Nested Clade Analysis of geographic structure in the morphologically variable *Themeda triandra* in South Africa

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Abstract

The use of phylogeography in plant systems has been on the increase in recent years with the use of chloroplast DNA to detect sufficient intraspecific variation to reach significant conclusions about plant species histories, both temporally and spatially. In this study, the geographic structure and possible origin of the morphologically variable *Themeda triandra* is explored. The *trn*F – *trn*C and *psb*D – *trn*S gene regions of the cpDNA were used to find 12 haplotypes found in 11 populations of *T. triandra* that encompass the species large distributional range. A haplotype tree was constructed that showed the relationship of the 11 haplotypes (haplotype H12 was excluded as it fell outside of the 95% confidence limit), with haplotype H6 inferred to be the ancestral haplotype. A nested clade analysis was performed with the results used to infer the geographic structure of *T. triandra* within South Africa. Significant results showed that there was restricted gene flow with nested clades involving the three Free State populations, indicating that there are barriers to gene flow with other haplotypes. The ancestral haplotype showed long distance colonisation, with a probable root of this colonisation being the Kruger National Park. This is the proposed point of introduction of *T. triandra* into South Africa, with results from this study supporting this proposal. A substantial amount of gene flow (25.49%; AMOVA) between populations is observed, with this probably being due to the widespread distribution of haplotypes H6 and H10. It is thought that *T. triandra* followed two migration routes within South Africa: one along the coast, with the other inland above the escarpment where populations became genetically isolated from populations below the escarpment. Further studies may look for a correlation between morphological variants of *T. triandra* and the cpDNA haplotypes found within the species.

Introduction

Plant phylogeography studies have been on the increase in recent years. Early plant phylogeographic studies struggled to find sufficient intraspecific variation using either chloroplast or mitochondrial DNA (Schaal *et al.* 1998; Olsen & Schaal 1999). Recent work has been discovering intraspecific variation in many more species (Schaal *et al.* 1998). Many successful plant phylogeography studies have been conducted on a wide range of plants, including ferns (Trewick *et al.* 2002), ivy (Grivet & Petit 2002), various Alpine plant species (Stehlik *et al.* 2002; Schönswetter *et al.* 2002), oaks (Lumaret *et al.* 2002; Cannon & Manos 2003) as well as a few grass phylogeography studies (Sahuquillo & Lumaret 1999; Olsen & Schaal 1999; Tyler 2002). Most of these studies have been done in the Northern Hemisphere often in an attempt to identify species refugia during periods of glaciation (see Grivet & Petit 2002; Schönswetter *et al.* 2002; Stelik *et al.* 2002; Trewick *et al.* 2002).

Various methods have been employed in grass phylogeography studies to try obtaining genetically informative DNA information. Tyler (2002) looked at allozyme variation in *Millium effusum*, but was unable to match variation found within the species with its geographic structure. The origin of the staple crop cussava (*Manihot esculenta*) was found to be the southern boarder of the Amazon basin (Olsen & Schaal 1999). This was determined through the use of single-copy nuclear DNA. Variation found using this genetic marker was found to be higher than either chloroplast or plant mitochondrial DNA at the intraspecific level (Olsen & Schaal 1999). The more traditional source of intraspecific variation in plants has been chloroplast DNA.

Themeda triandra is a widespread grass species, found predominantly in southern Asia, Africa and Australia (Clayton & Renvoize 1986; Jones 1999). *T. triandra* also has a wide distribution range in South Africa (Fig 1) although it is rarely found in the arid Northern Cape Province. Within South Africa, *T. triandra* is found predominantly in open grassland and bushveld regions of above average to very high summer rainfall (van Oudtshoorn 1992); hence it's more common distribution in the eastern half of South

Africa (Fig 1). *T. triandra* is a highly morphologically variable species, exhibiting great variation in both size and architecture (van Oudtshoorn 1992). There are many characteristics common to all varieties found within the species that allow for easy identification of *T. traindra*. These include pendent inflorescences, darkly coloured nodes and the compressed leaf sheaths (van Oudtshoorn 1992). At the end of the growing season, *T. triandra* individuals turn from blue-green to red in colour. This gives *T. triandra* its common name of "rooigras".

Taxonomically, *Themeda triandra* is a member of Andropogoneae, subfamily Panicoideae (Clayton & Renvoize 1986; Jones 1999). The origin of the Panicoideae is thought to be tropical (Clayton & Renvoize 1986), with southern Asia the most likely point of origin. This region is host to a large number of grass species, including very primitive ones, often an indication of a taxons point of origin (Hartly 1958). Members of the Andropogoneae, including *T. triandra*, migrated south into Africa, as well as Australia, during the late Tertiary (Jones 1999). The current distribution patterns of T. triandra suggest a migration of the species into Africa from southern Asia via tropical corridors (Liebenberg 1993). This corridor, most likely found along the east coast of Africa (Liebenberg 1993), may have facilitated the movement of *T. traindra* into South Africa, probably entering the country through the north eastern border, somewhere near the Kruger National Park. A proposed date of entry into South Africa is not known.

Theunissen (1992) performed transplant studies on *T. triandra* from various regions and grew the plants under uniform conditions, but the high amount of morphological variation within the species remained. It was conclude that there was a genetic basis behind the morphological variability seen in *T. triandra*.

Chloroplast DNA is a widely used region used in plant phylogeography studies (Schaal *et al* 1998). As with animal mitochondrial DNA, chloroplast DNA is nonrecombining and maternally inherited (Schaal *et al.* 1998; Grivet & Petit 2002) providing sufficient intraspecific variation (Schaal *et al.* 1998) and good geographical structure (Grivet & Petit 2002). In this study, haplotypes obtained from two regions within the chloroplast

genome along with the use of a Nested Clade Analysis if any geographical association exists between the haplotypes. If such an association does indeed exist, inferences about how the current geographic structure arose, be it through restricted gene flow, long distance dispersal or past fragmentation (Templeton *et al.* 1995), will be made. Due to its short dispersal ability (Clayton & Renvoize 1986), it is expected that many populations of *T. triandra* will possess haplotypes not shared with other populations, an indication of restricted gene flow (Maskas & Cruzan 2000). Any ancestral haplotype found is expected to exhibit a wide geographical range than any of the derived haplotypes.

Methods

Sampling

Populations of *Themeda triandra* were sampled across its geographical and morphological range in South Africa (Figure 3). Two populations were sampled from Hluhluwe/Umfolozi Game Reserve and Fort Hare University, Alice. Of the Alice populations, one population occurred in a controlled plot that is never burned by fire. The other plot is burned every two years. Other collection sites are listed in Table 1. Sampling occurred between April 2nd and April 12th 2004 for population 1 through 9 and the Kruger population. The Jonkershoek population was sampled on 23 May 2004. These sampling dates corresponded with flowering times of *T. triandra* and, especially for populations found in the Free State, occurred before the onset of frost (H. Snymann, pers. comm.).

One voucher specimen was collected from each population sampled. These specimens have been deposited in the Bolus Herbarium at the University of Cape Town. Plants were chosen at random from each population and three to four leaves were removed from the chosen plants. These leaves were stored in silica gel. In large populations ($n > 250$) plants greater than 20m away from each other were sampled. This was done to increase the chance of sampling genetic diversity. 20 plants from eleven populations were sampled in total, except for the Kruger population where only nine individuals were sampled. The number of sampled plants used in the DNA analysis varied from 4 – 7 individuals per population (see Table 1).

DNA Extraction, PCR amplification and Sequencing

Five centimeter lengths of leaf were ground in a pestle and mortar using bleached sand, PVP and 700µl of 2x CTAB and 1µl of β-mercaptoethanol. The samples were added to 1.5ml micro-centrifuge tubes and incubated at 65°C for 1 h. 600µl chloroform/isomyl alcohol 24:1 was added to the incubated samples which were inverted for 5 minutes to

mix thoroughly and subsequently centrifuged at 12 000 r.p.m. for 5 minutes. The supernatants were pipetted into fresh 1.5ml micro-centrifuge tubes and equal volumes of ice-cold isopropanol added to the samples which were stored in a freezer for a minimum of 1 hr. Further centrifuge spinning at 12 000 r.p.m for 5 minutes formed a DNA pellet. The isopropanol was discarded and the pellet washed with 75% ethanol and spun at 12 000 r.p.m for 3 minutes. The 75% ethanol was discarded and the pellet dried by placing the 1.5ml micro-centrifuge tube in silica gel. The DNA pellet was re-suspended in 50µl TE at ph 8.0.

Two noncoding regions of chloroplast DNA were chosen for the DNA analysis. These were *trn*F-*trn*C and *Psb*D-*trn*S (See Table 2 for a description of these primers). These regions were amplified using the Polymerase Chain Reaction (PCR). These reactions were carried out in 30µl volumes, comprising 1 X NH_4 buffer, 50mM $MgCl_2$, 0.1mM dNTPs, 0.3µM each of the forward and reverse primers, 0.75 units Super-Therm Taq, 17.65µl autoclaved PCR water and 3.0µl diluted DNA template. The thermal profile comprised an initial denaturation step at 94°C for two minutes, followed by 30 cycles of 94°C for one minute, 52°C for one minute and 72°C for two minutes, with a final polymerisation step of 72°C for seven minutes. Amplified products were then cleaned with the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences) protocol. To sequence the amplified DNA, 4µl of the amplified DNA was added to 2µl TRR (Big Dye® Terminator V3.1 cycle sequencing Ready Reaction Kit), 1µl 5x NH_4 buffer, 0.3µM primer and 2.84µl PCR water to create a reaction volume of 10µl.

Sequencing products were resolved on an ABI PRISM® 3100 Genetic Analyzer. Forward and reverse sequences were assembled and checked using SeqManII (LaserGene systems software, DNAstar, inc.). Checked sequences were manually aligned using MegAlign (LaserGene systems software, DNAstar, inc.).

Data analysis

Sequences of the two DNA regions were combined to define haplotypes for each individual sampled. Gaps within haplotypes of greater than 1 bp were treated as a single mutation and were coded as C if present and as a G if absent. These gap codes were inserted at the end of the DNA sequences. Contiguous gaps of variable length found in many haplotypes were excluded from the phylogenetic analysis and subsequent molecular analysis. Phylogentic relationships of these chloroplast DNA haplotypes were inferred using parsimony criteria. A neighbour joining tree was constructed enforcing the Kimura-2 parameters model in PAUP 4.0b10 (Swofford 2001), with bootstrap values based on 1000 replicates. Indels were treated as missing. A haplotype network was constructed in TCS ver. 1.13 (Clement *et al.* 2000) with a 95% confidence level. There was a single ambiguous connection within the haplotype network. This ambiguity was removed in order to perform a nested clade analysis. A decision was made to remove the connection between H2 and H1 and keep the connection between H1 and H10 (Figure 4). This decision was based on the higher frequency of individuals possessing H10 and its wider geographic distribution. The haplotype tree was then nested into clades.

The nested haplotype tree was incorporated into a Nested Clade Analysis, performed using Geodis ver. 2.2 (Posada 2001). A permutational contingency test is initially performed to test if there is any association between haplotypes and their geographic location. This test takes the form of a χ^2 test. If there is an association between haplotypes and their geographic location, haplotypes then undergo a geographic distance analysis. Clade distance, D_c, and nested clade distance, D_n, values are calculated. The interpretation of the evolutionary history of each nested clade was determined using the inference key of Templeton (1998).

The amount of chloroplast DNA variation within and among the 12 populations was determined using the analysis of molecular variance procedure (AMOVA) in Arlequin ver. 2.001 (Schneider *et al.* 2000) as well as Φ-statistics. Haplotypic correlation values

(F_{st}) were also correlated for all population pairs. The significance of these values was calculated using 110 random permutations with a significance level of 0.05.

Results

Phylogenetic analysis

The combined *trnC – trn F* and *psbD – trnS* regions formed an alignment of 1378 bp. The mid-point neighbour joining tree obtained is shown in Figure 4. The tree length was 32 steps and possessed a consistency index of 0.969 and a retention index of 0.984. Bootstrap support however was not very strong (Figure 4). The Kruger National Park 5 sample was highly divergent from all other samples as is indicated by its position on the tree.

The nested haplotype network obtained is shown in Figure 5, showing twelve distinct haplotypes. Haplotype H6 is identified as ancestral since 15 individuals possessed this haplotype. Increased sampling could change this result though, since haplotype H10 is found in 14 individuals. Both these haplotypes are found in six localities ranging from the Kruger National Park, along the 'coastal' populations through to the Jonkershoek population. The calculation of the haplotype network excluded haplotype H12 (KNP 5), as it did not fall within the 95% confidence level of 16 steps estimated by TCS ver. 1.13 (Clement *et al.* 2000). It was thus excluded from the subsequent nested clade and molecular analyses. The size of the haplotypes is proportional to the number of individuals possessing that haplotype. The permutational contingency test showed that all clades had an association between haplotypes and geographic location. D_c and D_n values were subsequently calculated (not shown). Using the inference key of Templeton (1998), either inferences of Past Fragmentation followed by Range Expansion or Restricted Gene Flow with Isolation by Distance explain the significance of clades with geographic localities (Table 3). Clades that showed no significance can be due to small sample size or inadequate sampling (Templeton, 1998).

The Pairwise Differences between all populations are shown in Table 4. Of the 55 comparisons, 36 were significant. The high amounts of significant Fst values (Table 4) show that there is little gene flow between populations in this study. The AMOVA results

(Table 5) show that 74.51% of the variation is due to variation within populations with the rest, 25.49%, is due to variation found among populations (Table 5). This fairly high among population variation is an indication of

Discussion

The use of a Nested Clade Analysis in phylogeography studies adds an extra dimension to those studies that rely on traditional population statistics (i.e. *F* statistics) which fail to make inferences regarding evolutionary histories (Templeton 1998). Nested Clade Analyses allow inferences to be made about the probable historical processes that may have caused current geographic structuring using haplotype information (Maskas & Cruzan 2000). One is also able to determine if the current geographic pattern of haplotypes is due to minimal gene flow between populations (Templeton 1995), often through the fact that a species is a poor disperser, with haplotypes restricted to single populations.

The nested haplotype network (Figure 5) indicates that there are six tip haplotypes. Of these haplotypes, five were found in only one population, with the six found in both Hluhluwe populations. With this low frequency of haplotypes one can assume that there is restricted gene flow between populations (Maskas & Cruzan 2000). Significant results from the Nested Clade Analysis (Table 3) and the F_{st} scores (Table 4) support this assumption. Many of the nested clades infer that there is restricted gene flow between populations of *T. triandra*. The amount of gene flow within species is determined by the distance that individuals are able to transfer their genes. In *T. triandra*, as with many grasses, seed dispersal distances are not very large (Clayton & Renvoize 1986). Environmental conditions also need to be correct for germination to take place. *T. triandra* is classified as a "Decreaser" grass, growing only in good grassland conditions, subsequent to the occupation of colonizer species (van Oudtshoorn 1992). The species is therefore unable to occupy unsuitable land that may occur between populations. The Free State populations in particular were located in regions with a large proportion of farmlands where highly disturbed land is not suitable for the movement of *T. triandra* populations and their genes.

Populations from the Free State (Bloemfontein and Colesburg including Hanover) all share very similar haplotypes (H1, H3 & H4; Figure 4). A single haplotype (H2) found in the Hanover population is shared with individuals found in both Hluhluwe populations. This sharing is probably due to long distance dispersal, possibly due to humans as the sampled Hanover population was found on the verge of a main highway. Further sampling in the intermediate areas between the two populations (i.e. the Drakensburg) may need to be conducted in order to see if this haplotype occurs between the two populations. This seems unlikely however, as this haplotype was not found in populations found closer to both Hluhluwe populations (i.e. Colesburg & Bloemfontein).

Migration of *T. triandra* populations is likely to have followed two routes: above or below the escarpment. Apart from the sharing of haplotype H2 between the Hluhluwe and Hanover populations, there is little gene flow between the Free State populations and other populations. These three haplotypes form a nested clade (2-2; Figure 5) and with the aid of the Templeton (1998) inference key, it was concluded that this clade experiences restricted gene flow. The calculated F_{st} values support the inference made using this key, showing, in particular the Colesburg and Bloemfontein populations, that the Free State populations are significantly different to the other *T. triandra* populations. The Colesburg population shows a single haplotype that occurs in no other population, an indication that there is no gene flow even with populations that are close to it. Similarly, the Bloemfontein population contains two haplotypes found nowhere else. The most likely explanation for this is that these haplotypes are very young and newly diverged. There is a possibility though that the reduction of genetic diversity is through the occurrence of a severe bottleneck (Maskas & Cruzan 2000). In order to accept this conclusion, information about possible historical events that would have cut of these populations need to be known. The effects of the Free State populations on the nested clade analysis are shown by many of the population inferences (Table 3), with many nested clades experiencing restricted gene flow. Increased sampling, especially towards the north of the country may provide a clearer picture of the geographic structure of *T. triandra*, as well as nullifying the effects of the Free State populations.

Of the genetic variation found in *T triandra*, the majority (74.51%) of it can be attributed to variation within populations, although a substantial amount (25.49%) is found among populations (Table 5). The occurrence of haplotypes belonging to single individuals within populations (i.e. haplotypes H7, H8, H9 & H10) as well as the occurrence of multiple haplotypes within populations could account for the high proportion of within population genetic variation. The Jeffrey's Bay population had five sequenced individuals, yet possessed four different haplotypes (Table 1). A similar situation was found in the Haga Haga population. The low frequency of these haplotypes is an indication that they are relatively young, probably diverging from the ancestral haplotype recently. An increase in the number of individuals sampled from these populations may provide a better understanding of the haplotype composition of these populations.

The inferred ancestral haplotype (H6; Figure 4) is found over a broad geographical area (Figure 3) from KNP in Mpumalanga to Jonkershoek in the Western Cape. Haplotype H10 follows a very similar geographical distribution (Figure 2), differing from H6 by a single mutational step (Figure 4). Due to its probable tropical origin (Hartley 1958; Clayton & Renvoize 1986; Liebenberg 1993 &Jones 1999), *Themeda triandra* is suspected to have migrated down Africa through the tropical east coast (Liebenberg 1993) in order to reach South Africa. The most likely point of entry into South Africa would therefore be towards the northeast, probably around the Kruger National Park (W. Bond pers. comm.). All KNP samples possessed either the H6 or H10 haplotype, except for the highly divergent haplotype H12. It is likely that the ancestral haplotype, H6, migrated from its probable introduction through the KNP along the eastern and southern coast of South Africa. This migration would have been facilitated by the tropical/sub-tropical conditions found along this coast. Clades 1-2 and 3-2 (Figure 5), to which H6 belongs, are inferred to have undergone long-distance colonization and past fragmentation respectively (Table 3). Haplotypes that survived periods of glaciation subsequently spread to widespread geographic localities due to range expansions (Trewich *et al.* 2002; Stehlik *et al.* 2002 & Honjo *et al.* 2004). Derived haplotypes, formed through mutations from the ancestral haplotype, are found in much narrower distribution ranges. A similar situation has occurred in *T. traindra*, with derived

haplotypes occurring in small ranges either along side or within the distributional range of the ancestral haplotype, H6. Tropical/subtropical conditions found along the coast of South Africa are most likely to have facilitated the wide distributional range of the ancestral haplotype. These conditions may have provided favourable opportunities for populations of *T. triandra* to establish, facilitating more gene flow between populations.

In general, the haplotype distribution map (Figure 2) shows the wide geographical distribution of the ancestral haplotype, with derived haplotypes forming in populations within its range. The Free State populations seem to have lost the ancestral haplotype and seem to be made up entirely of derived haplotypes.

Two populations from Alice in the Eastern Cape were sampled. These populations were under different controlled fire regimes. Alice2 was an unburned plot and Alice1 was burned every two years. All individuals, except for Alice1 2, had the same haplotype, H10. There is therefore no indication that fire, and plant responses to fire, may be due to the genetics of a plant.

Within this study, populations of *Themeda triandra* were not grouped to see if there was any significance between the haplotypes and any grouping variables. As is known, *T. triandra* is an extremely morphologically variable plant (Gibbs-Russel *et al* 1990, Theunissen 1992, van Oudtshoorn, 1992 & Liebenberg 1993). Morphological characteristics can be scored to allow the placement of individuals from each population into groups (i.e. tall vs. short). One would then be able to determine if the haplotype diversity within *T. triandra* is correlated with the high morphological variability within the species.

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References

Birmingham, E. & Moritz, C.1998. Comparative phylogeography: concepts and applications. *Molecular Ecology* 7: 367 - 369

Clayton, W.D. & Renvoize, S.A.1986. *Genera Graminum: Grasses of the World.* Royal Botanical Gardens, KEW

Grivet, D. & Petit, R.J.2002. Phylogeography of the common ivy (*Hedera* sp.) in Europe: genetic differentiation through space and time. *Molecular Ecology* 11: 1351 - 1362

Gibbs-Russell, G.E. & Spies, J.J.1988. Variation in important pasture grasses: I. Morphological and geographical variation. *Journal of the Grassland Society of Southern Africa* 5: 15 – 21

Gibbs-Russell, G.E., Watson, L., Koekemoer, M., Smook, L., Barker, N.P., Anderson, H.M. & Dallwitz, M.J.1990. *Grasses of southern Africa.* National Botanical Gardens/Botanical Research Institute, South Africa

Hartley, W. 1958. Studies on the origin, evolution, and distribution of the Gramineae I. The tribe Andropogoneae. *Australian Journal of Botany* 6: 115 - 128

Honjo, M., Ueno, S., Tsumura, Y., Washitani, I. & Ohsawa. Phylogeographic study based on intraspecific sequence variation of chloroplast DNA for the conservation of genetic diversity in the Japanese endangered species *Primula sieboldii. Biological Conservation* 120: 211 - 220

Jones, R. 1999. The Biogeography of the Grasses and Lowland Grasslands of South-eastern Australia. *In* R.N. Jones (ed.) The Great Plains Crash: Proceedings of a Conference on Victorian Lowland Grasslands and Grassy Woodlands, *Advances in Nature Conservation* 2: 11 - 18

Liebenberg, H. 1986. Cytotaxonomic studies in *Themeda triandra.* I. Chromosome numbers and microsporogenesis. *South African Journal of Botany* 52: 413 – 420

Liebenberg, H., Lubbinge, J. & Fossey, A.1993 Cytotaxonomic studies in *Themeda triandra* Forssk.:IV. A population cytogenetic study of a contact Zone between tetraploid and hexaploid populations. *South African Journal of Botany* 59: 305-310

Lumaret, R., Mir C., Michaud H. & Raynal V.2002. Phylogeographical variation of chloroplast DNA in holm oak (*Quercus ilex* L.). *Molecular Ecology* 11: 2327 - 2336

Maskas, S.D. & Cruzan, M.B.2000. Patterns of intraspecific diversification in the *Piriqueta caroliniana* complex in southern North America and the Bahamas. *Evolution* 54: 815 - 827

Oesterhold, M. & McNaughton, S.J.1988. Intraspecific variation in the response of *Themeda triandra* to defoliation: the effect of time of recovery and growth rates on compensatory growth. *Oecologia* 77: 181 - 186

Olsen, K.M. & Schaal, B.A.1999. Evidence on the origin of cassava: Phylogeography of *Manihot esculenta*. *Proceedings of the National Academy of Sciences USA* 96: 5586 - 5591

Sahuquillo, F. & Lunaret, R.1999. Chloroplast DNA variation in *Dactylis glomerata* L. taxa endemic to the Macronesian islands. *Molecular Ecology* 8: 1797 - 1803

Saltonstall, K.2001. A set of primers for amplification of noncoding regions of chloroplast DNA in the grasses. *Molecular Ecology Notes* 1: 76 – 78

Schneider, S.,Roessli, D., and Excoffier, L. (2000) Arlequin: A software for population genetics data analysis. Ver 2.000. Genetics and Biometry Lab, Dept. of Anthropology, University of Geneva.

Stehlik, I., Blattner, F.R., Holderegger, R. & Bachmann K.2002. Nunatak survival of the high alpine plant *Eritrichium nanum* (L.) Gaudin in the central alps during the ice ages. *Molecular Ecology* 11: 2027 - 2036

Taberlet, P., Gielly, L., Pautou, G. & Bouvet, J.1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* 17: 1105 - 1109

Templeton, A.R.1995. Seperating Population Structure from Population History: A Cladistic Analysis of the Geographical Distribution of Mitochondrial DNA Haplotypes in the Tiger Salamander, *Ambystoma tigrinum*. *Genetics* 140: 767 - 782

Templeton, A.1998. Nested clade analysis of phylogeographic data: testing hypotheses about gene flow and population history. *Molecular Ecology* 7: 381 - 397

Theunissen, J.D.1992. An ecosystematic investigation of *Themeda triandra* (Poaceae: Eragrostideae) in the semi-arid grasslands of southern Africa. *Journal of Arid environments* 23: 35 - 44.

Trewick, S.A., Morgan-Richards, M., Russel, J., Henderson, S., Rumsey, F.J., Pinter, I., Barrett, J.A., Gibby, M. & Vogel, J.C.2002. Polyploidy, phylogeography and Pleistocene refugia of the Rockfern *Asplenium ceterach*: evidence from chloroplast DNA. *Molecular Ecology* 11: 2003 - 2012

Van Oudtshoorn, F.1992. *Guide to Grasses of South Africa*. National Book Printers, Cape Town

Table 1: A table showing Population Numbers and the corresponding Localities. The numbers in this table correspond with those in Figure 1. Hluhluwe 1 is a collection site below the research centre at the Hluhluwe/Umfolzi Nature Reserve, while Hluhluwe 2 is located above the research centre. Alice 1 is an unburned fire plot belonging to the University of Fort Hare, while Alice 2 is a plot that is burned every two years. The GPS coordinates, the number of samples used and haplotypes found at each collection site is also included. The number of samples possessing a particular habitat are give in parenthesis after each haplotype.

Population Number	**Locality**	**GPS Coordinates**	**No. of samples examined**	**Haplotype**
1	Hanover	31°20.43S; 024°04.83E	5	H1(3),H2(2)
2	Colsburg	30°36.39S; 025°23.20E	5	H3(5)
3	Bloemfontein	30°36.41S; 025°23.19E	6	H1(3),H4(3)
4	Hluhluwe1	28°08.26S; 032°04.66E	5	H2(3),H5(1), H6(1)
5	Hluhluwe2	28 08.26S 032 04.66E	5	H2(2),H5(2), H6(1)
6	Haga Haga	32°45.92S; 028°14.52E	4	H6(1),H7(1), H10(2)
7	Alice1	32°40.03S; 026°52.52E	4	H10(3),H11(1)
8	Alice2	32°40.03S; 026°52.46E	5	H10(5)
9	Jeffery's Bay	33°57.69S; 024°57.17E	5	H6(2),H8(1), H9(1),H10(1)
10	Jonkershoek	33°57.70S; 024°57.17E	7	H6(6), H10(1)
11	Kruger National Park	025°10S; 31°00E	7	H6(4),H10(1), H12(1)

Table 2: A description of the primers used in this study.

Primer	**Sequence 5' – 3'**	**Reference**
*trn*C	CGAAATCGGTAGACGCTACG	Taberlet *et al.* (1991)
*trn*F	ATTTGAACTGGTGACACGAG	Taberlet *et al.* (1991)
*psb*D	CTATGGGGTCACARCCSAGG	Saltonstall (2001)
*trn*S	GCCGCTTTAGTCCACTCAGC	Saltonstall (2001)

Table 3: An interpretation of the nested clades in Figure 4 using the inference of Templeton (1998). Only significant inferences are shown.

Clade	Population inference
1-2	Long distance colonization
2-1	Restricted gene flow with isolation by distance
2-2	Restricted gene flow with isolation by distance
3-1	Restricted gene flow with isolation by distance
3-2	Past fragmentation followed by range expansion

Table 5: Analysis of Molecular Variance (AMOVA). The 12 localities were grouped together.

Source of variation	Degrees of freedom (d.f.)	Sums of squares	Variance	Percent variation
Among populations	10	40.946	0.501	25.49
Within populations	47	68.779	1.463	74.51

Table 4: Genetic differences between populations represented by an F_{st} matrix (± SD). Numbers in bold represent significant values (p = 0.05).

	Hanover	Bloemfontein	Colesburg	Hluhluwe1	Hluhluwe2	Haga Haga	Alice1	Alice2	Jeffrey's Bay	Jonkershoek	KNP
Hanover	*										
Bloemfontein	**0.00793+-0.0017**	*									
Colesburg	0.09719+-0.0052	**0.04959+-0.0038**	*								
Hluhluwe1	0.16628+-0.0063	**0.00793+-0.0015**	**0.00198+-0.0008**	*							
Hluhluwe2	0.09256+-0.0067	**0.00893+-0.0015**	**0.00331+-0.0011**	0.99967+-0.0000	*						
Haga Haga	**0.01884+-0.0024**	**0.00727+-0.0016**	**0.00298+-0.0009**	**0.01554+-0.0024**	**0.02281+-0.0024**	*					
Alice1	**0.00959+-0.0015**	**0.00727+-0.0013**	**0.02050+-0.0023**	**0.01785+-0.0025**	**0.01653+-0.0024**	0.41983+-0.0085	*				
Alice2	**0.00793+-0.0015**	**0.01025+-0.0021**	**0.00298+-0.0010**	**0.00793+-0.0016**	**0.00826+-0.0016**	0.16661+-0.0060	0.45124+-0.0085	*			
Jeffrey's Bay	**0.00661+-0.0016**	**0.00760+-0.0018**	**0.00198+-0.0009**	**0.02711+-0.0029**	**0.02678+-0.0029**	0.20463+-0.0074	**0.04430+-0.0040**	**0.04893+-0.0051**	*		
Jonkershoek	**0.00198+-0.0008**	**0.00132+-0.0006**	**0.00066+-0.0005**	**0.01091+-0.0018**	**0.00860+-0.0018**	0.18777+-0.0066	**0.00959+-0.0018**	**0.01653+-0.0027**	0.24826+-0.0070	*	
KNP	0.12826+-0.0070	**0.00231+-0.0009**	**0.00099+-0.0006**	0.41884+-0.0092	0.12926+-0.0054	0.61818+-0.0077	0.47636+-0.0093	0.45058+-0.0098	0.69488+-0.0096	0.56727+-0.0098	*

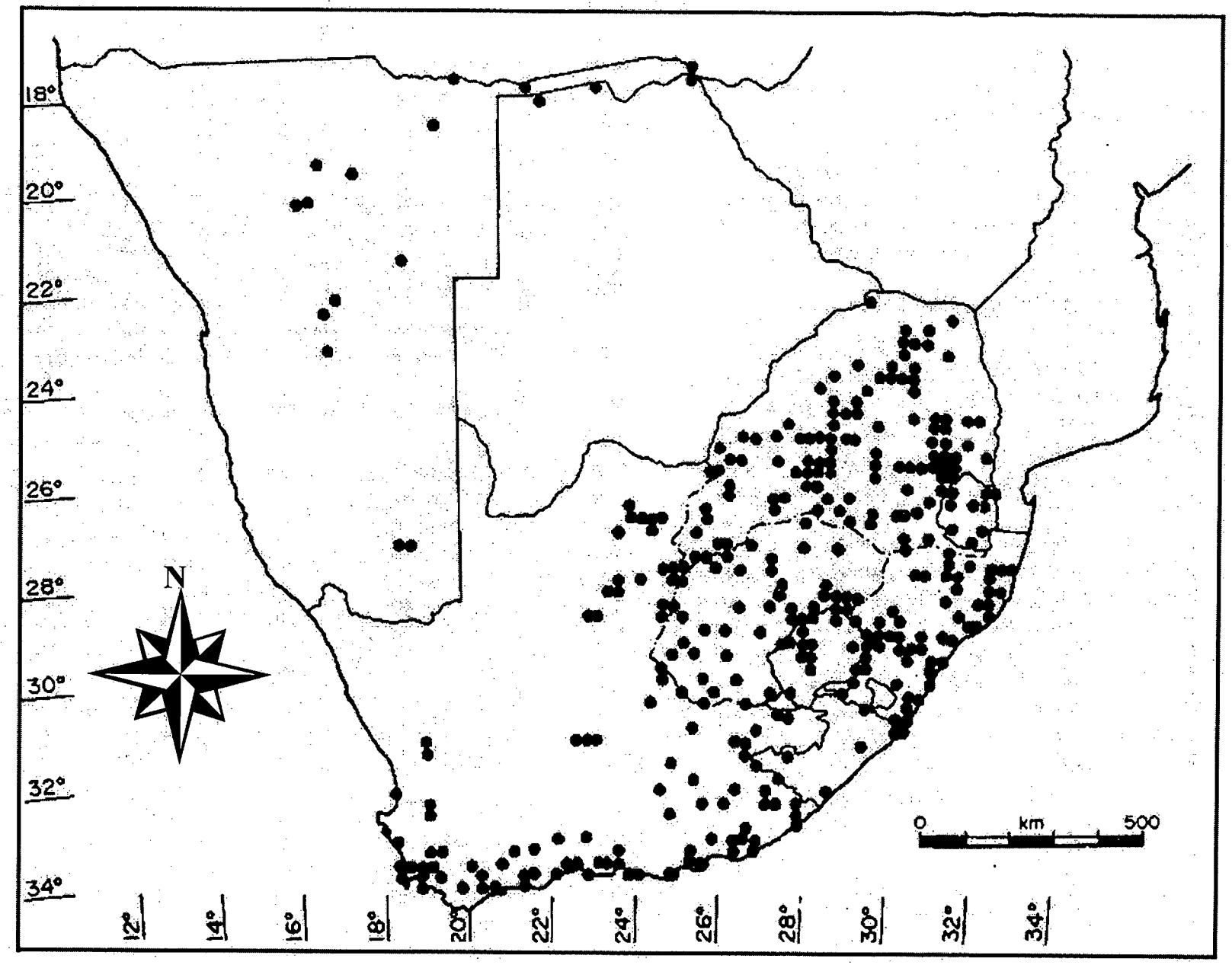

Figure 1: Distribution map of *Themeda triandra* in southern Africa (from Gibbs Russel and Spies 1988).

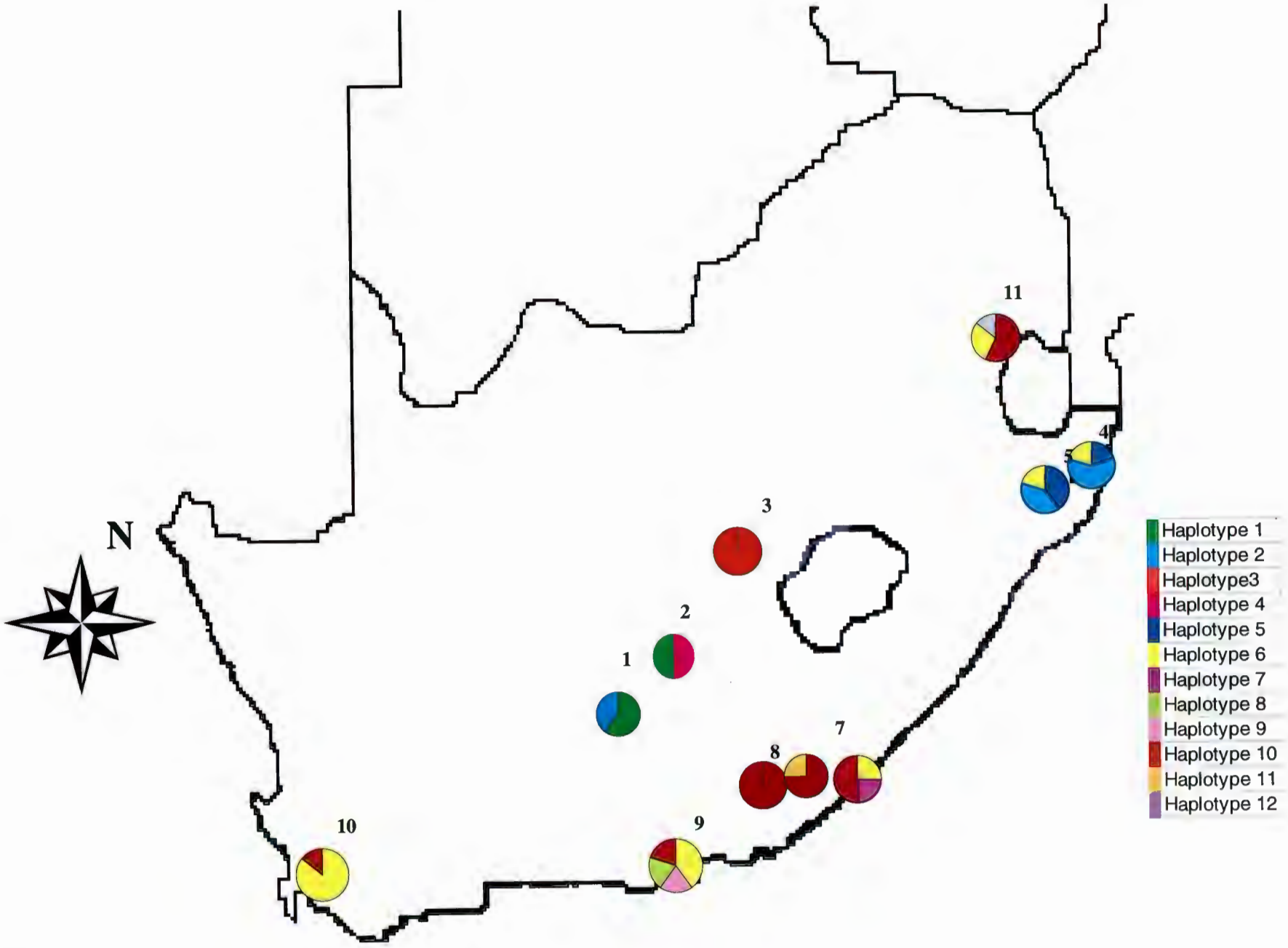

Figure 2: A map of South Africa showing the haplotype frequencies at the various sampling sites. Numbers/letters correspond with those in Table 1.

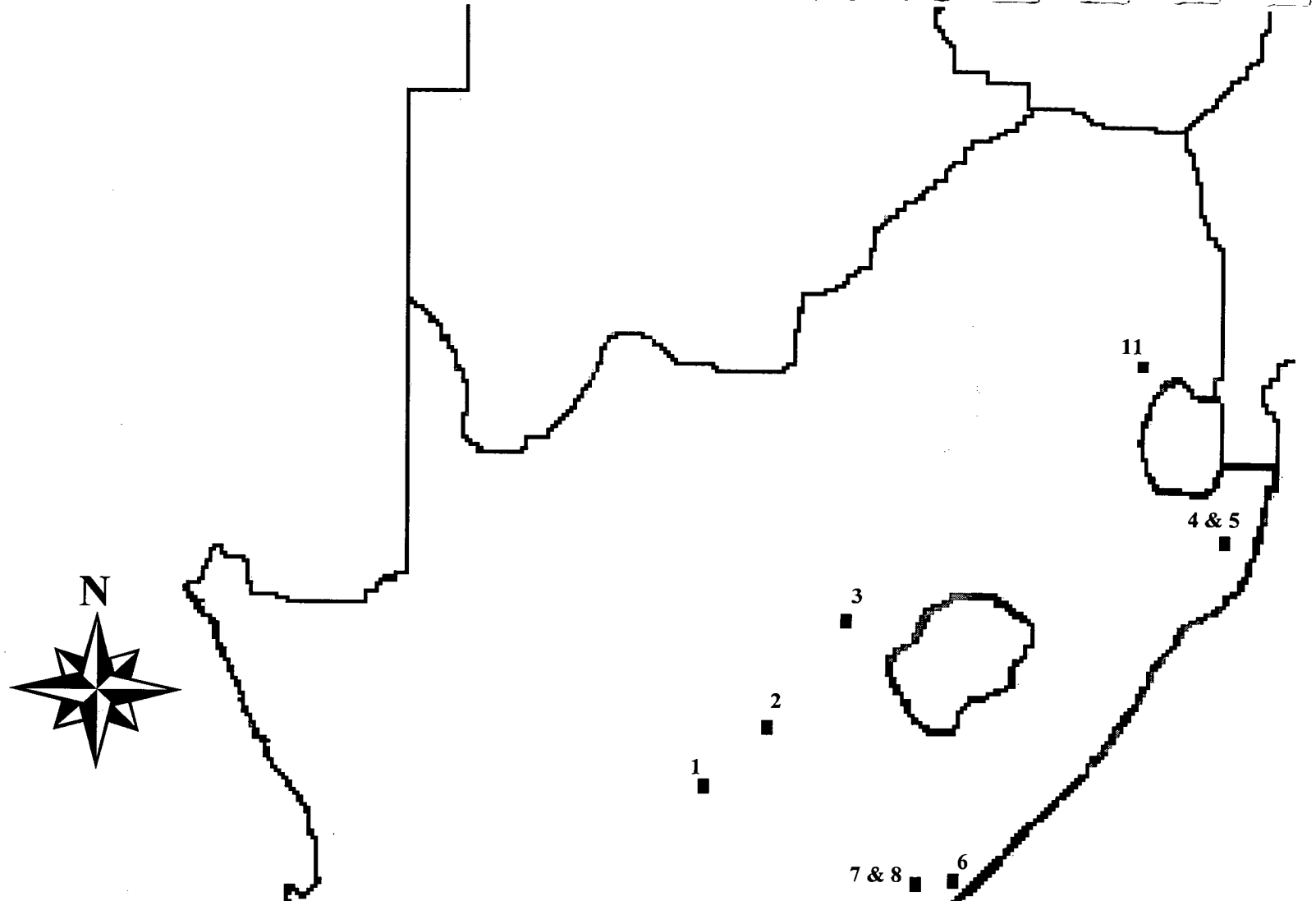

Figure 3: A map of South Africa showing sample sites of *Themeda triandra*. Numbers/letters correspond with those in Table 1, which contains the name of the collection sites and their GPS coordinates.

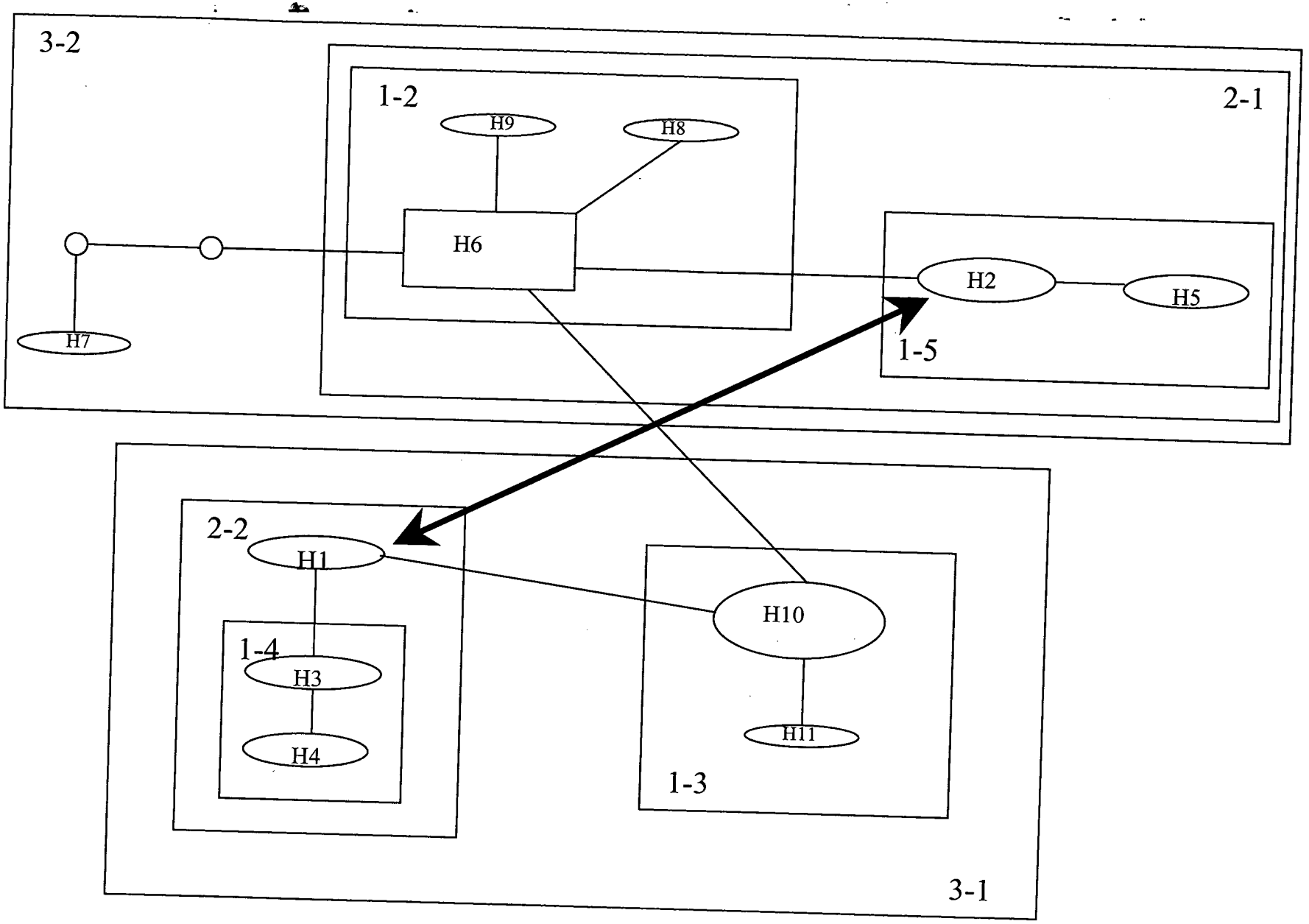

Figure 5: A nested haplotype network of data derived from *Themeda trinadra* cpDNA. The square represents the ancestral haplotype. The size of each haplotype is relative to the number of individuals possessing that haplotype. Haplotypes are represented by the same letters found in Table 1.Each line within the network represents a single mutational step. White circles represent haplotypes not found in any individuals. H12 is not included as it did not fall within the 95% confidence interval (16 steps). The removed ambiguous connection is indicated by the arrow.