GENETIC VARIATION OF *RANA FUSCIGULA* IN SOUTHERN AFRICA.

by

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Submitted in partial fulfilment of the requirements for the degree of Master of Science in the Department of Biochemistry, University of Western Cape.

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DEDICATION

This thesis is dedicated to my late father Yusuf, my mother Shariefa Arieff, my husband Nazeem and my children Taariq, Samiya and Rafiq.

DECLARATION

I, _____ , declare that my thesis on **"Genetic variation in *Rana fuscigula* in southern Africa"** is my own work and that all the sources I have used or quoted, have been indicated and acknowledged by means of complete references.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

bp	- Base pairs
kb	- kilobase
°C	- Degrees celsius
µCi	- micro Curie
dATP	- Deoxyadenosine triphosphate
dCTP	- Deoxycytidine triphosphate
dGTP	- Deoxyguanosine triphosphate
DNA	- Deoxyribonucleic acid
mtDNA	- mitochondrial deoxyribonucleic acid
rDNA	- Ribosomal deoxyribonucleic acid
α-[^{32}P]-dCTP	- ^{32}P-labelled deoxycytidine triphosphate
min	- minute
NTS	- nontranscribed spacer
ETS	- external transcribed spacer
ITS	- internal transcribed spacer

Restriction Enzymes :

A	- Asp 700/Xmn I
B	- Bam HI
c	- Bcl I
D	- Dra I
E	- Eco RI
g	- Bgl II
h	- KpnI
H	- Hind III
N	- Nco I
P	- Pst I
s	- Sac I
V	- Pvu II
X	- Xba I

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CHAPTER I

INTRODUCTION

Naturalists have long been engaged in describing and explaining diversity in the biological world. The discovery of the molecular basis of inheritance has led to a rapid increase in the use of biological macromolecules in these investigations. Scientists now routinely investigate the DNA of a range of organisms.

The relationships between taxa and the phylogeny of groups is determined by examining the differences and similarities between them. These differences are then appropriately analyzed. It is important to understand the natural variation within a group, before the differences between groups can be established.

This study aims to determine the molecular differences between individuals at the extreme edges of the distribution of a species. This will serve as a molecular baseline, from which other studies can proceed.

The experimental species is the frog *Rana fuscigula*, which has a range restricted to southern Namibia and South Africa. It was thus possible to collect material from the edge of the distribution assuming that maximum genetic difference would be found between individuals at the edge of the range.

1.1 The research question.

This study aims to determine the normal variation in ribosomal DNA of *Rana fuscigula*, using the technique of restriction site mapping. No previous molecular studies have been done on this species. *Rana fuscigula* is found from the south-western Cape and Namaqualand through to Natal and the Orange Free State, and southern Transvaal, with an isolated population in southern Namibia (Passmore and Carruthers, 1979; Wager, 1986).

1.2 The experimental animal: *Rana fuscigula.*

This species consists of large frogs, up to 125 mm in length. They are found on grassy edges of rivers and pools. The dorsal coloration of this species varies from brown to green. Dark spots and a light vertebral stripe are usually present. The jawline tapers gradually from behind the eyes to a slightly rounded snout. The ventral surface is smooth and white with dark mottling on the throat. The toes are webbed and the last phalanx of the fourth toe is free.

This frog is easily recognized, as it occurs with no other similar sized *Rana,* except for the eastern parts of its range where it is sympatric with *Rana angolensis*. It is easily distinguished from *R. angolensis* by differences in webbing

and vocalizations (Passmore and Carruthers, 1979).

Although adults are not easy to find, the tadpoles develop in pools for more than a year. The tadpoles can therefore be readily collected at all times of the year. Tadpole tail was the source of tissue for one of the localities in this study.

1.3 The experimental technique: Restriction Site Mapping.

There are many different molecular techniques available that are used in phylogenetic and population genetic studies. These include isozyme and allozyme studies (Ovenden *et al.*, 1993), immunological techniques such as immunodiffusion and microcomplement fixation (Hass and Hedges, 1991), restriction site mapping of DNA (Allard and Honeycutt, 1991; O'Ryan and Harley, 1993), and nucleotide sequencing (Stepien *et al.*, 1993).

The technique that I employed is restriction site mapping of ribosomal DNA. This technique has been successfully used to generate maps from which phylogenies have been inferred (Hillis and Davis, 1986).

Ribosomal DNA consists of three genes, a nontranscribed spacer region, an external transcribed spacer region and two internal transcribed spacer regions, repeated in tandem. Ribosomal DNA codes for the subunits of the ribosomes,

the sites where peptides are assembled. They are very conserved, and so yield data of long term changes accumulating in the genome. Between the ribosomal genes are sequences that are not transcribed, and hence not under any selection. These nontranscribed sequences are useful to examine short term changes, as they are free to accumulate sequence changes. Ribosomal DNA was selected as it offers information on both recent and ancient events.

The primary limitation of using restriction site mapping is that only about 500 base pairs of 12 000 base pairs of the rDNA molecule are involved. The only changes that can be detected are the loss or gain of a recognition sequence at a site, and major changes in the length of the repeat (HIllis and Davis, 1986). Gains of sites are more useful than losses, because losses can arise by a change in any of the six nucleotides in the recognition sequence. The acquisition of parallel, non-homologous losses is relatively more likely than gains. The extent of homoplasmy within the DNA arrays is limited as a result of their conservative nature (Hillis and Davis, 1986).

Restriction site mapping has the potential to rapidly sample sites associated with 12 000 or more bases on the rDNA genes. This information will be valuable to determine if there is variation, and to what extent this technique can be used for future phylogenetic studies in this genus.

CHAPTER 2

MATERIALS AND METHODS

2.1 Specimens and localities.

The different collecting localities of the biological material used are presented in Table I. The frogs were anaesthetized with ms-222 (3-Aminobenzoic acid ethyl ester, methane sulfonate salt) and the liver and skeletal muscle were dissected and frozen at -70°C until needed. The tadpoles were also anaesthetized with ms-222, frozen in liquid nitrogen during transportation and stored -70°C for several months. Two individuals were selected from each locality (Fig. 1). These localities were selected because they represented different parts of the range of the species.

TABLE: I. Source of biological material.

Ref. No. of Specimen	Locality
971	24° 10' S, 16° 15' E Naukluft (Namibia)
974	24° 10' S, 16° 15' E Naukluft (Namibia)
ac960	33° 32' S, 19° 08' E Bainskloof (Cape)
ac959	33° 32' S, 19° 08' E Bainskloof (Cape)
H/T71	29° 08' S, 26° 13' E Bloemfontein (O.F.S.)
H/T75	29° 08' S, 26° 13' E Bloemfontein (O.F.S.)

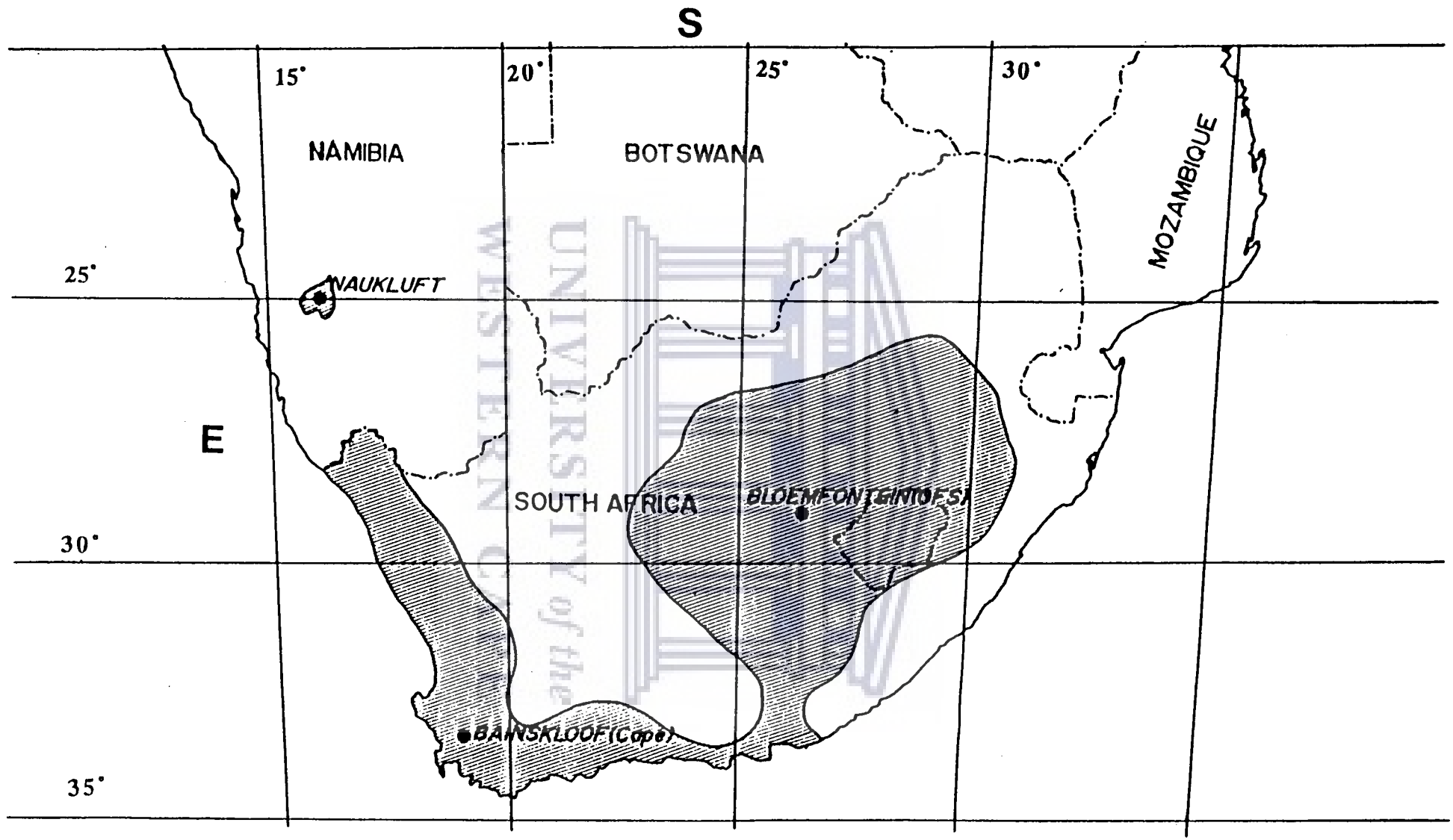

Fig. 1. Map indicating the distribution and collection localities of *Rana fuscigula* in southern Africa after Passmore and Carruthers (1979).

2.2 Overview of the method.

Differences in DNA between organisms can be studied using different methods, e.g. restriction fragment size comparisons, restriction site mapping and DNA sequencing. I used the technique of restriction site mapping.

Restriction site mapping of ribosomal DNA (Hillis and Davis, 1986) and mitochondrial DNA (Allard and Honeycutt, 1991) has been extensively used for evolutionary studies. Restriction site mapping of rDNA has the following advantages: (1) rDNA is mid to highly repetitive (Britten and Kohne, 1968), ensuring that multiple copies are present which facilitates hybridization. (2) The rDNA repeat length is within a range that can be examined by restriction site analysis. (3) rDNA contains both slowly evolving regions (the 18S, 5.8S and 28S rDNA genes) and more rapidly evolving regions (the transcribed and the nontranscribed spacer regions) and therefore information from various levels of evolutionary history can be recovered (Appels and Dvórak, 1982)(Fig. 2). (4) rDNA evolves in a concerted fashion (Dover and Coen, 1981), so that the rDNA of single individuals usually is representative of the species.

Restriction site mapping involved the extraction of DNA from either liver or muscle tissue using any of a number of methods. Once the DNA had been isolated it was quantitated and stored in the freezer at -20°C. The next step

Fig. 2. Diagram showing the structure of the ribosomal repeat unit. (Hillis and Davis, 1986). The abbreviations are as follows: NTS: nontranscribed spacer; ETS: external transcribed spacer and ITS: internal transcribed spacer.

was the digestion of 1 ug of DNA with a restriction enzyme in the appropriate digestion buffer. Thirteen different restriction endonucleases were used. Table 2 shows the enzymes and their respective cleavage sequence.

Each sample was digested singly with each restriction enzyme and was also digested with an enzyme in combination with Eco RI. The digested DNA samples were electrophoresed and transferred to a membrane using the Southern blotting technique.

Two rDNA probes were radioactively labelled using the nick translation method. These probes were obtained from D. Hillis (1986). The 18S rDNA probe was used to probe the 18S gene region and the NTS-ETS region. The 28S rDNA probe was used to probe the 28S gene region, 5.8S gene region and the ITS regions. The membrane was first hybridized with the one probe and then washed to reduce nonspecific binding of the probe. The membrane was exposed to x-ray film for 1-4 days.

The probe was then removed by stringent washing of the membrane and it was hybridized to the second labelled probe using the same procedure as above.

2.2.1 DNA Extraction.

DNA extractions were done using either the phenol method (Mayfield, 1984, Ausubel *et al.*, 1989, Hillis and Moritz, 1990) or the salt method (Miller *et al.*,1988). Pilot studies were done using three slightly different phenol extraction procedures on *X. laevis* muscle tissue. The DNA yields for all three phenol methods were very similar. I decided to use the Hillis and Moritz (1990) method because one could do extractions on as little as 100 ug of tissue. The other two phenol methods required one to use a much larger tissue sample for extraction. The NaCl method was a faster method for extracting DNA and did not involve the use of toxic chemicals. However the phenol extraction procedure gave a much higher yield of DNA (yield: mean=537,2; 317-866,7 ug/g; n=4) compared to the NaCl method (yield: mean=79,7; 83-76,3 ug/g; n=2). The results are illustrated in Table 3. Another advantage of the phenol method was that the restriction enzymes cut the DNA without any problems. Although the NaCl method was a very quick and easy extraction method, problems were experienced when trying to cut the DNA with different restriction enzymes.

DNA was extracted from either the liver or skeletal muscle of adult frogs, except in the case of the Naukluft samples (974 and 971) where DNA was extracted from the tadpole. Approximately 1 g of tissue was pulverized to a fine powder in liquid nitrogen with a prechilled mortar and pestle. See appendix I for more details of the different methods used.

2.2.2 DNA Quantitation.

DNA was firstly quantitated spectrophotometrically. The sample was appropriately diluted and the absorption was read at 260 nm (A_{260}) and 280 nm (A_{280}). The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An optical density (OD) of 1 corresponds to approximately 50 ug/ml for double-stranded DNA. The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA have OD_{260}/OD_{280} values of greater than 1.8. If there was contamination with protein or phenol, the OD_{260}/OD_{280} would be significantly less than the value given above, and accurate quantitation of the amount of nucleic acid would not be possible (Maniatis *et al.*, 1989). The equation is as follows :

$$\text{Concentration} = A_{260} \times 50 \times \text{Dilution factor} \quad (\text{ug/ml})$$

The second method of quantitating the DNA sample was to run it on an agarose minigel with a known amount of uncut lambda CI857 DNA and do a visual estimation of the quantity of DNA.

The reason for quantifying the DNA is that one requires a minimum of 1 ug of DNA on the gel when one is probing for restriction fragments otherwise the

autoradiography signal obtained is very faint. The minigel method of DNA estimation was preferred because the value obtained for the concentration of the DNA from the spectrophotometric determination included RNA contamination of the sample. The spectrophotometric method did not give a true value for the actual DNA concentration because the DNA sample was contaminated with RNA.

2.2.3 Single Digests.

Restriction enzymes or endonucleases are found in a wide range of bacterial species. They act on double-stranded DNA and cleave only at a specific sequence. These sequences are always short (four to six base pairs, occasionally more) and palindromic. Thirteen restriction enzymes obtained from Boehringer Mannheim were used in this study. One ug of DNA was digested in a total volume of 50 ul. The digestion mixture contained a volume of one tenth of the appropriate buffer, 5 units of the restriction enzyme and the rest of the volume was made up with sterile water. All the restriction enzymes used had a recognition sequence that included six specific nucleotides except for the enzyme, Xmn I or Asp 700. The enzymes that were used in this study, their cleavage sequences and the appropriate digestion buffer is given below in Table 2 (Ausubel *et al.*,1989).

TABLE: 3. The components and pH of the different digestion buffers.

Buffers	A	B	L	M	H
Tris-HCl	-	10	10	10	50
NaCl	-	100	-	50	100
$MgCl_2$	-	5	10	10	10
2-Mercaptoethanol	-	1	-	-	-
Dithioerythritol	-	-	1	1	1
Dithiothreitol-	0,5	-	-	-	-
Tris-acetate	33	-	-	-	-
K-acetate	66	-	-	-	-
Mg-acetate	10	-	-	-	-
pH	7,9	8,0	7,5	7,5	7,5

The composition of the various digestion buffers are in mmole/litre.

2.2.4 Double Digests.

Samples were cleaved with both enzymes simultaneously under normal conditions, using either voodoo or KGB buffer (McClelland, 1988), except in the

case of Bcl I where the DNA was first cleaved with Bcl I at 50°C and then with Eco RI at 37°C.

Double digests were used to locate specific restriction sites within the 28S and 18S ribosomal genes. The mapping of sites was accomplished by double digestions, using as reference points the Eco RI sites that are uniformly present in the vertebrates near the 5' ends of the 18S and 28S genes (Cortadas and Pavon, 1982).

2.2.5 Gel electrophoresis.

Cleaved DNA was electrophoresed at approximately 5 V/cm for 21 hours in 0,8% agarose gels (buffer system: 0,04 M Tris, 0,02 M sodium acetate, 0,018 M sodium chloride, 0,001 M EDTA, pH 8,0). The powerpack and the gel electrophoresis system used, was manufactured by Hoefer Scientific Instruments (model no. PS 500X). Lambda CI857 phage DNA cut with Hind III was included on each gel as a standard. Lambda CI857 phage DNA produced eight fragments when it was cut with Hind III. When run on a 0,8% agarose gel for 21 hours at 30 volts, the following size fragments are seen: 23 130 bp, 9 416 bp, 6 557 bp, 4 361 bp, 2 322 bp, 2 027 bp and 564 bp. The 125 bp fragment is not visible on the gel. The log molecular weight of the lambda-Hind III fragments were plotted against the distances migrated by the

The other side of the agarose gel is placed directly against a piece of hybond N filter. Dry 3MM Whatmann paper is placed against the Hybond N filter. Dry tissue paper and a weight is placed on top of the 3MM Whatmann paper. Fig. 3 shows the apparatus used in the Southern blotting technique.

DNA in the agarose gels was denatured in 1,5 M NaCl, 0,5 M NaOH for 1/2 to 1 hour and then neutralized in 1,5 M NaCl, 0,5 M Tris pH 5,5 for 1/2 hour. DNA fragments were then transferred to Hybond N filter, purchased from Amersham, using a modification of the Southern blot method (Southern, 1975). The modification is given in appendix I. The DNA was transferred to the Hybond N filter in such a way as to reproduce on the filter the distribution of fragments on the gel. Figure 4 is a flow diagram showing the steps in Southern blotting and hybridization. The filter was covered with 3 MM Whatmann paper and baked at 80°C for one hour. It was wrapped in plastic and stored at 4°C. After prehybridization it was hybridized with the radioactively labelled probe.

2.3.1 Preparation of the radioactive probe.

Because each gene has a unique nucleotide sequence, it can be detected with the aid of a radioactively labelled DNA fragment that is complementary to it, called a probe. The radioactive DNA probe is added to the filter paper, annealing only to the DNA containing the complementary gene, and the labelled

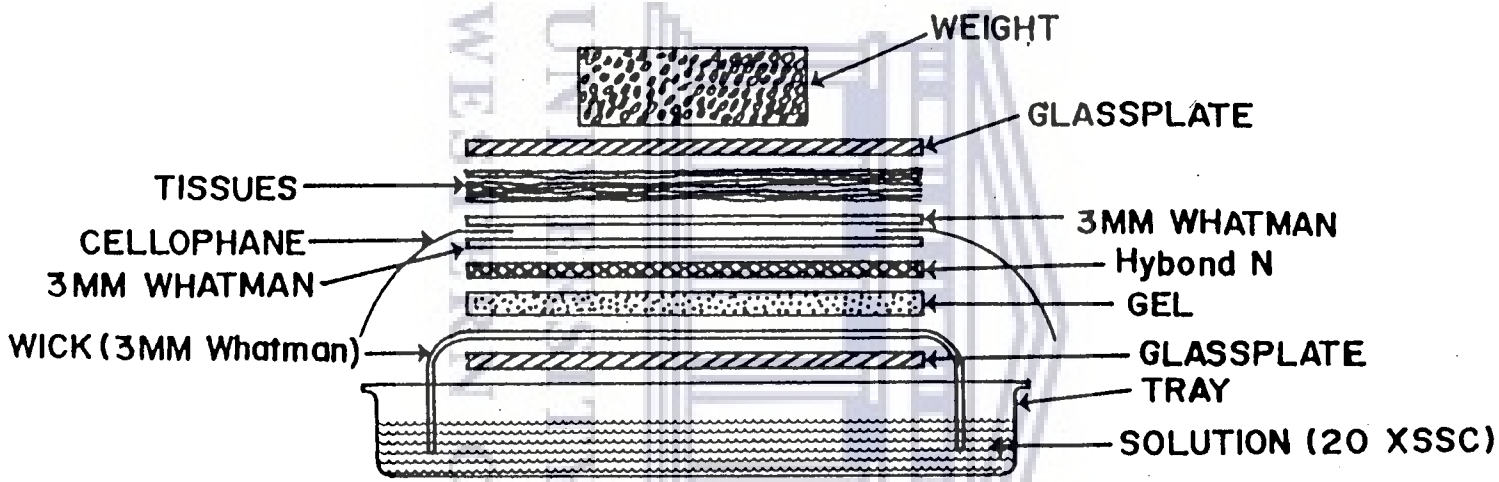

Fig. 3. A diagram showing the apparatus in the Southern Blotting Technique (Maniatis *et al.*, 1989).

fragments are visualised by autoradiography. Southern blots were probed radioactively using two different probes. The plasmid, pE2528, contained the internal transcribed spacers, 5.8S rDNA and most of the 28S rDNA gene of *Rana catesbeiana* in the vector pUC18 (Hillis and Davis, 1986; Hillis and Davis, 1987). The plasmid, designated pE2518, contained the rest of the rDNA gene including the 18S rDNA gene in the vector, pUC18.

The probes were labelled using the nick translation method. The nick translation kit was purchased from Boehringer Mannheim. The probes were labelled in the following way. Two ug of DNA were denatured by heating at 100°C for 10 minutes and were subsequently cooled on ice. The following components were added to microfuge tubes to make a final volume of 20 ul: 2 ug of denatured DNA corresponding to 2 ul, 1 ul dATP, 2 ul dGTP, 1 ul dTTP, 2 ul reaction mixture, 6 ul of sterile distilled water, 5 ul [α^{32}P]dCTP (50 µCi) and 2 ul Klenow enzyme (4 units). The mixture was incubated for 35 minutes at 15°C. The reaction was stopped by heating at 65°C for 10 minutes. The sample was then put through a Sephadex G50 gel filtration column. Gel filtration is a method which separates molecules on the basis of size. The smaller molecules of unincorporated deoxyribonucleotide triphosphate were trapped within the gel and were eluted last. The larger molecules, the labelled DNA, were eluted first.

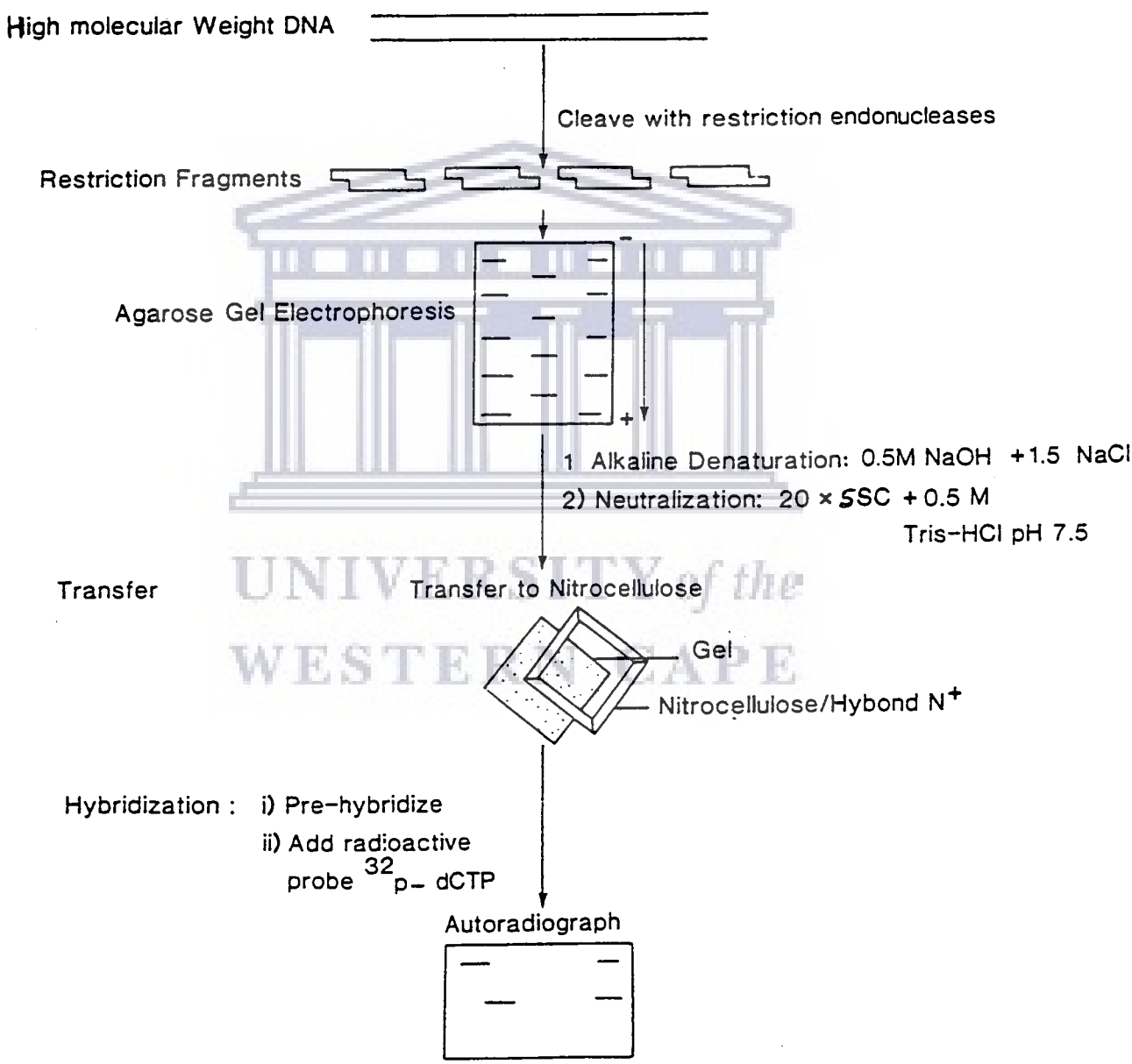

Fig. 4. Flow diagram showing the steps in Southern blotting and hybridization (Southern, 1975).

Uncut lambda C1857 DNA was labelled in a similar manner to the two rDNA probes. The standard or marker was lambda C1857-Hind III fragments. The bands of the standard were seen on the autoradiograph. This enabled me to size the other fragments on the autoradiograph by using the known fragment sizes of this standard for comparison.

2.3.2 Prehybridization, Hybridization and Washes.

The baked filter was cut into squares of 90 x 30 mm and placed in a perspex hybridization box with 10 ml of hybridization solution (Appendix I) for one to two hours at 65°C. Inadequate prehybridization can lead to high background. The reason for this is that the prehybrization solution contains high molecular weight compounds that will bind to the filter and thus act as a blocking agent. This will prevent the labelled probe from binding to the filter and causing a high background. When the probe is added it will only bind to the sequence in the DNA which is complementary to it. The labelled probe and 5 ul of labelled lambda CI857 DNA was denatured by boiling the mixture at 100°C for 10 minutes. This was added to the hybridization box (Fig. 5). The filter was hybridized at 65°C for 12-18 hours.The filters were washed twice in 50 ml of 2 x SSC containing 0,2% SDS for 15 minutes. The filters were then washed in 50 ml of 1 x SSC containing 0,1% SDS for one hour in a shaking waterbath. All the washes were

done at room temperature. The filters were rinsed in deionized water, dried and wrapped in plastic. They were placed in an x-ray cassette with a screen and exposed to 24 x 30 cm Cronex 4 x-ray film. The x-ray film, x-ray cassettes and screens were manufactured by E. I. Du Pont and Okamoto respectively. Hybridization conditions and washes were identical to those described by Sytsma and Schaal (1985).

2.3.3 Autoradiography.

The radioactively labelled filter was exposed to x-ray film for 1-4 days at -70°C.

2.3.4 Developing of x-ray films.

The developing of the x-ray film was done in a photographic dark room. The film was first placed in developing solution (Ilford Phenisol) for 3 minutes. The film was washed in water for 1 minute. The last step was to place the x-ray film in a diluted fixer solution (Super Amfix), for 3 minutes. The autoradiograph was thoroughly rinsed with water and air dried.

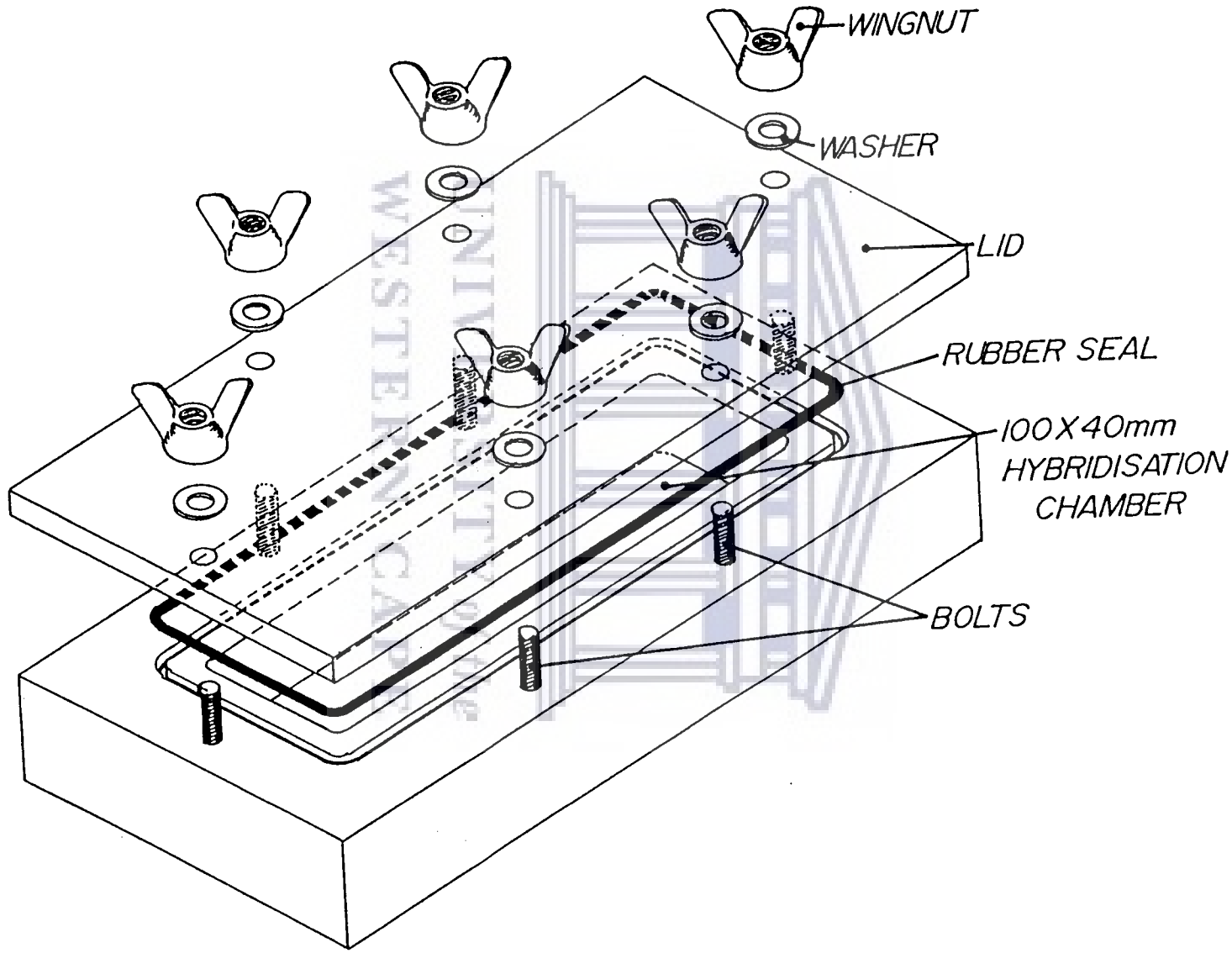

Fig. 5. Drawing of a hybridization box used during hybridization (Van den Plas, *et al.*, 1984).

2.6 Data Analysis.

Molecular data generated form this study was analyzed using a number of different programs.

The average size of the rDNA repeat length of the six individuals were calculated from five accurately sized sets of single and double digests.

Restriction site maps were drawn using the restriction data for all six individuals. The restriction site maps of the three different populations were compared. These maps were positioned in such a manner that the 5' Eco RI site in the 28S gene of each of the populations was aligned. Cortadas and Pavon (1982) have shown that the 18S and the 28S Eco RI sites at the 5' end are uniformly present in vertebrates. The differences in the maps between the three populations are listed in Table 9.

Genetic relationships were determined by comparing restriction site maps of the individuals with each other. The transformation of restriction endonuclease data to genetic distances was done using the formulae of Nei and Li (1979). These formulae are written into the RESOLVE program. The pairwise proportion of shared sites and the sequence divergence was calculated using the RESOLVE program.

CHAPTER 3

RESULTS

3.1 Results of extraction of DNA from the six individuals.

Two different methods of DNA extraction were used. The salt method (Miller *et al.*, 1984) and the phenol method (Hillis and Moritz, 1990). The yields of DNA obtained from the different tissue types and the different DNA extraction procedures are shown in Table 4.

TABLE 4. The tissue type, mass, extraction method, total DNA and yield for each of the six individuals analyzed in this study.

Sample	Tissue	Mass g	Extract Method	Tot.DNA ug	Yield ug/g
H/T75	SM	3,36	NaCl	279	83,0
H/T71	L & SM	1,0	Phenol	317	317
AC959	SM	1,0	Phenol	490	490
AC960	L	0,45	Phenol	390	866,7
974	Tad	1,0	Phenol	475	475
971	Tad	3,0	NaCl	229	76,3

H/T71 & H/T75 samples are from the Bloemfontein (O.F.S) area. The liver and skeletal muscle DNA of sample H/T71 were pooled. AC959 & AC960 samples are from BainsKloof (Cape) area. 974 and 971 are from the Naukluft area (Namibia) area. (SM=skeletal muscle, L=liver, Tad=tadpole)

The salt method gave low DNA yields (76,3 ug/g of tissue) and problems were experienced when cutting with different restriction enzymes. The problem was due to a high concentration of salt in the DNA sample and this inhibited the activity of the restriction enzymes. The only way to overcome this problem was to re-precipitate the DNA and redissolve it in the appropriate buffer. The phenol method gave much higher yields of DNA (475 ug/g of tissue) and I experienced no problems when cutting the DNA with the different enzymes.

3.2 Autoradiographic visualization.

Photographs of the autoradiographs of typical single and double digests of the six individuals are shown in Figures 6 to 8. Figure 6 is an autoradiograph obtained by probing a filter with the 28S rDNA probe. This filter contains DNA that had been digested with only a single enzyme. Figures 7 and 8 are autoradiographs obtained from a filter containing double digests which was first probed with the 18S rDNA and then the 28S rDNA. The lambda CI857 phage DNA cut with Hind III (marker) was clearly seen on all the autoradiographs.

Fig. 6. Autoradiograph showing the rDNA fragments which were generated when nuclear DNA was cut with various restriction enzymes. Six samples of DNA (1 ug) were digested with the restriction enzymes (A = Sac I, B = Nco I, C = Bcl I) and electrophoretically separated on an agarose gel. The cut DNA was transferred to nylon filters and probed with the 28S rDNA probe. Lane M is the molecular weight standard lambda C1857 DNA cut with Hind III. Sizes of marker fragments are shown. (Samples 1 & 2 are from the Naukluft population, samples 3 & 4 are from the Bainskloof population and samples 5 & 6 are from the Bloemfontein population).

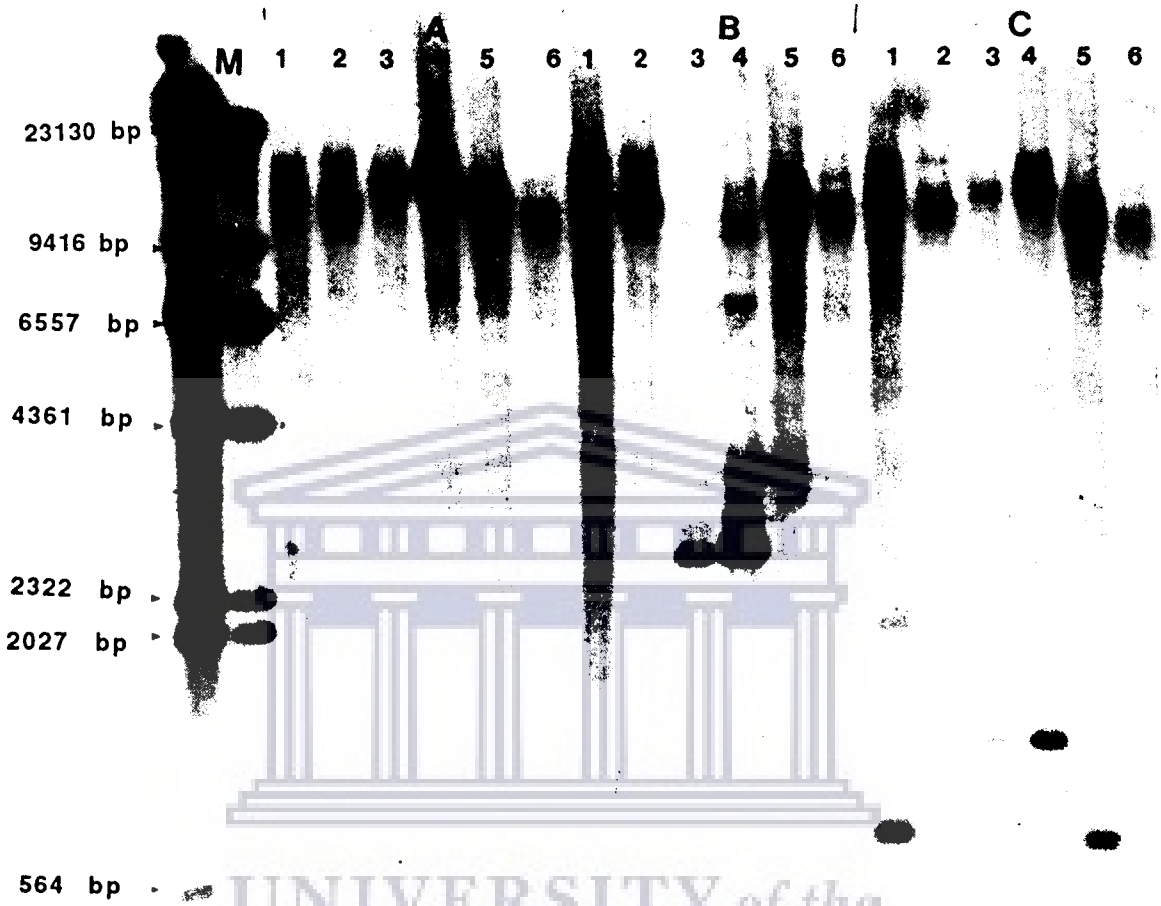

Fig. 7. Autoradiograph showing the rDNA fragments which were generated when nuclear DNA was cut with various restriction enzymes. Six samples of DNA (1 ug) were digested with two restriction enzymes simultaneously (A = Eco RI & Bcl I, B = Eco RI & Xho I, C = Eco RI & Kpn I) and electrophoretically separated on an agarose gel. The cut DNA was transferred to nylon filters and probed with the 18S rDNA probe. Lane M is the molecular weight standard lambda C1857 DNA cut with Hind III.. Sizes of marker fragments are shown. (Samples 1 & 2 are from the Naukluft population, samples 3 & 4 are from the Bainskloof population and samples 5 & 6 are from the Bloemfontein population).

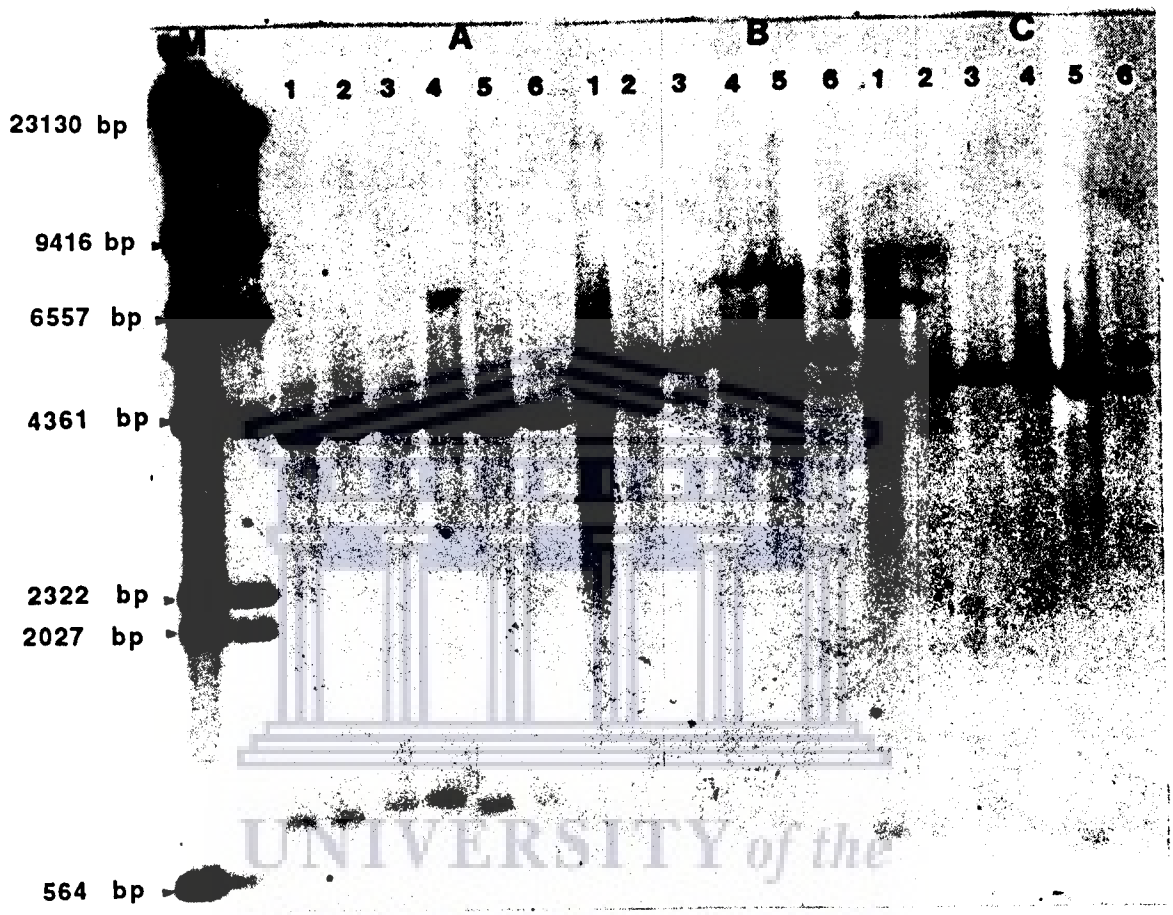

Fig. 8. Autoradiograph showing the rDNA fragments which were generated when nuclear DNA was cut with various restriction enzymes. Six samples of DNA (1 ug) were digested with restriction enzymes simultaneously (A = Eco RI & Bcl I, B = Eco RI & Xho I, C = Eco RI & Kpn I) and electrophoretically separated on an agarose gel. The cut DNA was transferred to nylon filters and probed with the 28S rDNA probe. Lane M is the molecular weight standard lambda C1857 DNA cut with Hind III. Sizes of marker fragments are shown. (Samples 1 & 2 are from the Naukluft population, samples 3 & 4 are from the Bainskloof population and samples 5 & 6 are from the Bloemfontein population).

3.3 Mapping of restriction sites.

Each sample was cut with thirteen restriction enzymes. The list of restriction enzymes used in this study is shown in Table 5. I was able to map 23 restriction sites for the Naukluft and the Bloemfontein samples and 24 restriction sites for the Bainskloof samples.

TABLE 5. Number of restriction sites per enzyme found in this study.

		NUMBER OF RESTRICTION SITES		
Locality		**Naukluft**	**Bloemfontein**	**Bainskloof**
Enzymes				
Bam HI	(B)	2	2	2
Nco I	(N)	2	2	2
Eco RI	(E)	2	2	2
Bgl II	(g)	1	1	1
Pst I	(P)	2	2	2
Kpn I	(h)	2	2	2
Bcl I	(c)	1	1	1
Xba I	(X)	2	2	2
Sac I	(s)	2	2	2

Table 5 continued.

Dra I	(D)	2	2	2
XmnI/Asp 700	(A)	3	3	3
Pvu II	(V)	1	1	2
Hind III	(H)	1	1	1

The letters in brackets represent the abbreviated form of the respective enzymes.

3.4 Size of rDNA repeat length of the six individuals.

The rDNA repeat length contains the 18S, 5.8S and 28S genes, transcribed and nontranscribed spacer regions (Fig. 2). There was no difference in the rDNA length of the two individuals used for each locality. The mean rDNA length of the Bloemfontein specimens was 17 101 bp (S.D.= ±352, N=5) and the mean rDNA length of the Naukluft specimens was 17 004 bp (S.D.= ±474, N=5). The mean genome size of the Bainskloof specimens was 19 414 bp (S.D.= ±450, N=5). Table 6 is a comparison of the size of the repeat length of the three populations using student's t test.

3.5 Restriction site maps.

Restriction sites maps were drawn independently for all six individuals using the restriction data listed in Tables 7 and 8. The data was entered into the program RESOLVE 3.3 (Harley, 1993) and with the aid of a Hewlett Packard plotter 7475A, all the restriction site maps were drawn (fig. 9).

To check for accuracy of alignment of restriction sites, the site alignment option of RESOLVE was used to align individual restriction enzymes for all six samples (fig. 10). This option gives an indication of the phylogenetically informative sites shared by the individuals. The number of shared sites were observed in the 18S rDNA and 28S rDNA gene of all the samples used in this study.

TABLE 7. Fragment lengths obtained when digesting the rDNA of the three populations with various restriction enzymes. The filters were probed with the 28S rDNA probe.

	Sizes of 28S rDNA Restriction fragments		
Locality	**Naukluft**	**Bainskloof**	**Bloemfontein**
Enzymes			
Bam HI	3 476	3 692	3 476
	13 528	15 722	13 625

Table 7 continued.

Nco I	4 599	694	4 599
	12 405	14 720	12 502
Eco RI	5 177	5 256	5 224
Bgl II	17 004	19 414	17 101
Pst I	6 605	8 322	6 605
		8 844	
Kpn I	5 138	6 023	5 138
Bcl I	17 004	19 414	17 101
Xba I	15 186	17 461	15 283
Sac I	1 229	1 232	1 229
	15 775	18 182	15 872
Dra I	4 034	4 435	4 034
	12 970	14 979	13 067
Xmn I/	1 171	609	1 171
Asp 700	4 100	4 700	4 100
Pvu II	17 004	7 692	17 101
Hind III	9 400	19 414	17 101

TABLE 8. Fragment lengths obtained when digesting the rDNA of the three populations with various restriction enzymes. The filters were probed with the 18S rDNA probe.

	Sizes of 18S Restriction fragments (bp)		
	Naukluft	Bainskloof	Bloemfontein
Enzymes			
Bam HI	3 476	3 692	3 476
	13 528	5 722	1 3625
Nco I	4 599	4 694	4 599
	12 405	14 720	12 502
Eco RI	11 827	14 158	11 877
Bgl II	17 004	19 414	17 101
Pst I	6 605	1 586	6 605
	10 399		10 496
Kpn I	5 138	6 023	5 138
	11 866	13 391	11 963
Bcl I	17 004	19 414	17 101
Xba I	1 818	1 953	1 818
	15 186		15 283
Sac I	15 775	18 182	15 872
Dra I	4 034	4 435	4 034

Table 8 continued.

	12 970	14 979	13 067
Xmn I/	11 733	4 700	11 830
Asp 700		4 105	
Pvu II	17 004	7 692	17 101
		11 722	
Hind III	9 400	19 414	17 101

3.5.1 Comparison of maps of samples from three different localities.

The repeat lengths of the rDNA of all three samples were found to be in the region of 17 000-19 500 bp (Table 5).

The restriction site maps of the rDNA of the Naukluft and Bloemfontein samples were identical as far as the number, position of restriction sites and the size of the repeat length of the rDNA were concerned. The only difference was that the Naukluft population had an additional Hind III site. The sizes of both the 28S and 18S Eco RI rDNA fragments were similar for both the Bloemfontein and Naukluft populations.

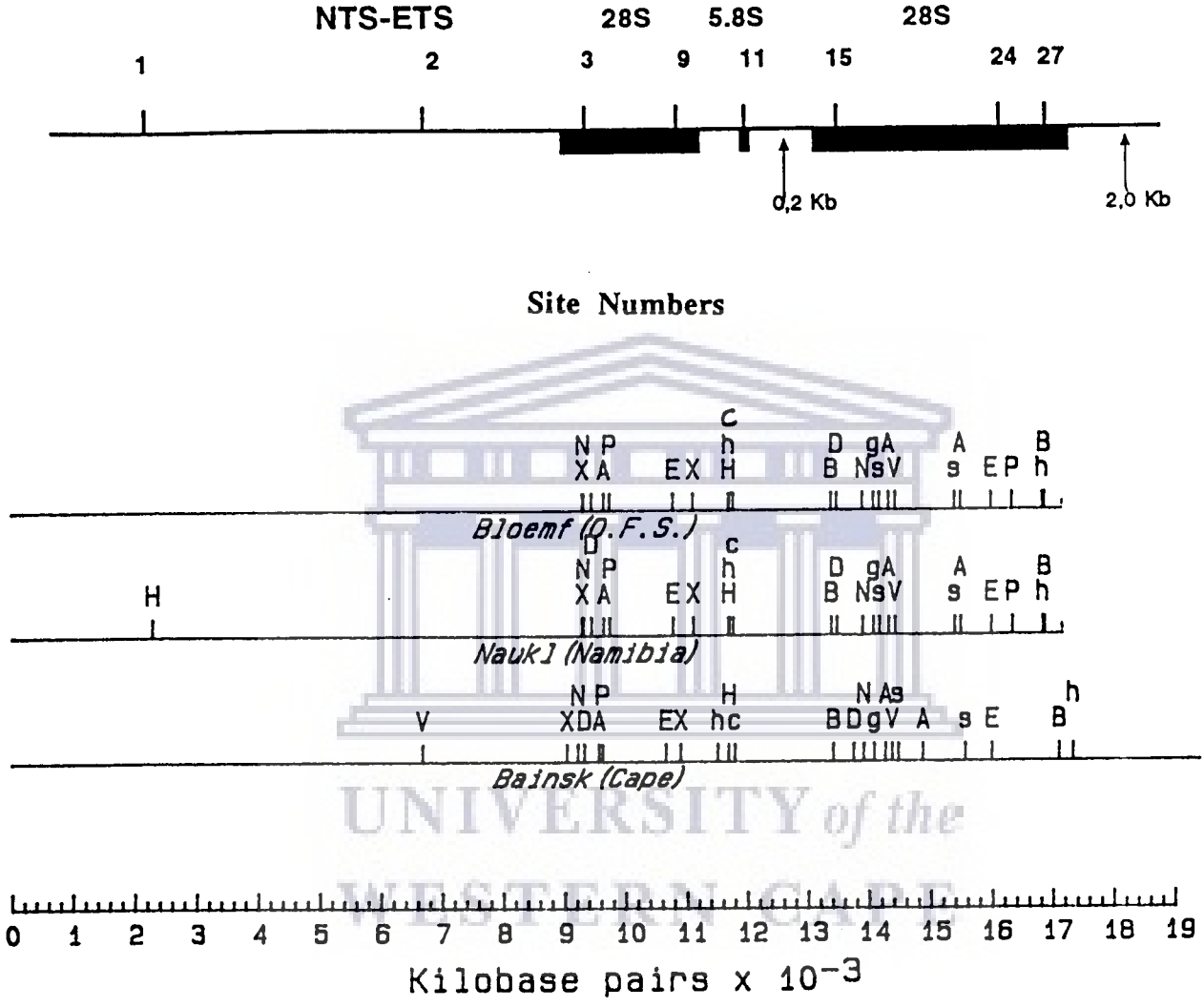

Fig. 9. Restriction site maps of the rDNA of the three different populations were drawn using the raw data. The program, Resolve 3.3 (Harley, 1993) was used. The site numbers correspond to the numbers in Table 9.

Table 9. Presence (+) or absence (-) of restriction sites is shown within the rDNA repeat of the six individuals used in this study. The site number refers to Fig. 10. Samples 974 & 971 are the Naukluft samples, ac959 & ac960 are the Bainskloof samples and h/t71 & h/t75 are the Bloemfontein samples. Twenty eight restriction sites/chararacters (C) were mapped. See abbreviations for enzyme names on page vii. The values +0.2 and +2 Kb indicate the insertions at the appropriate positions.

Enzymes	H	V	X	N	D	A	P	E	X	h	c	+0.2	H	V	B	D	N	g
Sites/C	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Sample																		
974	+	-	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+
971	+	-	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+
ac959	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ac960	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
h/t71	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+
h/t75	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+

Enzyme	s	V	A	s	A	E	P	B	h	+2.0
Sites/C	19	20	21	22	23	24	25	26	27	28
Sample										
974	+	+	-	+	+	+	+	+	+	-
971	+	+	-	+	+	+	+	+	+	-
ac959	+	+	+	+	-	+	-	+	+	+
ac960	+	+	+	+	-	+	-	+	+	+
h/t71	+	+	-	+	+	+	+	+	+	-
h/t75	+	+	-	+	+	+	+	+	+	-

The rDNA arrays of the six individuals were mapped with respect to restriction sites listed in table 9. Twenty six restriction sites were found using 13 different restriction enzymes. Six restriction sites were not present in all the individuals in this study (Fig. 9 and Table 9). Most of the conserved sites were located within the region that codes for 18S, 5.8S or 28S rDNA . Only three variable sites (sites 21, 23 and 25) were located in the 28S region.

Of the remaining three variable sites, two sites were located in the NTS-ETS region (sites 1 and 2) and one site was present in the ITS-2 region (site 14). Two insertions of 0,2 kb and 2,0 kb fragments and the three variable restriction sites were informative about the phylogeny of the three populations since the gene regions of the rDNA are very conserved.

3.6 Sequence Divergence.

In this study the data were manipulated in the following way. The different rDNA size for each locality was used. Using the RESOLVE program, the total number of characters/sites, the proportion of shared restriction sites, the number of conserved sites and the sequence divergence values were determined. The formulae of Nei and Li (1979) enabling one to calculate

sequence divergence were included in the program, RESOLVE 3.3. The sequence divergence data were used to construct a distance matrix for all three localities.

3.6.1 Using the different rDNA array size for each locality.

The restriction site maps (Fig. 9) and the site alignment of the three different localities for individual enzymes were compared (Fig. 10). The informative sites were estimated with 2% error of site matching. The 2% error of site matching is a function of the Resolve program 3.3 (Harley, 1993). The total number of different sites was 33. There were 14 conserved sites (present in the same place in all the populations) and seven shared sites (present in all the populations but in different places). Three sites were not present in all three populations.

The % pairwise sequence divergence with 2% error of site matching was calculated and is shown in Table 10.

Table 10. Measurement of % pairwise sequence divergence using Nei and Li's method (1979) for the three different localities.

	1	2
1. Bloemfontein		
2. Naukluft	0,3	
3. Bainskloof	2,3	2,6

These values indicate that the Naukluft and Bloemfontein populations are relatively closely related. The Bainskloof population is different to the other two populations.

CHAPTER 4

DISCUSSION

The genus *Rana* contains close to 300 species (Frost, 1985), among which the morphology is for the most part conservative. It is an old genus and its numerous species are distributed across the world. My study involved looking at *Rana fuscigula* using the restriction site mapping technique to determine the genetic variation of populations at the edges of the range of the species. The populations in three different localities were investigated: Naukluft (Namibia) in the north-west, Bainskloof in the south and Bloemfontein in the east.

The technique of restriction site mapping was used in this study to compare the genetic variation of the three populations with each other. Restriction site mapping is useful for studying phylogeny of relatively closely related species (Hillis and Davis, 1986). Restriction site maps of individuals are generated using a variety of restriction endonucleases. At least 10 or more are necessary to obtain a useful number of sites. The potential errors of this approach include: (i) Restriction sites are situated very close to each other and fragments of very similar sizes are produced. This can result in the inversion of sites. (ii) The production of fragments of 1 kb or less by restriction cutting which can sometimes run off the gel if the gel is run for a long time. (iii) The restriction

enzymes do not cut the DNA because the DNA is not clean or the restriction enzyme is inactive. These potential errors can be minimised by using many enzymes, using enzymes which are six base cutters, checking the activity of an enzyme before doing a digestion and repeating digestions. The primary limitation of using restriction site mapping is that only about 500 base pairs of 12 000 base pairs of the rDNA repeat unit of *Rana* is examined in this study (Hillis and Davis, 1986). In addition, the only changes that can be detected involve the gain or loss of a recognition sequence at a site and major changes in length of the repeat. Loss of restriction sites can be accomplished by a change at any of the six nucleotides in the recognition sequence, so the acquisition of parallel, non-homologous losses is relatively likely (Hillis and Davis, 1986). However, the extent of homoplasy within the rDNA arrays is limited as a result of their conservative nature (Hillis and Davis, 1986).

Xenopus laevis was used as a control throughout to keep a check on all the practical procedures eg. DNA extraction and restriction site mapping. The map which I obtained for *Xenopus laevis* was very similar to the map of *Xenopus* drawn by Hillis and Davis' (1986). An additional Pst I and Nco I site was found in the NTS-ETS region. The differences in the rDNA maps probably represent normal variation in a widely distributed species.

Two individuals were used from each locality. This sample size might be

This study showed that there is no significant difference between the rDNA length of the Bloemfontein and the Naukluft populations (Table 6). The only difference between the two populations is that the Naukluft population has an extra Hind III site located in the NTS-ETS region.

When comparing the Bainskloof population with the other two populations a number of differences were observed. These variations were: (i) Three variable sites were present in the 28S gene region. These sites were sites 21, 23 and 25 (Table 9). (ii) Three variable sites were present in the non-coding regions (sites 1, 2 and 14). (iii) Two insertions or deletions of approximately 2 kb and 200 bp were found in the NTS-ETS and ITS region respectively. Restriction sites in the coding regions e.g. 18S, 28S and 5.8S genes are very conserved (Hillis and Davis, 1986). The sites in the conserved regions of the rDNA repeat unit were therefore not very useful when one wanted to make inferences about the phylogeny of organisms. Using the three restriction site maps in Fig. 9, Table 9 was drawn up. Table 9 showed the absence and presence of restriction sites and where they occurred in the rDNA repeat. Of a total of 26 sites, 22 sites (sites 3 to 9 in the 18S gene region, sites 10 and 11 in the 5.8S gene region and sites 15 to 27 in the 28S gene region) were present in the coding regions of all three populations. Only three sites out of the 22 sites were variable. These were sites 21, 23 and 25. This was only 13,6% of the sites in the coding region. Hillis and Davis (1986) found that only 7,4% of the variable sites occurred in the

the 5.8S gene regions of the rDNA of the three populations were conserved. Hillis and Davis (1986) have previously shown similar findings in their work with 32 different ranid species. The change in the size of rDNA is due to either deletions or insertions in the ITS and the NTS regions of the rDNA repeat.

The similarity between the Naukluft and Bloemfontein populations could be due to the fact that the Orange and Fish rivers are in the same river drainage system (the Orange river draining westward) and since frogs can move along the river courses, gene flow would be expected. Bainskloof is on a different drainage system separated by the dry Karoo. If there is no frog movement between the river systems then one would not expect gene flow. Gene differences will accumulate in these separate populations.

4.2 Genetic distance as an indicator of species status.

There are many conflicting viewpoints as to whether genetic distances/sequence divergence calculated from molecular data should be used as the only indicator to decide whether or not an organism is of a different species (Crowe, 1988). Although this debate is not yet resolved, I regard populations with significant genetic differences as representing different gene pools, and hence different species. The formulae of Nei and Li (1979) were used to convert restriction data to genetic distance values so that a decision on the species status of the

Hillis and Davis (1986) found rDNA sequence divergence values as low as 1,3% between *Rana palmipes* and *Rana sierramadrensis* and a value of 4,4% between *Rana palmipes* and *Rana sylvatica.* rDNA sequence divergence of 3 - 12,8% was recorded between different species of rodents in the genus *Onychomys* (Allard and Honeycutt, 1991).

Sequence divergence values for mitochondrial DNA have been determined for a number of organisms including frogs. Many of these studies cast doubt on the subspecific status of some populations. Carr *et al.* (1987) recorded mitochondrial sequence divergence values of 3 - 7% for subspecies of *Xenopus laevis* and 1 - 39% for different species of *Xenopus*. Honeycutt *et al.* (1987) found that mtDNA sequence divergence values for three subspecies of *Cryptomys hottentotus* were higher than the values recorded for separate species in this genus. An average sequence divergence value of 15,8% was recorded for the subspecies and an average value of 7,5% for separate species in this genus. George and Ryder (1986) used restriction site mapping of the mtDNA for different species of the genus *Equus* and recorded sequence divergence values that ranged between 0 - 7,8%. Sequence divergence values of subspecies of Canada geese of 0,04 - 2,54% and 8% for two separate species were recorded (Van Wagner and Baker, 1990). A comparison of sequence divergence values between two species of rhinoceroses was estimated to be 6,79% (O'Ryan and Harley, 1993). The high sequence divergence values for

mtDNA can be explained by the observation of Brown *et al*. (1979) that mtDNA has a higher sequence divergence rate than nuclear DNA.

The low rDNA sequence divergence value of 0,3% between the Naukluft and the Bloemfontein populations indicate that these frogs belong to the same species. I, therefore support the current view that the Naukluft and the Bloemfontein population are conspecific. However, the DNA sequence divergence values of 2,3% for the Bloemfontein and Bainskloof populations and 2,6% for the Naukluft and the Bainskloof population suggest that the Bainskloof population belongs to a different species. Since this study has only examined the populations in the mountainous regions at Bainskloof I suggest that the populations in the low lying areas be investigated before a definite conclusion is drawn on the species status of the Bainskloof population.

sequence divergence values as low as 1,3% between *Rana palmipes* and *Rana sierramadrensis* and a value of 4,4% between *Rana palmipes* and *Rana sylvatica.*

My findings support the current conspecific status of the Naukluft and the Bloemfontein populations. The high sequence divergence value of 2,3% and 2,6% suggests that the frogs from the Bainskloof population belongs to a different species. Since this study has only examined the populations in the mountainous areas at Bainksloof I suggest that the populations in the low lying areas be investigated.

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APPENDIX I

A. DNA EXTRACTION METHODS

METHOD 1 (PHENOL METHOD) (Mayfield, 1984)

MATERIALS:

1. CELL LYSIS BUFFER

320 mM sucrose	10,95 g/100 ml
1% (v/v) triton X-100	1 ml/100 ml
5 mM $MgCl_2$	0,102 g/100 ml
10 mM tris-HCl pH 7,6	0,121 g/100 ml

Dissolve the sucrose, $MgCl_2$ and tris-HCl in 80 ml water. pH the solution to 7,6. Add triton X-100. Check the pH. Make the solution up to volume. Sterilize by autoclaving. Store in the freezer.

2. SALINE EDTA

25 Mm EDTA	0,9306 g/100 ml
75 Mm NaCl pH8,0	0,438 g/100 ml

Dissolve the EDTA and NaCl in 80 ml water. Increase the pH by using 4 M NaOH. Sterilize by autoclaving.

3. SDS 10% (w/v)

10 g SDS in 100 ml sterile water.

4. PROTEINASE K (10 mg/ml)

Dissolve 1 mg proteinase K in 0,1 ml sterile water. Make only when needed.

with loose pestle (carefully 4 strokes up and down). Pellet nuclei by spinning at 5 000 rpm for 20 minutes.at 4°C.

3. Add 8 ml saline EDTA to nuclei pellet and add 0,8 ml 10% SDS. Vortex briefly for approximately 15 seconds on Whirli mix.

4. Add 50 ul proteinase K and incubate at 37°C overnight (gooey and semi-transparent).

5. Add 0,5 ml 5 M Na perchlorate.(1/10 total volume).

6. Add equal volume of phenol. Mix gently. Separate phase by centrifugation at 10 000 rpm for 5 minutes at 10°C. Remove aqueous phase (top phase) with wide bore pipette. Repeat phenol extractions 3 times.

7. Extract top phase with $CHCl_3$:isoamyl alcohol. Mix gently and separate as in step 6. Remove and keep top phase.

8. Precipitate DNA from aqueous phase by adding 2 volumes of cold absolute EtOH. Lift out DNA with sealed end of pasteur-pipette and place in microfuge tube. Spin pellet to get off excess EtOH, suck off excess EtOH.

9. Shake into 1 ml of 1 X TE buffer. Dissolve overnight at 4°C with gentle mixing.

METHOD 2 (PHENOL METHOD) (Hillis and Moritz, 1990)

MATERIALS:

1. STE

   0,1 M NaCl

   0,05 M tris-HCl pH 7,5

   0.001 M EDTA

Autoclave.

2. 20% SDS

20 g SDS in 100 ml sterile water.

3. PHENOL/ CHLOROFORM/ ISOAMYL ALCOHOL(25:24:1)

Mix well.

5. CHLOROFORM:ISOAMYL ALCOHOL (24:1)

96 ml chloroform

4 ml isoamyl alcohol

Shake up well. Allow solution to stand overnight.

6. Proteinase K

Dissolve 1 mg proteinase K in 0,1 ml sterile water. Make up freshly for use.

7. 2 M NaCl

8. 70% EtOH and 95% EtOH

9. 10 x TE

100 mM tris-HCl, pH7,5	1,21 g/100 ml
10 mM EDTA	0,372 g/100 ml

Dissolve in 80 ml of H_20 and pH to 7,5. Make up to volume. Sterilize.

METHOD:

1. Weigh 100 mg of sample per tube. Grind tissue sample with a pestle and mortar using liquid N_2. Add 500 ul of STE.
2. Add 25 ul of a 10 mg/ml stock proteinase K. Mix well.
3. Add 25 ul 20% SDS. Mix gently.
4. Incubate for 2 hours at 55°C. Mix occasionally to keep tissue

suspended.

5. Add 500 ul PCI, mix gently at room temperature for 5 mins.

6. Centrifuge at 7 000 g for 5 mins.

7. Carefully remove aqueous (top) layer with micropipette and a wide bore tip and transfer to a clean tube.

8. Re-extract the aqueous phase again with PCI. Repeat steps 5-7.

9. Add equal volume of CI, mix gently, and incubate at room temperature for 5 mins. Centrifuge for 3 mins at 7 000 g.

10. Carefully remove aqueous (top) layer with micropipette and wide bore tip and transfer to a clean tube. Do not disturb the interface.

11. Re-extract the aqueous phase with CI. Repeat steps 9-10.

12. Add 45 ul of 2 M NaCl and 1 ml cold absolute EtOH (-20°C) to precipitate the DNA. Incubate on ice for 10-20 mins.

13. Spin down the DNA precipitate for 1 min at 7 000 g.

14. Wash with 70% EtOH (-20°C). Repeat this step.

15. Wash with 95%, decant and dry DNA.

16. Dissolve DNA in 50-100 ul 1xTE.

17. Leave at 4°C overnight. Freeze away.

METHOD 3 (SALTING OUT METHOD)(Miller *et al.*, 1988)

MATERIALS:

1. CELL LYSIS BUFFER

320 mM sucrose	10,95 g/100 ml
1% (v/v) triton X-100	1 ml/100 ml
5 mM $MgCl_2$	0,102 g/100 ml
10 mM tris-HCl, pH 7,6	0,121 g/100 ml

Dissolve sucrose,$MgCl_2$, and tris-HCl in 80ml water. pH solution to 7,6. Add triton X-100. Check pH. Make up to volume. Sterilize by autoclaving. Store in

freezer.

2. NUCLEI LYSIS BUFFER

10 mM tris-HCl

400 mM NaCl

2 mM Na_2EDTA, pH 8,2

3. 10% SDS

4. PROTEINASE K (10 mg/ml)

5. 6 M NaCl

6. EtOH at room temperature

METHOD:

1. 0,5-1 g tissue ground with liquid N_2 in mortar and pestle.
2. Add 60 ml cell lysis buffer. Homogenise in loose-fitting Potter.
3. Centrifuge at 4 000 rpm for 20 minutes at 4°C.
4. Add 3 ml nuclei lysis buffer to nuclei pellet.

Mix well.

5. Add 0,2 ml 10% SDS.
6. Add 50 ul proteinase K incubate at 37°C overnight.
7. Add 1 ml 6 M NaCl. Shake vigorously for 15 seconds.
8. Centrifuge at 8 000 rpm for 15 minutes.
9. Discard the protein pellet. To the supernatant add 2 volumes of room temperature EtOH.
10. Remove strands with sterile sealed pipette ends.
11. Dry DNA. Add 200 ul sterile water and mix overnight.

12. Read concentration.

METHOD 4: (PHENOL METHOD) (Ausubel *et al.*, 1989)

MATERIALS:

1. DIGESTION BUFFER pH 8.0

100 mM NaCl	0,2922 g/50 ml
10 mM tris	0,061 g/50 ml
25 mM EDTA	0,465 g/50 ml
0,5% SDS	0,25 g/50 ml
0,1 mg Proteinase K	5,0 mg/50 ml

2. PHENOL/ CHLOROFORM/ ISOAMYL ALCOHOL (25:24:1)

Mix well.

3. 7,5 M NH_4Ac

4. 70% and ABSOLUTE EtOH AT -20°C

5. 10 X T.E. Buffer

100 mM tris-HCl, pH7,5	1,21 g/100 ml
10 mM EDTA	0,372 g/100 ml

Dissolve in 80 ml. pH to 7,5. Make up to volume. Sterilize.

METHOD:

1. Weigh frog skeletal muscle and record the weight (0,5 -1,0 g suggested).
2. Transfer muscle tissue to mortar and pestle.

3. Add liquid N_2 and pulverise while continuously adding N_2.

4. Transfer powdered tissue to tube and keep on dry ice.

5. Suspend 100 mg of powdered tissue in 1,2 ml of digestion buffer.

6. Incubate with shaking at 50°C for 12 - 18 hours in tightly capped tubes.

7. Extract samples with equal volume of phenol/chloroform/isoamyl alcohol::25/24/1. Centrifuge for 10 min. at 10 000 rpm. Repeat this extraction 3 times.

8. Extract with chloroform/isoamyl alcohol::24/1 once.

9. Transfer the top aqueous phase and add 1/2 volume of 7.5 M NH_4Ac.

10. Add 2 volumes 100% EtOH.

11. Recover DNA by centrifugation at 10 000 rpm for 5 mins.

12. Decant EtOH. Rinse pellet with 70% EtOH and air dry.

13. Resuspend DNA in 1xTE. Shake at room temperature or 65°C or DNA can be stored indefinitely at -70°C.

RE-EXTRACTION METHOD FOR DNA USING SALT METHOD

If the DNA pellet from the EtOH precipitation does not look clean (white), it can be cleaned in the following manner.

REAGENTS:

1. 5 M SODIUM PERCHLORATE

   35,12 g in 50 ml water.

Sterilise by autoclaving. Store at 4°C.

2. NUCLEI LYSIS BUFFER

   10 mM tris-HCl

400 mM NaCl

2 mM Na_2EDTA, pH 8,2

3. 10% SDS

4. PROTEINASE K (10 mg/ml)

5. 6 M NaCl

6. Cold Absolute EtOH

METHOD:

1. Dissolve DNA in 3 ml water.
2. Add 1/10th volume 5 M Na perchlorate.
3. Add ice cold EtOH. Leave in freezer to precipitate (15 min).
4. Spin down DNA at 9 000 rpm for 1 minute.
5. Dry pellet.
6. Dissolve pellet in 3 ml nuclei lysis buffer.
7. Add 0,2 ml 10% SDS.
8. Add 50 ul proteinase K (10 mg/ml).
9. Incubate at 37°C overnight.
10. Add 1 ml 6 M NaCl. Shake vigorously for 15 seconds.
11. Centrifuge at 8 000 rpm for 15 minutes.
12. Discard the protein pellet. To the supernatant add 2 volumes of room temperature EtOH.
13. Spin at 9 000 rpm for 1 minute.
14. Dry DNA. Add 200 ul sterile water and mix overnight.
15. Read concentration.

b. DOUBLE DIGESTS

Voodoo Buffer

Buffer A

0,1 M Dithiothreitol

Buffer B

1% Digitonin

Buffer C

0,2 M Tris pH 7,5

0,7 M Nacl

0,2 M KCl

0,1 M Mg Cl_2

0,5 mM Spermine

0,125 mM Spermidine

1:1000 dilution of Aprotinin

Voodoo buffer = 5 ul of buffer A + 5 ul of buffer B + 5 ul of buffer C

Method:

Add 1 ug DNA to 15 ul of voodoo buffer. Add sterile water to make up the volume. Add 1 ul of each restriction enzyme (10 U/ ul). Total volume 50 ul Incubate at 37°C for 2 hours. Add another 1 ul of each enzyme and incubate for another 4 hours. Add 5 ul loading/stop buffer and mix. Freeze. Run 6 ul (0,1 ug) of incubate on minigel to check if enzymes has cut the DNA. Always run 0,1 ug of uncut DNA alongside digests.

D. Lambda Hind III marker

1.	Lambda DNA (0,25 ug/ul)	200 ul
2.	Hind III (10 U/ul)	15 ul
3.	Boehringer buffer B	25 ul
4.	Sterile water	10 ul
	Total volume	250 ul

Incubate at 37°C for 2 hours. Run 0,1 ug of cut lambda on a minigel. If the DNA is not properly cut, add an extra 5 ul of Hind III enzyme. Incubate for another 1 hour at 37°C. Add 50 ul of loading buffer.

E. LABELLING OF THE PROBE (18S OR 28S rDNA) USING NICK TRANSLATION METHOD

Add the following components to a sterile eppendorf tube:

(i) 2 ul (2 ug) of denatured probe (heated for 10 min at 95°C and quickly cooled on ice)

(ii) 1 ul dATP
1 ul dGTP
1 ul dTTP

(iii) 2 ul reaction mixture (solution 6)

(iv) 5 ul (50 µCi) [α-^{32}P]dCTP

(v) 7 ul sterile H_2O

Spin down components for 1 minute.

(vi) 1 ul enzyme mixture (solution 7)

Incubate at 15°C for 35 minutes. Stop the reaction by heating to 65°C for 10 minutes.

(vii) Remove unincorporated label in the following way:

In a 1,0 ml syringe , pack a Sephadex G-50/TE column. Place a plug of glasswool in the tip of the syringe, up to 0,05 ml mark. Using a pasteur-pipette, take from the bottom slurry of G-50 and add to the column. Centrifuge at 3 000 rpm for 3 min. using a decapitated microfuge tube as a drain. Repeat the above 2 steps till the syringe is nearly full. Add 100 ul 1 x TE and centrifuge as before. Repeat until 100 ul eluant is collected. Add 90 ul 1 x TE. Mix and add to column. Centrifuge as before, collecting fractions in a clean decapitated tube. Boil cleaned up probe for 10 min. and snapcool. Open up the hybridization chamber and add the labelled probe to the hybridization mixture. Hybridize the filters with the probe overnight.

F. SOUTHERN BLOTTING, HYBRIDIZATION AND WASHES

Blotting and Hybridization Solutions

1. 20 x SSC

   3 M NaCl

   0,3 M Citrate

   175,3 g NaCl/litre

   88,2 g $Na_3Citrate.2H_2O$/litre

   pH to 7 with HCl

2. Denaturing Solution

   0,5 M NaOH

   1,5 M NaCl

Mix 300 ml 5 M NaCl/litre with 100 ml 5 M NaOH/litre. Add 600 ml H_2O.

3. Neutralizing Solution

0,5 M Tris

1,5 M NaCl

87,66 g NaCl/litre

60,57 g Tris/litre

pH to 5,5 with HCl (approx. 100 ml conc HCl)

4. Hybridization Solution

5 x SSC

O,5% SDS

5 x Denhardt's (DEN)

25 mM KPO4

For 100 ml

20 x SSC	25 ml
20% SDS	2,5 ml
100 x DEN	5,0 ml
0,5 M KPO4 , pH 6,5	5,0 ml
Sterile H_2O	62,5 ml

100 x Denhardt's Solution

2% Ficoll 400`

2% PVP-360 (polyvinyl pyrrolidone)

2% Bovine Serum Albumin

Dissolve in sterile H_2O.

PROCEDURE FOR SOUTHERN BLOTTING

1. Shake gel for 1/2 hr - 1 hr in 400 ml denaturing solution at room temperature.
2. Rinse in deionized water.
3. Shake gel for 1/2 hr in 500 ml neutralizing solution at room temperature.
4. Soak nylon and 2 pieces 3 MM Whatmann paper (same size as gel) in 2 x SSC.
5. Soak 3 MM Whatmann paper wick (larger than gel) in 20 x SSC.
6. Layer on glass plate in the following order:
   - Wick
   - gel
   - nylon
   - 1st piece of Whatmann paper (same size as gel)
   - Klingwrap on sides of Whatmann paper
   - 2nd piece of Whatmann (same size as gel)
   - Box of tissues
   - Centre glass plate over tissues
   - Weight (500 ml bottle half filled)
7. Blot overnight.
8. Mark wells onto nylon filter and label.
9. Place the filter between 2 pieces of 3 MM Whatmann paper and bake at 80°C for 1 hour.
10. Store in fridge at 4°C.

HYBRIDIZATION OF SOUTHERN BLOTS

1. Prehybridize baked filters in 15 ml of hybridization solution in hybridization box at 65°C for +2 hours.
2. Remove prehybridized filters and add them to the box containing the probe.
3. Hybridize at 65°C in a waterbath for 12-18 hours (overnight).
4. Wash filters as follows :

Solution I	Solution II
100 ml 20 x SSC	50 ml 20 x SSC
10 ml 20% SDS	5 ml 20% SDS
to 1 litre water	to 1 litre water

a) Place filters in a small tupperware with lid.

b) Soak for 15 min in 200 ml Solution I behind plexiglass screen. Wash will be very hot.

c) Repeat step (b).

d) Rinse with 100 ml Solution II.

e) Soak with shaking in 200 ml Solution II at 37°C for one hour.

f) Repeat step (e).

g) Rinse filters with deionized water, dry on paper towels and expose to x-ray film with double intensifying screens at -80°C.

Note : Do not allow filters to dry out totally if they have to be reused.

APPENDIX 2

Resolve Version 3.3, Harley- Unpublished

Resolve, a computer program which allows easy handling and manipulation of restriction mapping data was originally written for the Hewlett-Packard model 86 but has subsequently been re-written in True Basic, in an IBM compatible format.

The program offers three main functions :

(i) Construction of restriction maps of DNA fom single and double digestion data.

(ii) The management of sets of mapped data from different DNA's after catalogueing, editing and manipulating, where necessary.

(iii) The comparison of maps from related taxa and the construction of sequence divergence tables and phylogenetically informative sites.

RAW DATA

Sequence of Restriction Sites for figures 10 and 11.

Sequence of restriction sites for Bloemfontein (O.F.S.)

1.	X 9288	2.	N 9304	3.	D 9437	4.	A 9644
5.	P 9736	6.	E 10774	7.	X 11106	8.	H 1160
9.	h 11701	10.	c 11764	11.	B 13387	12.	D 13471
13	N 13903	14.	g 14078	15.	s 14168	16.	A 14317
17.	V 14417	18.	s 15397	19.	A 15488	20.	E 16000
21.	P 16341	22.	h 16839	23.	B 16863		

Sequence of restriction sites for Naukluft (Namibia)

1.	H 2290	2.	X 9288	3.	N 9304	4.	D 9437
5.	A 9644	6.	P 9736	7.	E 10774	8.	X 11106
9.	H 11690	10.	h 11701	11.	c 11764	12.	B 13387
13	D 13471	14.	N 13903	15.	g 14078	16.	s 14168
17.	A 14317	18.	V 14417	19.	s 15397	20.	A 15488
21.	E 16000	22.	P 16341	23.	h 16839	24.	B 16863

Sequence of restriction sites for Bainskloof (Cape)

1.	V 6669	2.	X 9035	3.	N 9212	4.	D 9314
5.	A 9561	6.	P 9613	7.	E 10651	8.	X 10895
9.	h 11505	10.	H 11690	11.	c 11786	12.	B 13412
13	D 13749	14.	N 13906	15.	g 14078	16.	A 14261
17.	V 14361	18.	s 14463	19.	A 14870	20.	s 15566
21.	E 16000	22.	B 17104	23.	h 17334