarius* specimens in $\delta^{13}C$, but there was a difference in $\delta^{15}N$ (Table 8: $\delta^{13}C$: N= 12, 12, $p > 0.05$, $\delta^{15}N$: N= 12, 12 , $p < 0.05$). The upper leaf bases from the control *E. arenarius* specimen were lower in $\delta^{13}C$ compared to the middle and lower leaf bases (Figure 16; Table 9: N = 12, $p < 0.05$), and $\delta^{15}N$ was not different within the control specimen and had a value of around -2.5 ‰ (Figure 16; Table 9: N = 12, $p > 0.05$). The upper leaf bases for the relocated *E. arenarius* specimen were significantly lower in $\delta^{13}C$ and higher in $\delta^{15}N$ compared to the middle and lower leaf bases (Figure 16; Table 9: $\delta^{13}C$: N = 12, $p < 0.05$, $\delta^{15}N$: N = 12, $p < 0.05$). Although, the upper leaf bases were significantly different in $\delta^{13}C$ from the middle and lower leaf bases for both the control and the relocated specimens, the relocated specimen was different by >2.5 ‰

compared to a difference of <1.5 ‰ for the control. The greater difference in $\delta^{13}C$ for the relocated specimen is possibly because the cycad was relocated to a different environment. The higher $\delta^{15}N$ value for the upper leaf base in the relocated *E. arenarius* is also suggestive that these leaf bases were grown in a different soil compared to the middle and lower leaf bases, supporting the shift seen in $\delta^{13}C$ (Figure 16).

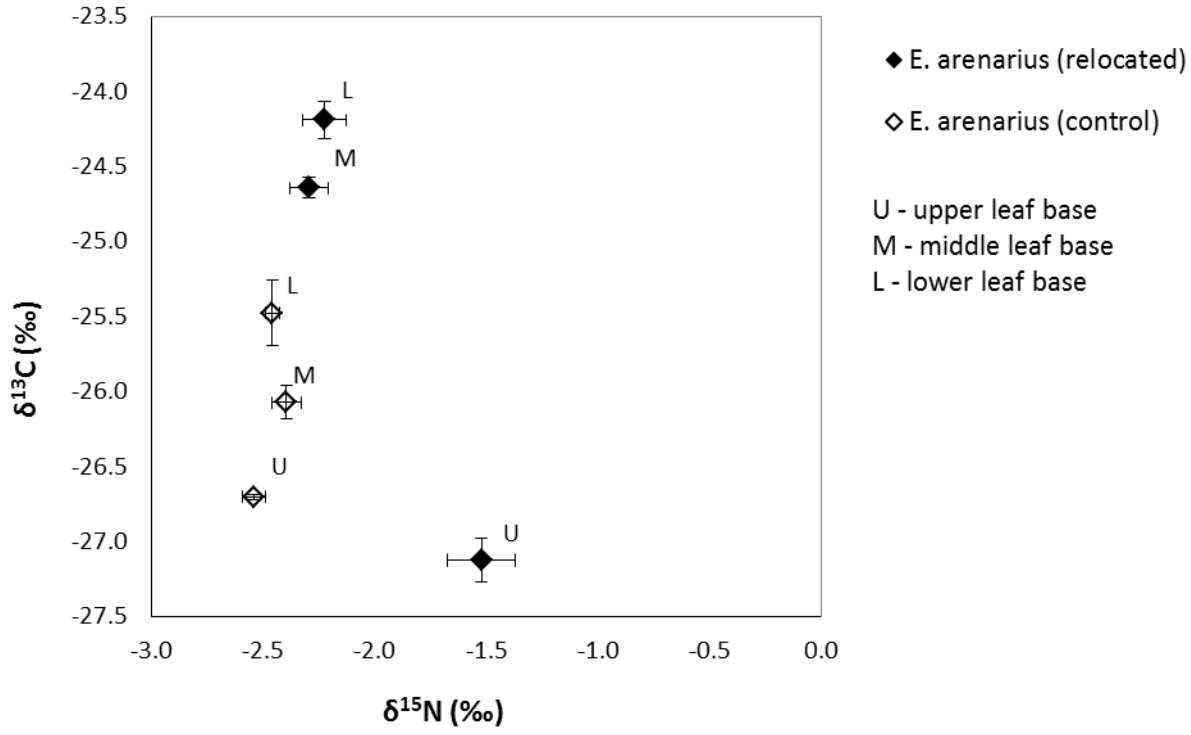

Figure 16. The $\delta^{13}C$ and $\delta^{15}N$ isotope values with standard error for the relocated and control *E. arenarius* specimens.

Table 8. Mann-Whitney U test for $\delta^{15}N$, $\delta^{13}C$ and $\delta^{34}S$ stable isotopes between the relocated and control specimen for *E. lebomboensis* and *E. arenarius* leaf bases.

Mann-Whitney U test	**Median**	**N**	**U**	**P**
$\delta^{15}N$				
E. lebomboensis(relocated)	-1.36	9	89	> 0.05
E. lebomboensis(control)	-1.18	12		
E. arenarius (relocated)	-2.18	12	205	**< 0.05**
E. arenarius (control)	-2.48	12		
$\delta^{13}C$				
E. lebomboensis(relocated)	-22.86	9	116	> 0.05
E. lebomboensis(control)	-23.46	12		
E. arenarius (relocated)	-24.65	12	174	> 0.05
E. arenarius (control)	-26.14	12		
$\delta^{34}S$				
E. lebomboensis(relocated)	16.36	9	149	**< 0.05**
E. lebomboensis(control)	13.86	12		

Table 9. Kruskal-Wallice test for $\delta^{15}N$, $\delta^{13}C$ and $\delta^{34}S$ between the upper, middle and lower leaf bases for the relocated and control specimens of *E. lebomboensis* and *E. arenarius*.

Kruskal-Wallis test	**N**	**Df**	**H**	**p**
$\delta^{13}C$				
E. lebomboensis (relocated)	9	2	5.6	> 0.05
E. lebomboensis (control)	12	2	2.9	> 0.05
E. arenarius (relocated)	12	2	9.88	**< 0.05**
E. arenarius (control)	12	2	9.04	**< 0.05**
$\delta^{15}N$				
E. lebomboensis (relocated)	9	2	1.16	> 0.05
E. lebomboensis (control)	12	2	7.04	**< 0.05**
E. arenarius (relocated)	12	2	7.39	**< 0.05**
E. arenarius (control)	12	2	2.42	> 0.05
$\delta^{34}S$				
E. lebomboensis (relocated)	9	2	4.36	> 0.05
E. lebomboensis (control)	12	2	8.35	**< 0.05**
E. arenarius (relocated)	12	2	4.27	> 0.05

The relocated *E. lebomboensis* specimen was higher in $\delta^{34}S$ compared to the control specimen (Figure 17; Table 8: N = 9, 12, P < 0.05), potentially indicating that they were incorporating $\delta^{34}S$ from different soils. $\delta^{34}S$ increased from the lower leaf bases to the upper leaf bases for the control *E. lebomboensis* specimen, however there was no noticeable difference between the different leaf bases (Figure 17). The upper leaf bases for the control *E. lebomboensis* specimen were lower in $\delta^{34}S$ compared to the middle and lower leaf bases (Figure 17: Table 9: N = 12, $p < 0.05$). The differences seen in $\delta^{34}S$ within the same specimen could be due to changes in sulphur availability within the soil or selective absorption of sulphur by the plant (Trust and Fry, 1992), because the control specimens remained within the same locality.

The relocated *E. arenarius* specimen was lower in $\delta^{34}S$ compared to the control specimen (Figure 18), separating the two specimens from each other. The upper leaf bases were lower in $\delta^{34}S$ compared to the middle and lower leaf bases for the relocated *E.arenarius* specimen (Figure 18; Table 9: N = 12, H = 4.27, $p < 0.05$). This may be an indication that the upper leaf bases were grown in a different soil, given that the middle and lower leaf bases for the relocated plant were similar to the control in isotopic value. However, this is uncertain due to the missing upper leaf base value for the control specimen.

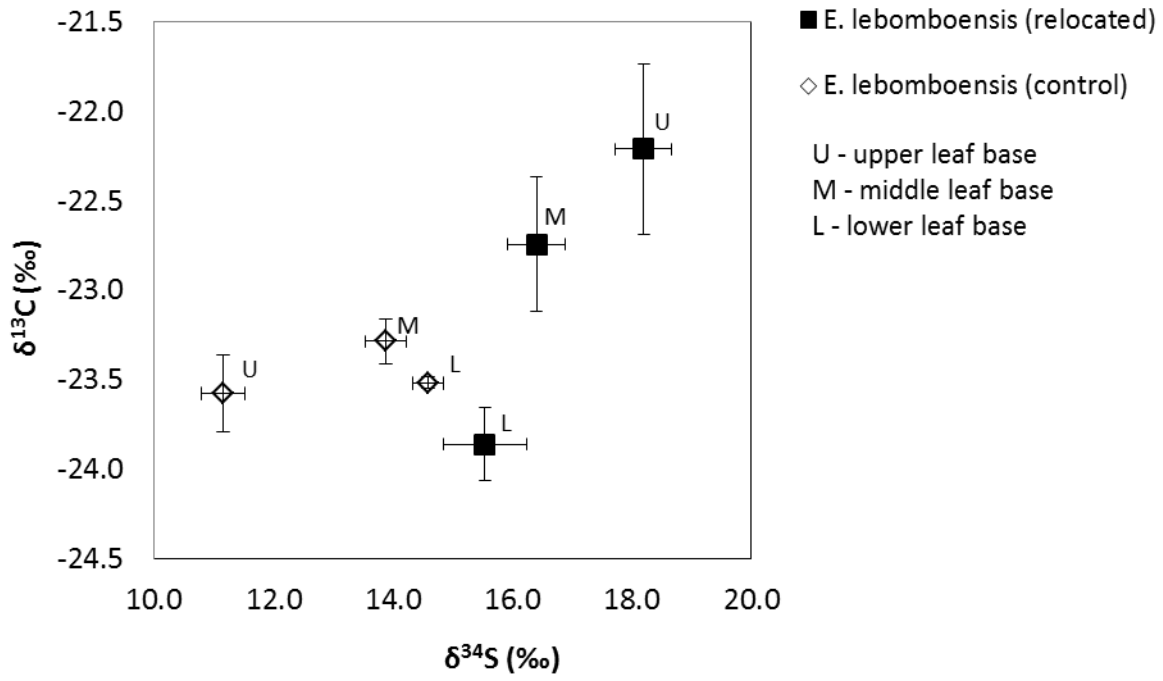

Figure 17. The $\delta^{13}C$ and $\delta^{34}S$ isotope values with standard error for the relocated (solid markers) and control (open markers) *E. lebomboensis* specimens.

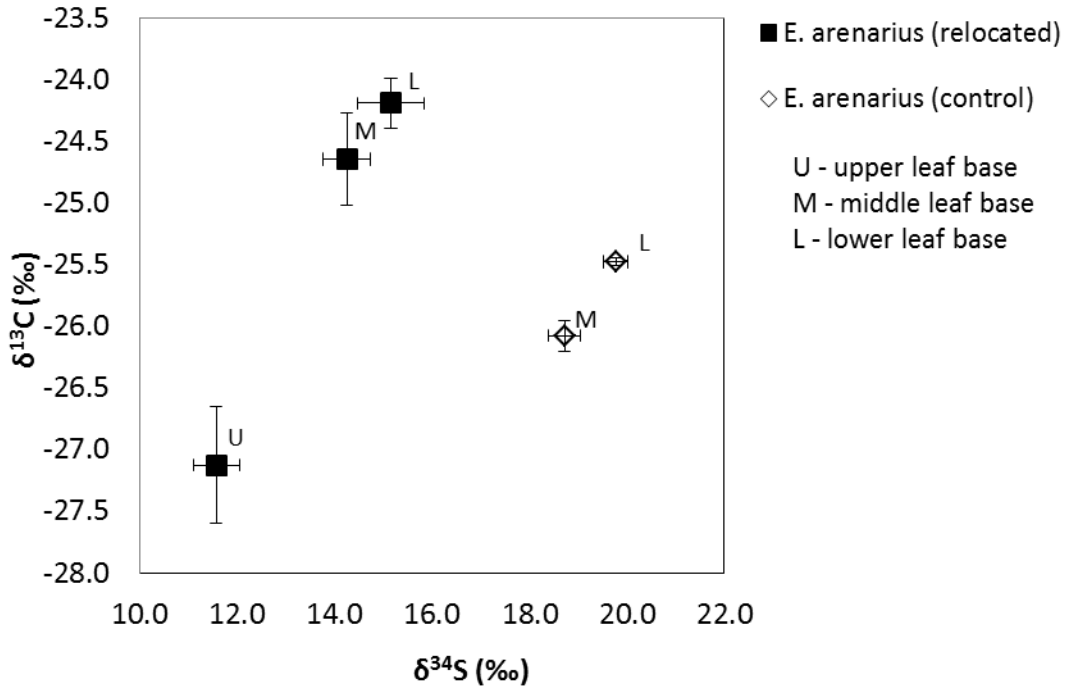

Figure 18. The $\delta^{13}C$ and $\delta^{34}S$ isotope values with standard error for the relocated and control *E. arenarius* specimens.

3.4.2 Lead and Strontium

There was a significant difference in $^{87}Sr/^{86}Sr$ between the relocated and control specimens for *E. lebomboensis* (Figure 19; Table 10), suggesting that these specimens grew in different soil types. The difference in $^{87}Sr/^{86}Sr$ between the control and relocated *E. lebomboensis* specimens might be attributed to the proximity of the relocated specimen to the ocean. Sea spray has been known to influence the $^{87}Sr/^{86}Sr$ significantly in organic matter growing close to the ocean (Rummel et al., 2010). Ocean water has a $^{87}Sr/^{86}Sr$ ratio of 0.7092 which was similar to the $^{87}Sr/^{86}Sr$ ratio of the relocated plant (Cape Town) with a mean of 0.710876 ± 0.00005 (SE) (Figure 19).

There was no difference in the $^{87}Sr/^{86}Sr$ between the upper, middle and lower leaf bases for the relocated *E. lebomboensis* specimen (Figure 19; Table 11: $N = 6$, $Df = 2$, $p > 0.05$). This is indicative that the leaf bases from the relocated *E. lebomboensis* specimen were grown in the same soil type. The $^{87}Sr/^{86}Sr$ for the middle leaf bases was significantly lower than the upper and middle leaf bases within the *E. lebomboensis* control specimen (Table 11: $N = 9$, $Df = 2$, $p < 0.05$). However, this difference is most likely due to extremely small variance between the different leaf bases.

There was a difference in $^{87}Sr/^{86}Sr$ between the relocated and control specimens for *E. arenarius* (Figure 20; Table 10).This was because the upper leaf bases for the relocated *E. arenarius* specimen had significantly higher $^{87}Sr/^{86}Sr$ ratio than the middle and lower leaf bases (Figure 20; Table 11). The middle and lower leaf bases from the relocated *E .arenarius* specimen had almost the same $^{87}Sr/^{86}Sr$ ratio as the control specimen's leaf bases (Figure 20). This strongly suggests that the middle and lower leaf bases from the relocated plant grew in the same soil type as the control specimen. The proximity to the ocean can also explain the $^{87}Sr/^{86}Sr$ ratio for the middel and lower leaf bases for the relocated, as well as the control *E. arenarius* plants. This is because $^{87}Sr/^{86}Sr$ ratios varied around the ocean water $^{87}Sr/^{86}Sr$ ratio of 0.7092 (Rummel et al., 2010) and it is known that the *E. arenarius* specimen came from an environment that was within 15 km from the coast.

Table 10. Summary of Mann-Whitney U test between the relocated and control *E. lebomboensis* and *E. arenarius* specimens for the $^{87}Sr/^{86}Sr$, $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{207}Pb$ ratios.

Mann-Whitney U test	Median	N	U	p
$^{87}Sr/^{86}Sr$				
E. lebomboensis (test)	0.71091	6	21	**< 0.05**
E. lebomboensis (control)	0.7231	9		
E. arenarius (test)	0.71061	9	126	**< 0.05**
E. arenarius (control)	0.70922	9		
$^{206}Pb/^{207}Pb$				
E. lebomboensis (test)	1.144	6	49	> 0.05
E. lebomboensis (control)	1.143	9		
E. arenarius (test)	1.202	9	85	> 0.05
E. arenarius (control)	1.200	9		
$^{208}Pb/^{207}Pb$				
E. lebomboensis (test)	2.095	6	45	> 0.05
E. lebomboensis (control)	2.107	9		
E. arenarius (test)	2.042	9	86	> 0.05
E. arenarius (control)	2.050	9		

Table 11. Summary Kruskal-Wallice test comparing the $^{87}Sr/^{86}Sr$, $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{207}Pb$ ratios for the upper, middle and lower leaf bases for the relocated and control *E. lebomboensis* and *E. arenarius* specimens.

Kruskal-Wallice	**N**	**Df**	**H**	**p**
$^{87}Sr/^{86}Sr$				
E. lebomboensis (relocated)	6	2	4.57	> 0.05
E. lebomboensis (control)	9	2	7.2	**< 0.05**
E. arenarius (relocated)	9	2	6.49	**< 0.05**
E. arenarius (control)	9	2	5.96	**< 0.05**
$^{206}Pb/^{207}Pb$				
E. lebomboensis (test)	6	2	2.57	> 0.05
E. lebomboensis (control)	9	2	0.36	> 0.05
E. arenarius (test)	9	2	2.76	> 0.05
E. arenarius (control)	9	2	2.49	> 0.05
$^{208}Pb/^{207}Pb$				
E. lebomboensis (test)	6	2	3.71	> 0.05
E. lebomboensis (control)	9	2	0.09	> 0.05
E. arenarius (test)	9	2	3.47	> 0.05
E. arenarius (control)	9	2	2.49	> 0.05

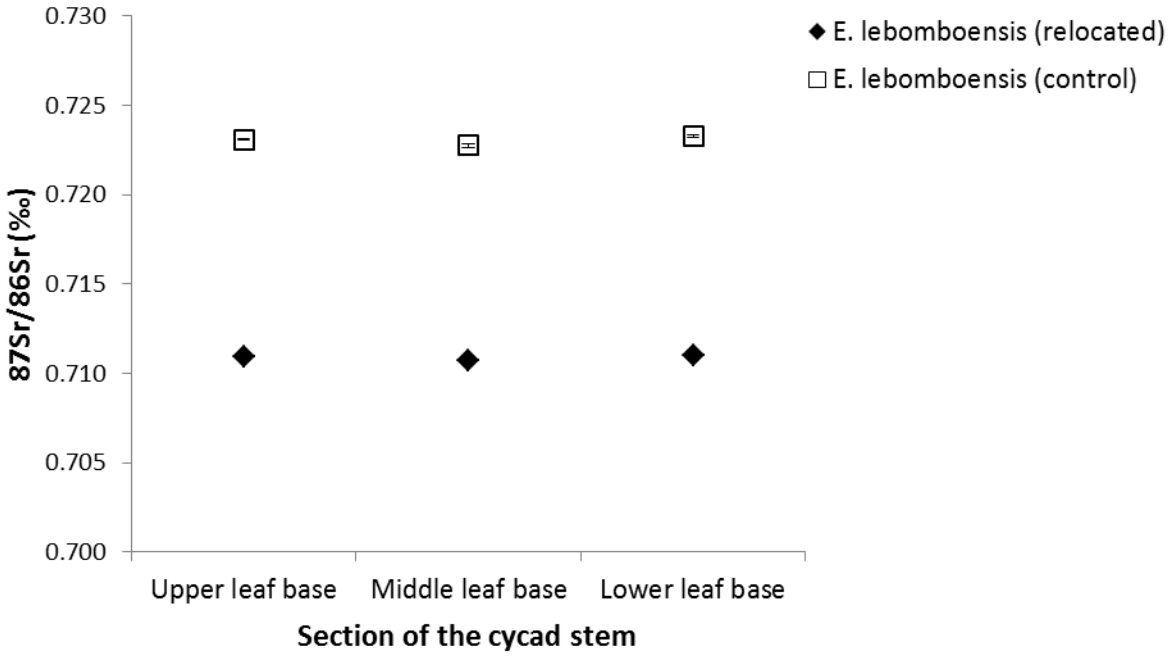

Figure 19. The $^{87}Sr/^{86}Sr$ ratio for the upper, middle and lower leaf bases from the relocated and control *E. lebomboensis* specimens.

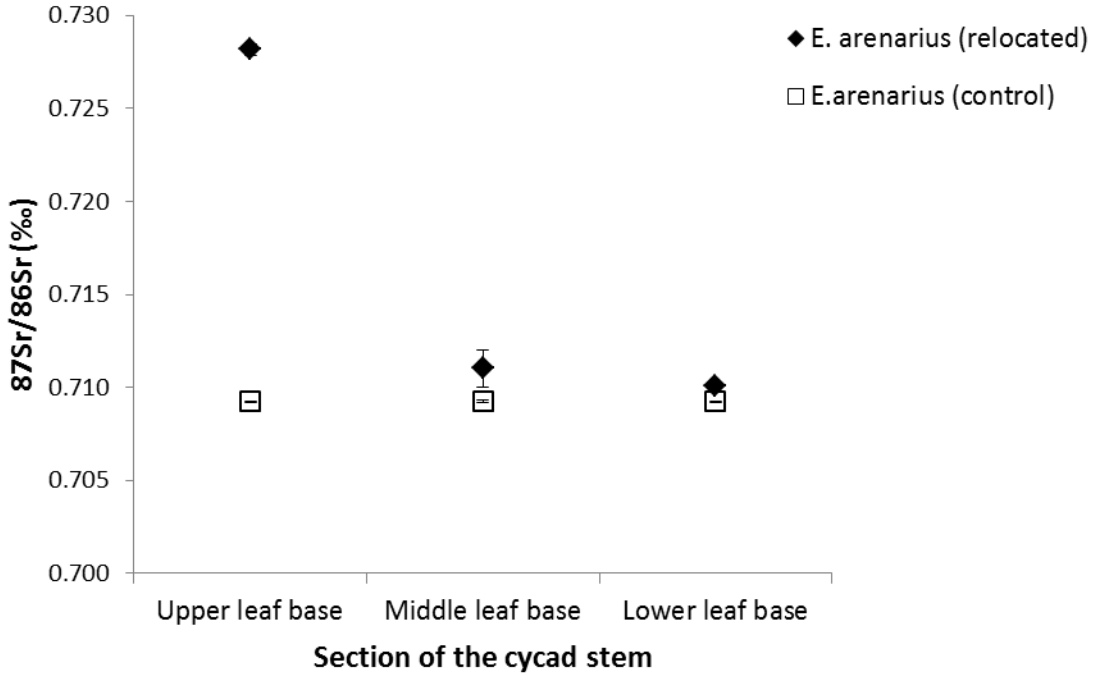

Figure 20. The $^{87}Sr/^{86}Sr$ ratio for the upper, middle and lower leaf bases from the relocated and control *E. arenarius* specimens.

There was no difference in $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{207}Pb$ between the control and relocated *E. lebomboensis* and *E. arenarius* specimens (Figures 21 and 22; Table 10). The $^{206}Pb/^{207}Pb$ ratio varied between 1.10 and 1.18 and $^{208}Pb/^{207}Pb$ ratios varied around 2.4 for the *E. lebomboensis* leaf bases (Figure 21). These lead isotopes ratios concur with lead isotope ratios sampled in vegetation originating from the Cape Peninsula Formation (Soderberg and Compton, 2007), Phillippi vegetation (Govender, 2002), and average crust (Faure, 1986). Due to the large variation in $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{207}Pb$ for the *E. arenarius* specimens, the general geology that these specimens originated from could not be inferred.

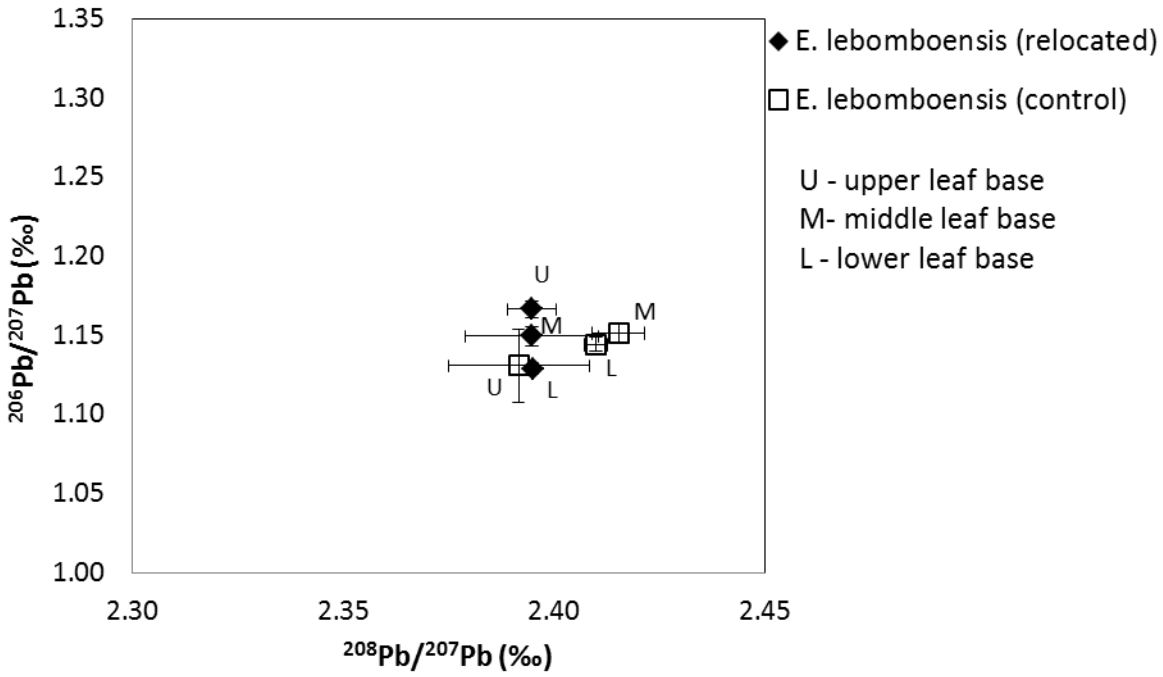

Figure 21. The mean $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{207}Pb$ ratio and standard error of for the relocated and control *E. lebomboensis* specimens.

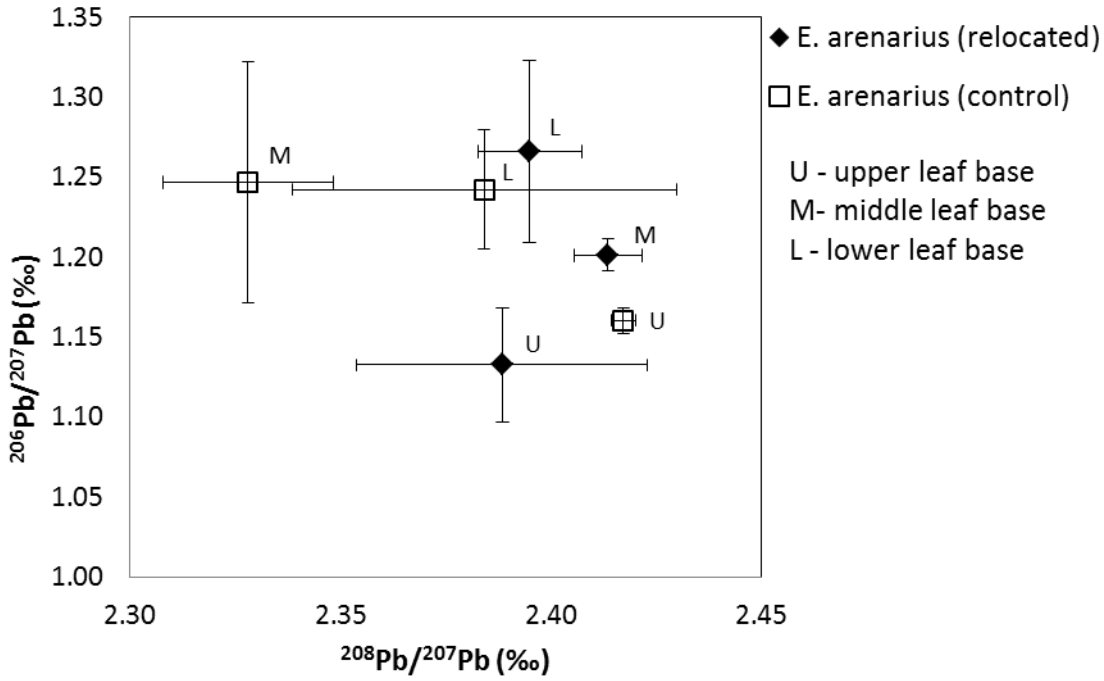

Figure 22. The mean $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{207}Pb$ ratio and standard error of for the relocated and control *E. arenarius* specimens.

3.5 Principal component analysis

3.5.1 Leaf bases

The PCA for *E. lebomboensis* separated the relocated and the control specimen into two distinct groupings (Figure 23). Based on the first component which accounted for 52.66 % of the variance, the $\delta^{13}C$, $\delta^{34}S$ and $^{87}Sr/^{86}Sr$ ratios were the most influential isotopes for distinguishing between the control and relocated *E. lebomboensis* specimens (Figure 23). The $\delta^{15}N$ isotopes explained the variation seen in the control specimen based on the second component which only accounted for 20.92 % of the variance (Figure 23). The $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{207}Pb$ ratios were not as useful in explaining the differences in spatial distribution between the relocated and control leaf bases (Figure 23). These results indicate that the control and relocated specimens originated from different environments based on $\delta^{13}C$, $\delta^{34}S$, $\delta^{15}N$ and $^{87}Sr/^{86}Sr$.

The PCA plot for *E. arenarius* grouped the upper leaf bases from the relocated specimen together, and the middle and lower leaf bases with the control specimen (Figure 24). The first component, which explained 59.83 % of the variance, grouped the upper leaf bases from the relocated specimen together according to $^{87}Sr/^{86}Sr$ and $\delta^{15}N$. The rest of the leaf bases were

grouped together according to $\delta^{13}C$ and $^{206}Pb/^{207}Pb$ (Figure 24). The $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{207}Pb$ ratios explained the distribution of the control *E. arenarius* specimen based on the second component, which accounted for 23.77 % of the variance (Figure 24). These results indicate that the upper leaf bases from the relocated plant originated from a different environment (Nelspruit), compared to the middle and lower leaf bases, which were grouped with the control specimen indicating that they originated from the wild (Eastern Cape).

$\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$ and $^{87}Sr/^{86}Sr$ in combination showed great potential for distinguishing between the different environments and could be useful as a forensic tool (Figures 23 and 24).

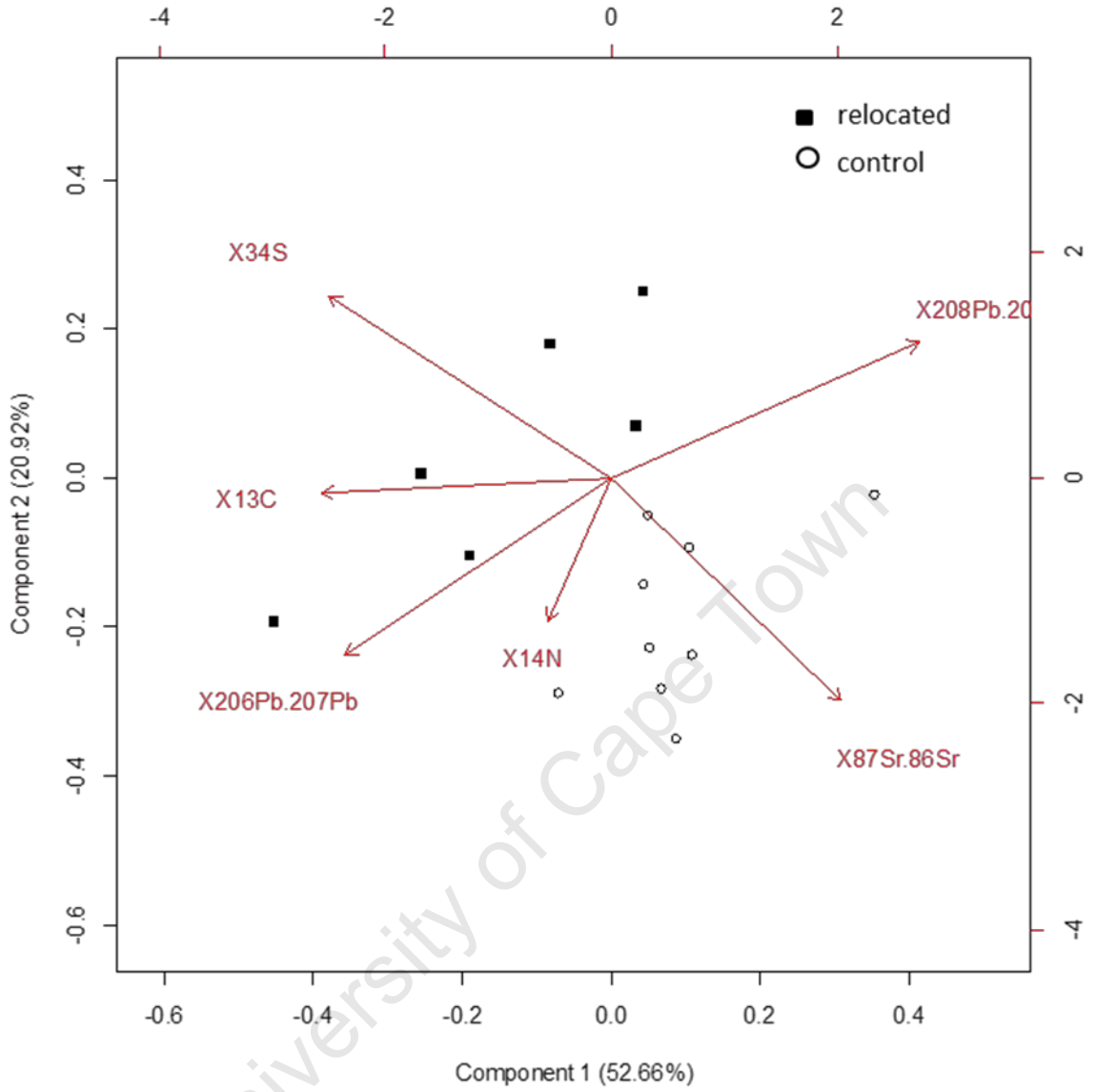

Figure 23. The principal component analysis for the leaf base samples collected for the relocated *E. lebomboensis* (Cape Town) and the control *E. lebomboensis* (KwaZulu-Natal). The red arrows indicate which isotopes explain the variance within the different samples for the first two components with the percent variance in brackets.

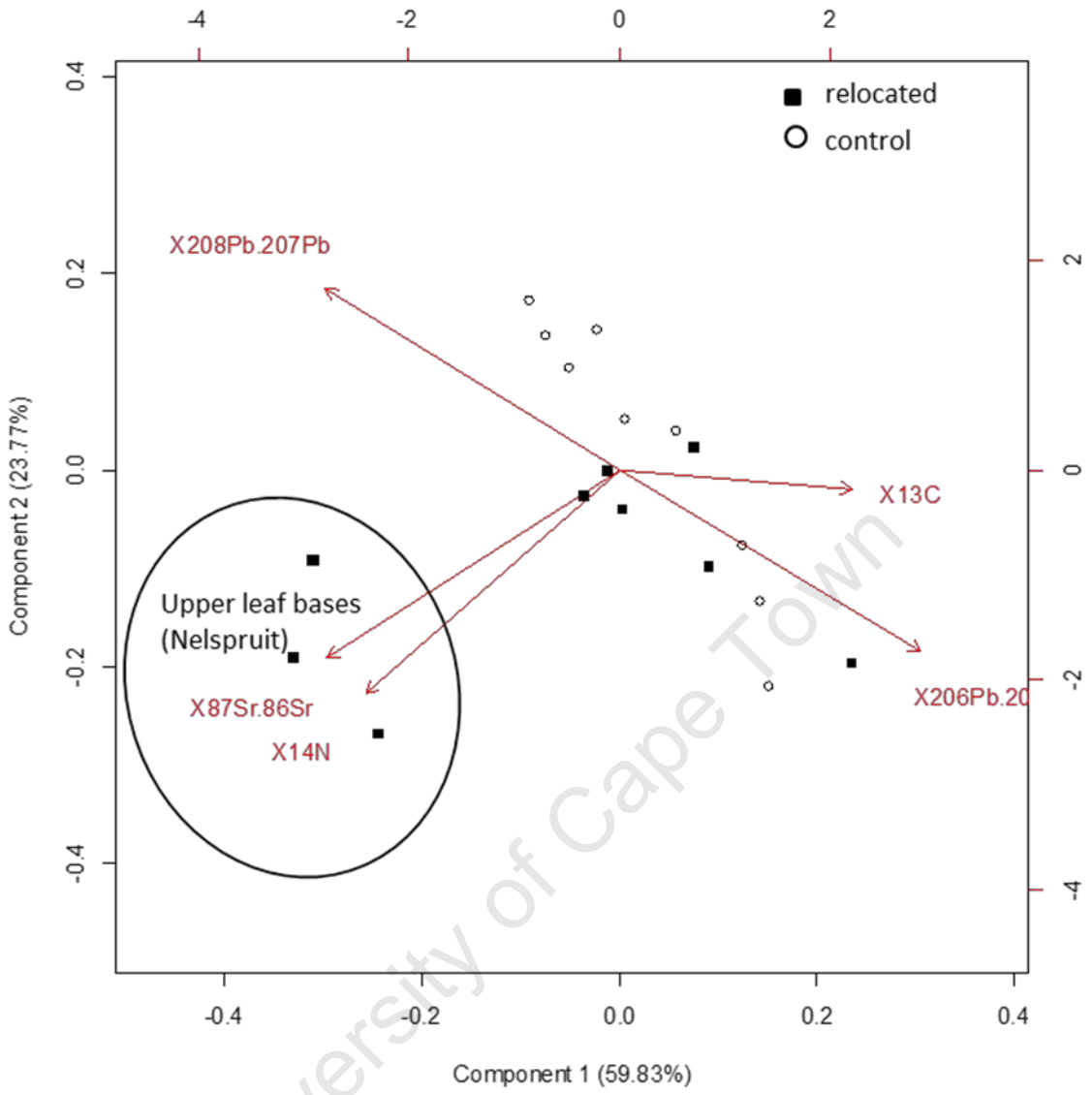

Figure 24. The principal component analysis for leaf base samples collected for the relocated *E. arenarius* (Nelspruit) and control *E. arenarius* (Eastern Cape). The red arrows indicate which isotopes explain the variance within the different samples for the first two components with the percent variance in brackets.

3.5.2 Trace element concentrations

Figure 25 shows the PCA plot for the trace element concentrations with the relocated and control *E. lebomboensis* specimens. The first component represented 35.97 % of the variance and separated the two specimens from each other with the relocated *E. lebomboensis* on the right and the control on the left of the plot (Figure 25). The upper, middle and lower leaf bases for the control specimen were correlated to Zr, Y, Ba, and Ga trace element concentrations and were distributed closely together on the plot (Figure 25). The leaf bases for the relocated *E. lebomboensis* specimen were distributed across the plot with the upper leaf bases correlated to Cu, Li and Rb, the middle leaf bases correlated to Cr, Zn and Pb, and the lower leaf bases correlated to La, Ce and Nd trace element concentrations (Figure 25).

The first component for the trace element concentrations in the *E. arenarius* specimens represented 52.05 % of the variability and separated the control specimen from the middle and lower leaf bases of the relocated specimen (Figure 26). The middle and lower leaf bases from the relocated specimen were correlated to the Cu, Li and Rb trace element concentrations and the second component explained the variance in the upper leaf bases through the Pb and Zn trace element concentrations (Figure 26). The variability in the upper leaf bases for the control specimen were explained by Zr, Y, Nd, Ce, Ba, Ga and La trace element concentrations, whereas the middle and lower leaf bases were explained by Cr and V trace element concentrations.

The trace element concentrations appear to separate the relocated and control specimens from each other (Figure 25 and 26). The upper leaf bases for *E. lebomboensis* and *E. arenarius* relocated specimens were both spatially separated from the middle and lower leaf bases by the second component (Figure 25 and 26). Based on the *E. lebomboensis* radiocarbon results the upper leaf bases were grown in the same locality as the lower leaf bases (Table 2), therefore the reason for this spatial separation is uncertain. In *E. arenarius* the movement of the plant to a new locality may explain the spatial separation of the upper leaf bases from the relocated specimen in the PCA plot (Figure 26).

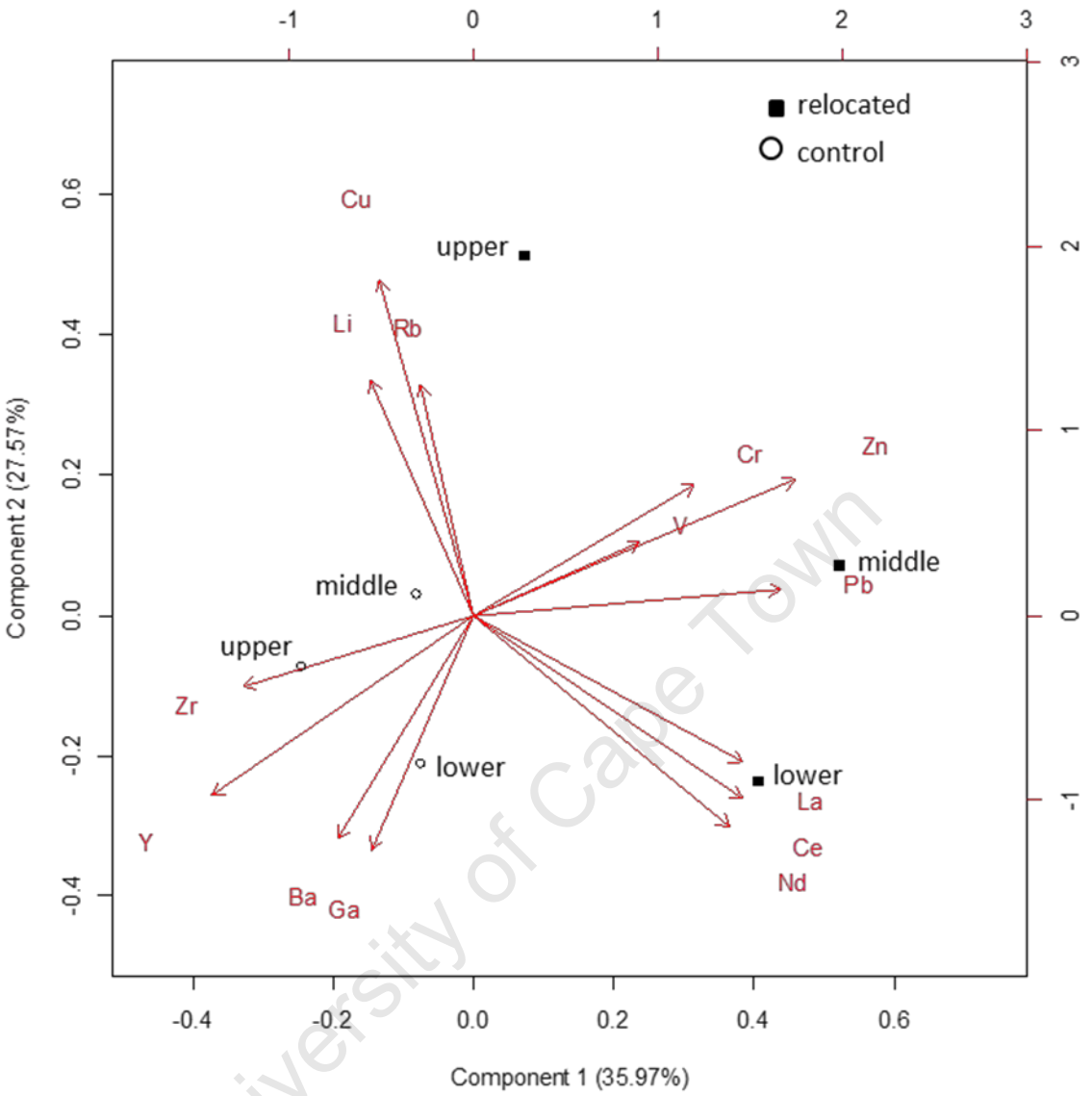

Figure 25. The principal component analysis for the upper, middle and lower leaf base samples collected for the relocated and the control *E. lebomboensis*. The red arrows indicate which trace elements explain the variance within the different samples for the first two components with the percent variance in brackets.

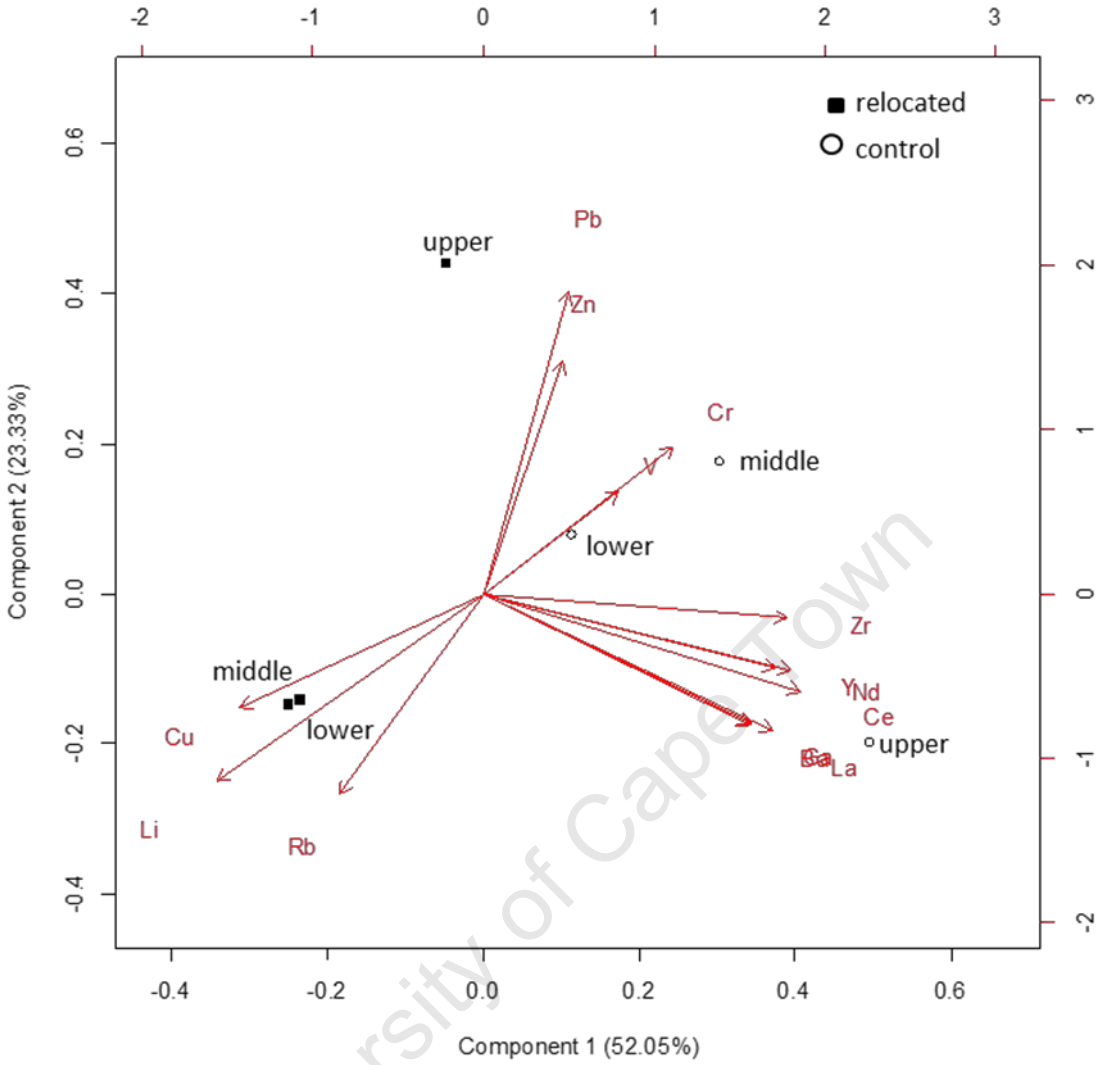

Figure 26. The principal component analysis for the upper, middle and lower leaf base samples collected for the relocated and control *E. arenarius*. The red arrows indicate which trace elements explain the variance within the different samples for the first two components with the percent variance in brackets.

4. Discussion

4.1 Establishing a chronology for forensic purposes

Our results indicate that using stable isotopes as a forensic technique to trace the origins of poached cycads has great potential. The effectiveness of this method relies on how fast cycad tissue turns over and the period since the relocation occurred that is detectable. Based on the *E. lebomboensis* radiocarbon results, the poached cycad should be relocated between 6 and 30 years ago, however, this estimate is from one specimen and more replicates are necessary on different species to understand the variability in tissue age. Due to the difficulties associated with interpreting and being able to predict cycad age, the use of radiocarbon dating is essential for establishing a chronology of time since the relocation occurred for a forensic technique to be valid.

The *E. lebomboensis* specimen sampled at Kirstenbosch National Botanical Garden was in excess of 80 years old, and we sampled across the range of vascular tissues at the base. Thus, it seems probable that the oldest tissue in the stem was sampled. Yet, the oldest radiocarbon date obtained was 30 years old. This could be explained by the mixing of new tissue with old tissue dropping the mean age of the cycad stem, or that the stem sampled was a new stem that replaced the original relocated stem from the wild. We think the latter explanation is the most parsimonious. This is supported by the stable isotopic results, which strongly suggest that the relocated *E. lebomboensis* plant was grown in a different locality to the control specimen. If cellulose from the wild locality were still present and the isotopic signature was contaminated with new plant material, then a greater amount of variance in isotopic values and some overlap with the control specimen's isotopic signatures, as seen with the relocated *E. arenarius* specimen, would be expected (Figure 13). *E. lebomboensis* seeds planted 20 years ago in the Lowveld National Botanical Garden as part of their genetic seed bank project are now equivalent in size to the specimen sampled at Kirstenbosch National Botanical Garden (W. Froneman pers. comm., 2012). Thus, it seems plausible that the cycad stem sampled at Kirstenbosch garden grew to the size observed within 30 years.

Our results suggest that relocations from less than 30 years ago will be feasible to determine that *E. lebomboensis* specimens originated from a different environment, due to their potentially fast growth rates. The same time frame for detecting a shift in location may be true for *E. arenarius*, which was relocated 20 years ago. However, this can only be confirmed

once we receive the radiocarbon dates for this specimen. Previous radiocarbon ages have been dated to 150 years old for *E. transvenosus* (Vogel et al., 2002) and 210 years old for *E. eugene-maraisii* (Vogel and van der Merwe, 1995), therefore the detectable period since the poaching event may be greater than 30 years ago in slower growing species. Previously, it was expected that the use of isotope forensics would only be effective in cycads grown within the relocated area for a long period of time, due to their slow growth rates. The upper leaf bases for *E. lebomboensis* were grown 6 to 10 years ago, therefore sampling of upper leaf bases from specimens that have been growing in a new location for only 6 years should be feasible (Figure 11).

The potential contamination of leaf bases and vascular rings with new material, did not affect our results, because enough old tissue remained to detect a difference in isotopic signal indicating that the relocated *E. arenarius* specimen was removed from the wild. Therefore, the absolute age of the cycad tissue being sampled may not be very important for tracing the movement of a poached cycad.

The retention of old tissue from the wild environment in the cycad, which is dependent on how long ago the plant was relocated, the turn over time and growth rate for that species, will determine whether or not this forensic technique will work. Within this limitation the following potential forensic techniques could be informative in determining if a plant has been removed from the wild.

4.2 Feasibility of tree coring as a forensic technique

4.2.1 Practical limitations

The chronology of tree rings in hard wood species is easy to interpret with annually increasing rings. In cycads, this is not the case, thus radiocarbon dating is a prerequisite for determining the age of the vascular tissues. The interweaving of new vascular tissue with old may occur within the vascular rings, giving younger ages than expected (Figure 11).This may be problematic with cycads relocated long ago, due to the attenuation of the isotopic ratio from the wild environment by the new plant tissue grown in the new locality.

The minimum amount of time required for a poached cycad to have grown in a new location for this technique to be effective is uncertain, thus, the upper vascular tissue should be aged. New vascular rings are not associated with annual growth, number of crowns or cones produced, or the number of seasons (Chamberlain, 1911). Thus, we expect that vascular rings

would take longer than leaf bases to incorporate enough new tissue for a shift in isotopic signature to be detectable. Furthermore, the time required for new tissue to be included into the vascular rings may not be consistent and predictable over time or similar between individuals. Thus, further development for using tree coring as a forensic technique should include a detailed assessment on the histology of vascular rings.

The cellulose extraction required larger quantities of material than what we anticipated, thus, numerous cores were necessary for one sample, or a larger increment corer is required for future sampling. Future research should quantify the risks associated with this technique through long term monitoring of damage done to the stem. Furthermore, reducing the vascular rings to cellulose limits us to only sampling $\delta^{18}O$ and $\delta^{13}C$, because all the other elements are removed from the tissue.

4.2.2 Key findings

The use of $\delta^{18}O$ and $\delta^{13}C$ in combination could serve as a feasible forensic technique based on the separation of the *E. lebomboensis* specimens, which supported our findings from the radiocarbon dating (Figure 12). In addition, the greater variability within the relocated *E. arenarius* specimen, which overlapped in $\delta^{18}O$ and $\delta^{13}C$ values with the control specimen, was indicative of a plant that grew in two different environments (Figure 13). Especially the upper vascular rings from the relocated plant, which were greater in $\delta^{18}O$ values compared to the lower vascular rings and the control (Figure 13). Evaluating the variables driving the differences seen between the relocated and control specimens will help highlight the future usefulness of $\delta^{18}O$ and $\delta^{13}C$ as a forensic tool and which of these variables will be most informative to support results when repeating this technique.

4.2.3 Evaluating results based on changes in the environment

Although stable isotope ratios, such as $\delta^{18}O$ vary spatially and can be predicted using models such as OIPC (Bowen, 2013) and other spatial data sets (Schulze, 2000; Bowen and Wilkinson, 2002; West et al., 2011), local variability is often the dominant factor influencing stable isotope ratios within the plant, which cannot be determined from large scale modeling or general climatic data. This is because multiple variables that counter act each other could be responsible for the changes seen in isotopic signature (West et al., 2010). Thus, direct measurements of variables that influence stable isotope values need to be sampled in order to understand why we see changes between the different localities, which can then be used in

models (West et al., 2010; Kahmen et al., 2011). This is beyond the scope of this study, therefore general inferences to explain the patterns seen will be made.

E. lebomboensis

Generally inland sites have lower $\delta^{18}O$ due to increasing altitude and continental effects, causing lower $\delta^{18}O$ within the source water, which is known as the "rain-out effect" (Bowen and Wilkinson, 2002; Bowling et al., 2003). The control specimen was growing at a higher elevation (Table 1: 1 100 m; Google Earth, 2011) compared to the relocated specimen (Table 1: 150 m; Google Earth, 2011), potentially explaining why the relocated plant had higher $\delta^{18}O$ values, indicating that $\delta^{18}O$ may be sensitive to changes in $\delta^{18}O$ source water with increasing elevation.

Seasonal differences in source water $\delta^{18}O$ may be causing the changes seen between the control and relocated plants, given that there is a difference of ~2 ‰ between the two cycads (Figure 12). This difference is equivalent to the change in mean $\delta^{18}O$ precipitation between the two localities for both summer (Table 1: January) and winter (Table 1: June). This suggests that both specimens have a growth sprout during summer and seasonal changes in source water (winter versus summer rainfall) are causing the change in isotopic composition.

Areas with higher precipitation are generally associated with higher $\delta^{13}C$ in plant cellulose (more positive $\delta^{13}C$ value; Warren et al., 2001). This was not consistent with our results for *E. lebomboensis* where $\delta^{13}C$ values for the control specimen (Table 1: mean annual precipitation of 1053 mm; Schulze, 2000) were higher than the relocated specimen (Table 1: mean annual precipitation of 1400 mm; Harris et al., 2010). The control specimen could have been water stressed, because it grew on the edge of a cliff face in minimal soil, therefore limited moisture would be retained during dry periods explaining the higher $\delta^{13}C$ values. Thus, seasonality, local variables as well as elevation were potentially causing the difference in $\delta^{13}C$ and $\delta^{18}O$ values between the relocated and control specimens.

E. arenarius

Across South Africa there is large spatial variation in seasons, with gradients between winter, summer and year round precipitation (Mucina and Rutherford, 2006). $\delta^{18}O$ was sensitive to seasonal changes in climate, therefore has great potential for tracing cycads relocated between areas with different seasons. Precipitation seasonality changing from all round precipitation in the Eastern Cape to summer precipitation in Nelspruit, possibly caused the

higher $\delta^{18}O$ values seen in the upper vascular rings for the relocated plant (Figure 13; Mucina and Rutherford, 2006). This is because Nelspruit experiences a dry season, resulting in higher $\delta^{18}O$ values (Table 1: -1.4 ‰; Bowen, 2013) during winter, potentially causing the $\delta^{18}O$ values in the cellulose to be higher. The humidity during the dry season was lower for Nelspruit (Table 1: 58 %; Schulze, 2000) compared to the Eastern Cape (Table 1: 67 %; Schulze, 2000), which would increase the evaporative enrichment within the leaf, thereby causing higher $\delta^{18}O$ values in the plant cellulose compared to what would be expected from the source water (Figure 1; Roden et al., 2005).

Changes in $\delta^{13}C$ values are difficult to interpret due to the number of variables involved in photosynthesis that counter act each other and changes within the local environment, such as shade, nutrient and water avaiblaility (Warren et al., 2001). Therefore, $\delta^{13}C$ values were not as sensitive to changes in seasonality as seen with the $\delta^{18}O$ isotopes. The $\delta^{13}C$ values for the upper vascular rings in the relocated specimen were similar to the lower vascular rings, possibly because the mean annual precipitation was similar between Nelspruit and the Eastern Cape (Table 1), however, additional information would be necessary for further explaination.

4.2.4 Summary

$\delta^{18}O$ and $\delta^{13}C$ in combination with general environmental data can inform us of which variables may be affecting the isotopic composition seen as discussed above for the *E. lebomboesis* and *E. arenarius* specimens. How is this information useful for determing if a poached specimen was relocated? Sampling of poached specimens would be expected to show higher variation in $\delta^{18}O$ as seen in the *E. arenarius* specimen. Inferences can be made based on shifts seen in $\delta^{18}O$ using general environmental data from the region that the specimen was relocated to. If the shift in isotopic signature cannot be explained through general climatic data for that region, this would indicate that the plant grew in a different locality before it was placed in the garden.

There are weaknesses with this method, due to the multiple physiological and environmental variables that could be influencing the $\delta^{18}O$ and $\delta^{13}C$ within cellulose. Sampling of $\delta^{18}O$ in the stem water and/or precipitation will provide more information on whether the isotopes seen are dominantly influencd by source water, and sampling of VPD within the leaves would provide information on transpiration rates and water stress within the plant. This additional information can then be used to make better inferences using models (West et al.,

2006; West et al., 2010; Kahmen et al., 2011) to determine which variables are causing the change seen in the cellulose isotopic values. These models can then refute which isotopic values in the plant tissue could not have grown within the area the poached plant was moved to.

4.2 Sampling petioles to trace cycad movement

4.2.1 Limitations with sampling petioles

Petioles do not record a chronology of plant tissue to compare recently incorporated tissue from a new locality to old plant tissue grown within the wild environment. Directly comparing the petioles (new tissue) to the leaf bases (older tissue) as a reference for the isotopic signatures in the wild environment is not feasible for certain isotopes, because petioles and leaf bases are composed of different plant tissues that have different degrees of isotopic fractionation. The control specimen may be different from the relocated specimen for a number environmental or biological variables (see Evans, 2001; Warren et al., 2001; Dawson et al., 2002) that influence certain isotopes, such as $\delta^{13}C$ and $\delta^{15}N$, other than the relocation of the poached plant. Our inability to analyse $\delta^{34}S$ in petioles from the *E. arenarius* control specimen, suggests that low sulphur concentrations may limit the usefulness of this isotope.

4.2.1 Evaluating usefulness and future development

The advantage of using petiole samples as a forensic technique is that you do not have contamination of old tissue with new tissue, because the petioles represent recently grown tissue. Thus, sampling petioles allows us to determine the origin of more recently poached cycads compared to leaf bases or vascular rings, which take longer to assimilate new tissue. Cycads generally flush a new set of leaves every 1 to 3 years, depending on the species (Norstog and Nicholls, 1997), therefore petioles could trace specimens that have been relocated within the past 1 to 3 years instead of 6 to 10 years when sampling the leaf bases (Figure 11).

Comparing petioles between a relocated and control cycad, or between the petiole and leaf bases within a relocated cycad may be valid for $^{87}Sr/^{86}Sr$ ratios, because strontium is relatively invariable within a location and generally not affected by different fractionation rates within different tissues as other isotopes are (Rummel, et al., 2010). This was reflected in our results were the $^{87}Sr/^{86}Sr$ ratios within the petioles of the *E. lebomboensis* specimens

were the same as the $^{87}Sr/^{86}Sr$ ratios within the leaf bases (Figure 14 and 19). The $^{87}Sr/^{86}Sr$ ratios within the petioles were the same as the upper leaf bases for the relocated *E. arenarius* specimen. Furthermore, the petioles from the control *E. arenarius* specimen were the same as the middle and lower leaf bases from the relocated specimen, suggesting that $^{87}Sr/^{86}Sr$ ratios remain unaltered within different plant tissues (Figure 14 and 20).

Replication of this method on other cycad species using $^{87}Sr/^{86}Sr$ ratios would be required for further development this method. Future research could determine the feasibility of comparing $^{87}Sr/^{86}Sr$ ratios within the petioles to ratios found within leaf bases. Analysing the surounding vegetation and soil within the new environment could serve as a control to determine if the petioles grew in the new location.

4.3 Feasibility of leaf base sampling to trace cycads

4.3.1 Practical limitations

The contamination of new tissue surrounding the older leaf bases as the stem grows in girth or is damaged, is thought to give radiocarbon ages that are younger than expected, as found by Vogel and van der Merwe (1995) and Raimondo and Donaldson (2003). Although this may be the case, there was still enough old tissue within the leaf bases to detect an isotopic signal from the wild. What we do not know is how much contamination can occur for this method to still be feasible and if the amount of contamination within the leaf bases is more prominent after a certain age?

4.3.2 The bulk isotopes

Poached cycads usually end up in private gardens and are likely to be exposed to more fertile soils, fertilization and irrigation within the garden compared to the wild. Based on our results from *E. arenarius*, the use of $^{87}Sr/^{86}Sr$ ratios, $\delta^{15}N$ and $\delta^{13}C$ isotopes in combination may be a powerful tool to show that a cycad originated from a less fertile soil or water stressed environment than what would be expected in private gardens. Soil fertility from the underlying geology may influence the $^{87}Sr/^{86}Sr$ ratios between different habitats, with higher ratios expected in fertile soils (Rummel et al., 2010). The higher $^{87}Sr/^{86}Sr$ ratios in the upper leaf bases for the relocated *E. arenarius* specimen are most likely due to a change in soil type, possibly with a corresponding change in soil fertility, which is consistent with the change in $\delta^{15}N$ values seen in Figure 16.

Fertilization or richer soils is a plausible explanation for the change in $^{87}Sr/^{86}Sr$ ratios within the upper leaf bases, because the *E. arenarius* plant was moved to a botanical garden. Lower $\delta^{13}C$ for the upper leaf bases in the relocated plant may indicate that the plant was not water stressed, possibly due to irrigation within the garden. Nelspruit receives similar mean annual precipitation to the Eastern Cape (Table 1), thus irrigation, especially during the dry season, would support the lower $\delta^{13}C$ seen in Figure 18 for the upper leaf bases in the relocated plant.

The most robust isotope ratio with the greatest potential to be used independently for tracing cycad movement was $^{87}Sr/^{86}Sr$, which reflected differences between locations for both species clearly (Figures 19 and 20). The proximity of the cycad to the ocean appeared to affect the $^{87}Sr/^{86}Sr$ ratios for the relocated *E. lebomboensis* and control *E. arenarius* specimens, which both had ratios similar to that of the ocean. Sea spray or mist coming off the ocean may cause $^{87}Sr/^{86}Sr$ ratios to be lower than expected for a particular soil type (Rummel et al., 2010). In future, it would be interesting to test if such a large difference in the $^{87}Sr/^{86}Sr$ ratios is observed within a cycad that was moved between two inland or two coastal locations.

The higher $\delta^{34}S$ values in the relocated *E. lebomboensis* specimen could potentially be explained by increased sulfate inputs from aerosol pollution within Cape Town (Figure 17). Although $\delta^{34}S$ may be useful to trace cycad movement in relation to pollution source, this was not consistent with the *E. arenarius* specimen, which had much lower $\delta^{34}S$ values for the upper leaf bases grown in Nelspruit (with numerous polluting industries within the area) than in the Eastern Cape (wild). Whether pollution is in fact influencing $\delta^{34}S$ values could be tested by measuring the $\delta^{34}S$ in precipitation, which can then be compared to $\delta^{34}S$ values in the leaf bases. Another possibility is that the different soils may have different $\delta^{34}S$ values, therefore testing of the $\delta^{34}S$ in the soil would determine if $\delta^{34}S$ values in the cycad is reflecting that of the soil or atmospheric pollution.

The sampling of leaf bases was informative in tracing the relocation of the *E. arenarius* specimen and separating the *E. lebomboensis* specimens from different localities, which was consistent with our radiocarbon, and $\delta^{18}O$ and $\delta^{13}C$ analyses. The most explanatory isotopes were $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$ and $^{87}Sr/^{86}Sr$ ratios, especially when combined together using PCA plots. Based on our results, the lead isotopic ratios were less appropriate for tracing the origins of cycads, because they were not sensitive enough to changes in geology (Figures 21 and 22).

4.3.3 Trace element concentrations

Our results show that trace element concentrations could potentially distinguish between individual plants and determine the dominant trace element concentrations within the cycad (Figure 25 and 26). Trace element concentrations may be a reflection of the physiological needs of the plant and not necessarily the availability of trace elements within the soil (Kabata-Pendias, 2004). This may explain the large differences in dominant trace elements seen between leaf bases within the same specimen.

Another explanation is that the trace element concentrations sampled were not representative of the variation found within the plant due to the lack of replication. Only one replicate for each sample was analysed for the trace element concentrations, because we had to be conservative when removing material from the cycads, due to their threatened status, and the ashing of the samples for the strontium, lead and trace element concentrations required larger amounts of material than anticipated. Understanding how trace element concentrations vary between different localities, and increasing sample sizes to see how variable they are within the plant, would provide more information on their viability as a tracer. We only analysed a few selected trace elements based on their higher concentrations, therefore it is plausible that these trace elements were uninformative and other trace elements may be more useful. Additional information on how trace element concentrations are affected when absorbed by the plant (i.e. selectivity of elemental uptake) in comparison to which elements are available in the soil, may justify the use of trace element concentrations as a tracer in future.

4.4. Using multiple tracers

Using multiple isotopes in PCA plots reduces the natural variability in isotopic composition and highlights potential patterns or groupings in the data. This was shown with the two *E. lebomboensis* specimens, which were separated from each other in the PCA plot (Figure 23) based on $\delta^{15}N$, $\delta^{34}S$, $\delta^{13}C$ and $^{87}Sr/^{86}Sr$, even though the $\delta^{15}N$ and $\delta^{13}C$ values were similar between specimens when analysed individually (Figure 15). The clear grouping of the upper leaf bases from the relocated *E. arenarius* specimen and the grouping of the middle and lower leaf bases from the relocated specimen with the control specimen provides a beautiful example of how multiple isotope tracers can be used to trace a relocated cycad (Figure 24).

When using multivariate analyses more replicates can improve our confidence in the patterns seen, because the probability of sampling all of the variance within the plant is greater when

the sample size is increased. However, the effectiveness of multivariate analyses is dependent on how informative the tracers are, because not all isotopes will be appropriate tracers. Thus, for multivariate analyses to be robust, we need to be selective in which isotope tracers are used. Based on our findings $\delta^{15}N$, $\delta^{34}S$, $\delta^{13}C$ and $^{87}Sr/^{86}Sr$ ratios showed great potential for mulitvariate analyses in cycads. However, further testing is recommended with other species of known relocation history to determine if these isotopes are still effective tracers.

5. Conclusion

Our findings suggest that the use of radiocarbon dating is essential to develop a chronology of tissue age. Based on the radiocarbon ages from the *E. lebomboensis* leaf bases, a relocated cycad needs to be growing within the new locality for 6 years to incorporate tissue from the new environment, and a maximum of 30 years to retain tissue from the previous environment in order for leaf base sampling to be effective. Sampling of $\delta^{18}O$ in the vascular rings was useful for tracing the relocated *E. arenarius* specimen and showing the separation of the two *E. lebomboensis* specimens. $\delta^{18}O$ can be modelled to determine whether the isotopic composition in the new locality is consistent with the surrounding environment.

Sampling of leaf bases and multivariate analyses using $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$ and $^{87}Sr/^{86}Sr$ ratios were effective for tracing the movement of cycads, whereas $^{206}Pb/^{207}Pb$, $^{208}Pb/^{207}Pb$ and trace element concentrations were less effective for tracing cycad movement. The $^{87}Sr/^{86}Sr$ ratios showed great potential within the petiole samples for development of a method to sample the most recent plant material and compare it to a specimen in the wild (control). Future development of $^{87}Sr/^{86}Sr$ ratios could include comparing leaf bases that contain material from the old environment to the petioles or soil within the new environment. Based on our study, the use of radiocarbon dating and stable isotope ratios has huge potential to be used as a forensic technique.

Therefore, future research should focus on developing the leaf base and petiole sampling methods further, and explore the possibility of analysing $\delta^{18}O$ within the leaf bases. This is because the use of tree coring is more prone to contamination with new tissue compared to the leaf bases. Recommendations and ideas for future development of this method are discussed further in Chapter 3. This study has provided an overview for how radiocarbon dating, stable isotopes and trace element concentrations could be useful as a forensic tracer of poached cycads, however, further investigation and replication is required before these methods will be applicable.

Chapter 3:

Synthesis and recommendations for future research

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1. Recommendations and future research

This study determined the potential for using stable isotopes, trace element concentrations and radiocarbon dating to trace poached cycads back to the wild. The results from this study have provided a good basis for future work in cycad forensics. From the two sampling methods explored, the leaf base and petiole sampling methods were the most effective. This provides a simple tool for authorities to use in future when sampling a suspects garden and does not require expensive equipment or damage to the cycad.

Tracing of the relocated *E. arenarius* specimen was successful; therefore, this scenario needs to be repeated with other species so that cycads with an unknown origin can be traced. The way forward from this study would be selecting and adapting the methods discussed in Chapter 2 to develop a robust forensic method with the least limitations. Further investigation into using spatial forensics to trace poached cycads back to their original populations or regions would be the next challenge and may require data from each population or region, depending on findings from other cycad species.

1.1 Refining future sampling

Focussing future development of this forensic method on leaf base and petiole sampling would provide fewer limitations and more options for isotope ratio sampling. The sampling of the petioles will be key in determining if a cycad was recently removed from the wild. Petioles are likely to have newly grown within a suspect's garden. Therefore, the development of a future sampling method should focus on determining how the petioles differ in isotopic composition compared to the leaf bases.

The $^{87}Sr/^{86}Sr$ ratios showed great potential, because little or no fractionation within different plant tissue occurs, therefore it is a true reflection of the soil type that the cycad grew in and could be used alone as a forensic method without sampling other isotopes. However, this requires further testing on other species with known relocation histories. If $^{87}Sr/^{86}Sr$ ratios do not work, because there is little geological difference between the old and the new environments then further testing can be done using $\delta^{34}S$, $\delta^{15}N$ and $\delta^{13}C$, and potentially $\delta^{18}O$. Comparing different tissues (i.e. comparing isotopes in the petioles to the leaf bases) that fractionate isotope ratios differently may be problematic when analyzing $\delta^{34}S$, $\delta^{15}N$, $\delta^{13}C$, and $\delta^{18}O$, therefore these isotopes may only be useful when comparing between leaf bases.

The use of $\delta^{18}O$ is informative for tracing cycads across the landscape based on precipitation gradients and comparing results to established $\delta^{18}O$ precipitation maps and models (Bowen, 2013; Bowen et al., 2005). Investigation into the feasibility of analysing $\delta^{18}O$ in whole and cellulose extracted leaf bases and petioles could provide the same information gained from sampling the vascular rings. The effectiveness of analysing $\delta^{18}O$ within the leaf bases and petioles, will determine the necessity for sampling vascular rings as an additional method. Further research into the histology of the vasculature in cycads would provide a better understanding of contamination with new tissue, giving more confidence in results retrieved. Thus, sampling of vascular rings has potential as a forensic method, however, if $\delta^{18}O$ can be sampled within the leaf bases and petioles, it would provide a more effective and efficient forensic method.

Further research is required to know how long a cycad needs to be growing within the new environment. Sampling of more cycads with different periods since they have been relocated is needed to get a better estimate of the time since the relocation event occurred that will be valid for using isotope forensics.

1.2 Control specimens and spatial maps

The control specimen was necessary to validate that the shift seen in isotopic signature within the relocated plant was not simply due to environmental variation at a given location. When repeating this study on other species a control specimen from the wild would be recommended for comparison to the relocated cycads. Once this method has been further tested a control specimen may not be necessary to determine if there was a shift in isotopic signature. Instead, creating a spatial map of isotopic signatures for cycads in the wild could be used as a reference and serve the equivalent function of a control specimen by determining the potential areas that a poached cycad in question originated from. When sampling control specimens from the wild the information gained could contribute to a spatial data base for different regions where cycad populations occur.

The sampling of plant material from the surounding vegetation within the new locality that the cycad was moved to could provide a second control. This would give an isotopic ratio for the new locality that can be compared to the isotopic ratio within the cycad in question. The isotopic ratios of plants within the garden should match the petioles or leaf bases grown in the new locality and be different from the leaf bases grown in the wild.

Our study showed considerable isotopic variation between localities, indicating that our method may be successful in spatial forensic applications (West et al., 2010). The most promising isotope ratio based on our study was $^{87}Sr/^{86}Sr$, as this is a signal of the underlying geology and would appead to be the starting point for any forensic investigation. However, in cases where cycads were relocated to a common geology, $^{87}Sr/^{86}Sr$ would not work and an alternative isotopic tracer would be more appropriate. Our study indicated variation in $\delta^{18}O$, $\delta^{34}S$, $\delta^{15}N$ and $\delta^{13}C$ between locations and variation in these isotopes might be most useful when combined using multivariate analyses. Future directions of cycad forensics would include an Isoscapes approach of spatially characterizing cycad ranges and utilizing process models to predict and test isotope ratios expected within the new and wild localities.

In practice, individual forensic applications may require a specific set of isotope tracers, depending on the variation between wild and relocated localities. The experimental design of sampling may be specific to the individual cycads sampled as well. The method of sampling can be adapted to the specific scenario, providing the method is based on the theory of comparing new plant material grown within the new locality to old plant material grown within the wild locality. For example, sampling of petioles from the new locality and comparing them to old petioles from the wild environment, rather than comparing them to old leaf bases. In some cases, the cycad may not have a large enough stem to sample leaf bases from the upper, middle and lower sections of the stem and only sampling of the petioles from the new locality and upper leaf bases from the old locality can be done.

2. Limitations

One limitation with this study was working with endangered plants, thus, the amount of tissue sampled had to be restrictive. For this reason, only the minimum number of replicates necessary was collected to get an indication of which plant tissue and stable isotopes would be effective in tracing relocated cycads to their origin. Another limitation was the reduction of material during the ashing of leaf base samples for lead, strontium and trace element concentration as well as extracting cellulose for $\delta^{13}C$ and $\delta^{18}O$ analyses, which required more material than anticipated. We know now which tissues and isotopes reflect the movement of the cycad best, thus, selective sampling in future will allow more material to be available.

Increased sample sizes will make statistics more reliable, for example with the principal component analysis, because more variability within the cycad would be included. However,

this depends on which isotopes are analysed as some isotopes, such as $\delta^{13}C$, $\delta^{34}S$, and $\delta^{15}N$ are more variable within the cycad and would require larger sample sizes than others, such as $^{87}Sr/^{86}Sr$ ratios.

There is a large cost involved with analysing just one cycad, this limits the number of cycads that can be processed. Once the sampling method is refined and costs are reduced, more cycads can be sampled. Reduced cost would make this method more available for authorities to implement.

3. Impact on the illegal cycad trade

This sampling method could potentially transform court cases by providing external evidence that the cycad originated from the wild when trying to prosecute poachers and collectors. One advantage is that poachers generally cut the leaves of the cycad before transporting it to a new locality. Thus, petioles within a suspect's garden are likely to have newly grown within the new locality. Recently poached cycads from the wild that have not incorporated an isotope ratio from the new environment can be left to grow new petioles, which can then be sampled. The effective use of this method in court will make cycad collectors and poachers aware that they can be caught a number of years after the illegal cycad was purchased, prohibiting or at least reducing illegal trade.

This study has confirmed that the use of radiocarbon dating and isotope ratios are valid. Future sampling using the petiole and leaf base methods on another two specimens with well-documented relocation histories within the past 30 years is necessary to show that this method is repeatable. Sampling on other highly collected species will provide additional support for this technique. The next step would be using this method on specimens with unknown relocation histories to show that they are from the wild, and could provide evidence in court cases at the same time. We hope that the successful use of this forensic method in future will deter collectors from buying illegal cycads.

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