GENETIC VARIATION BETWEEN TWO SUBSPECIES OF REEDFROGS IN THE GENUS *Hyperolius* (ANURA: Hyperoliidae)

by

Anthony Jacobus Hess

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

Promoter: Dr. Denver Hendricks

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DEDICATION

This thesis is dedicated to my mother Joybell, my father Henry, my brothers Timothy, LLowellyn, and Benjamin, my sister Gina and my grandmother Alice Kellerman.

DECLARATION

I, _____, declare that **"Genetic variation between two subspecies of reedfrogs in the genus *Hyperolius* (Anura: Hyperoliidae)"** 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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My thanks and appreciation go to Beulah Hendricks and Tasneem Mohamed for assistance in the formatting and printing of this thesis, respectively. Also to Rosemary for her encouragement. Finally, to the Almighty God for giving me the strength and perseverance to complete this study.

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CHAPTER 1

INTRODUCTION

The frog genus *Hyperolius* is the largest of 19 genera in the Hyperoliidae (Channing, 1989). The members of the genus display considerable morphological homogeneity with a diversity of dorsal colour patterns. The genus is endemic to Africa and is distributed throughout the wetlands south of the Sahara excluding the western plateau slopes and South African plateau. They are found in close proximity to water, on reeds, sedges and also on water-lily leaves (Lambiris, 1989). The South African forms are commonly referred to as reedfrogs (Passmore and Carruthers, 1979). The members of the genus bury themselves in the ground and under leaves during the dry season and emerge after the first rains (Lambiris, 1989). The eggs are laid in water, attached to waterplants, but certain species deposit eggs above the water level or between water-lily leaves (Lambiris, 1989). Characters that distinguish the genus from other African genera are as follows: The pupil is horizontal to round; Vomerine teeth are absent; The fingers and toes are webbed; The oral disc is ventrally situated (Passmore and Carruthers, 1979).

Taxonomy of *Hyperolius marmoratus*

The genus contains more than 120 species which show great variation in dorsal colour and colour pattern. This variation has been used as an important

taxonomic character in the classification of the various species. However, many species show polychromatism as well as sexual dichromatism and the colour and patterns of juveniles often differ from adults of both sexes (Poynton, 1964). This has led to conflicting opinions on the taxonomy of the genus with researchers giving different names to the same populations. These problems are especially prominent within the *Hyperolius marmoratus* complex. The frogs that belong to this complex are morphologically very similar and are currently classified under the superspecies *H. marmoratus* (Frost, 1985). The forms within this superspecies share certain morphological characteristics that distinguish them from other members of the large genus (Schiøtz, 1971). The superspecies was earlier classified under the name *H. viridiflavus* (Laurent, 1951) and later as *H. marmoratus* (Poynton, 1964). Laurent (1951) divided the superspecies into the species *H. marmoratus, H. tuberculatus* and *H. viridiflavus*, whereas Schiøtz (1971), classified all the *H. marmoratus* forms as *H. viridiflavus* and also recognized two additional species; *H. parallelus* and *H. marginatus*. There is still no consensus about the subdivision of the superspecies *H. marmoratus*.

This study is primarily concerned with a group of frogs, within the species *Hyperolius marmoratus* (Poynton, 1964), which occurs along the east coast of southern Africa. According to Poynton, (1964), this group "lies in a very tightly knit gradient" stretching from the south of Malawi down to the Tsitsikama area in South Africa. He divided the group into the following subspecies: *H. m. taeniatus, H. m. broadleyi, H. m. swynnertoni, H. m. marginatus, H. m. marmoratus* and *H. m. verrucosus*. Poynton and Broadley (1987) recorded

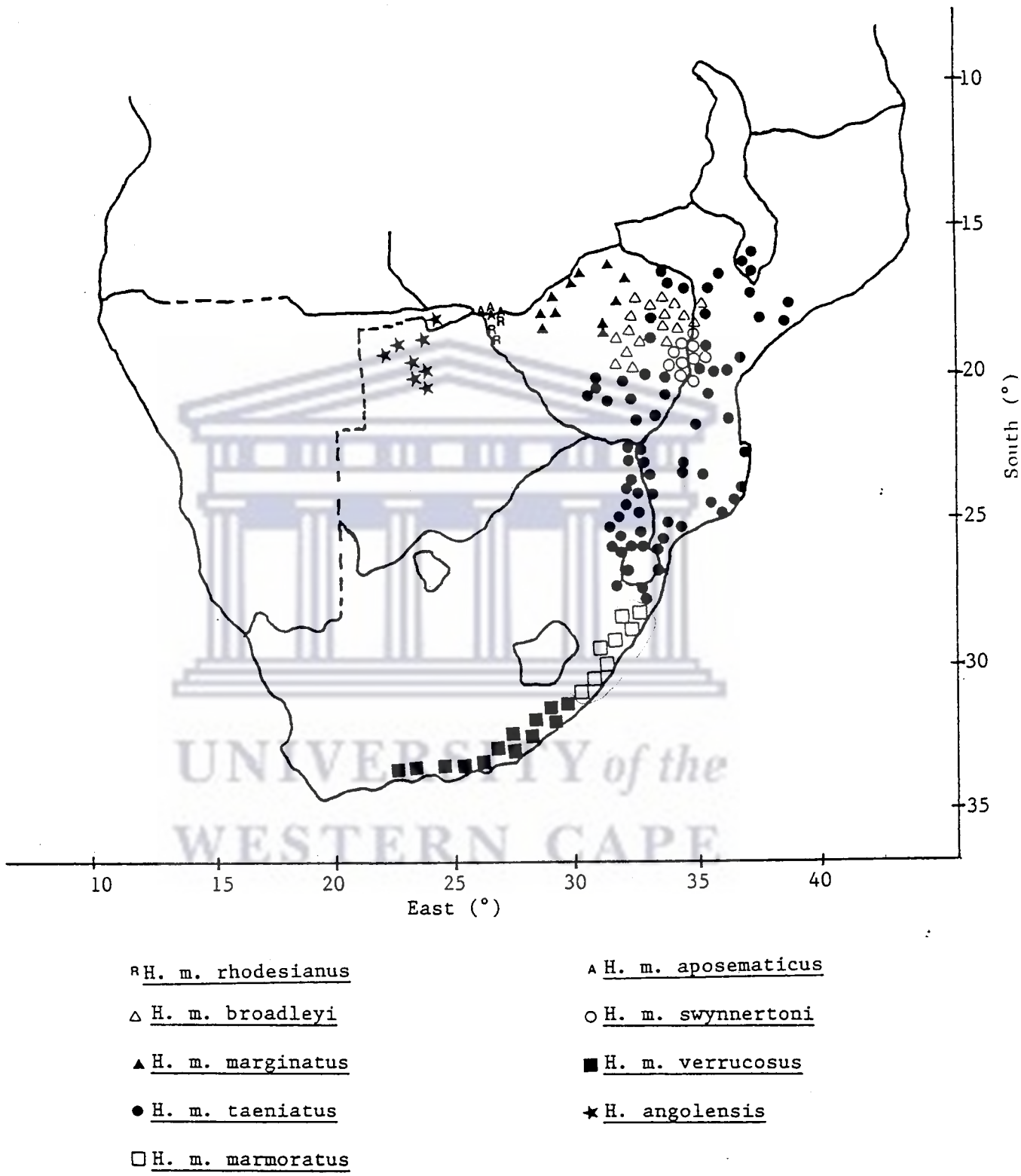

Figure 1. Map of the distribution of the subspecies of *Hyperolius marmoratus* which occur along the east coast of Southern Africa (Poynton, 1964; Poynton and Broadley, 1991). Also included is the distribution of the reference group, *H. angolensis*.

another two subspecies in southern Africa, called *H. m. rhodesianus and H. m. aposematicus*. These two subspecies however, do not form part of the gradient along the east coast. Figure 1 gives the distribution of the subspecies. Variation in dorsal colours and patterns occurs between, as well as within the various groups, but over-all patterns correlate to some extent with geographic distribution (Passmore and Carruthers, 1979). An overlap occurs at the interfaces of distribution ranges of the various subspecies with suggestions of interbreeding at these zones (Poynton, 1985).

The demarcation of the southern African *Hyperolius marmoratus* species into subspecies, by Poynton (1964) was not accepted by Schiøtz (1975), nor by Laurent (1976). Schiøtz (1975) divided the group into the species *H. parallelus, H. marginatus and H. verrucosus* with *H. m. taeniatus* being classified a subspecies of *H. parallelus*. Laurent (1976), on the other hand divided, the group into the species *H. marginatus* and *H. marmoratus*. The species were further divided into subspecies by both authors. Classification of species and subspecies was based on dorsal colour and colour pattern. A problem that contributes to the different classifications is sexual dichromatism and also the colour of the juveniles that differs from that of the adults.

The description and geographical distribution of the subspecies gradient of *H. marmoratus* are as follows: *H. m. marmoratus* has a characteristic mottled pattern and occurs within a region stretching from Transkei to about St Lucia. Uniformity in colour is absent but they all have a tendency towards longitudinal

[GURE 2. Photographs indicating the colour patterns of five subspecies E *Hyperolius marmoratus* that occur in the gradient along the east coast of outhern Africa. Also included is a photograph of the reference group, *Hyperolius ngolensis*.

Key

A = *Hyperolius marmoratus verrucosus*

B = *Hyperolius angolensis*

C = *Hyperolius marmoratus marmoratus*

D = *Hyperolius marmoratus taeniatus*

E = *Hyperolius marmoratus swynertoni*

F = *Hyperolius marmoratus broadleyi*

hotographs: courtesy of Prof. Alan Channing, Department of Biochemistry, niversity of the Western Cape, Private Bag X17, Bellville, 7535)

striping (Passmore and Carruthers, 1979). Photographs indicating the colour patterns of the subspecies are presented in Figure 2. *H. m. verrucosus* is a spotted reedfrog with a distribution range that stretches from the Tsitsikamma area in Knysna to Transkei.

A brown colour is characteristic with small pale, black-edged spots. *H. m. taeniatus* is a striped frog, lacking colour uniformity. They are distributed northwards of St Lucia through Natal and the eastern Transvaal. Their adult pattern consists of four longitudinal black stripes alternating with three white stripes, of equal width and thin yellow, orange or orange-red lines usually contained within the white lines (Lambiris, 1989). Interbreeding in the overlap zone between *H. m. taeniatus* and *H. m. verrucosus* had been suggested by Poynton and Broadley (1987). *H. m. broadleyi*, commonly known as Broadley's reedfrog is distributed over the eastern and central highveld of Zimbabwe. Dorsal colours and patterns are variable within this group but they are commonly black dorsally with three longitudinal white stripes, each containing a thin red line. Poynton (1985), reported interbreeding between individuals of *H. m. broadleyi* and *H. m. taeniatus* which occur in the overlap zones of eastern Zimbabwe and adjoining Mozambique respectively. *H. m. swynnertoni*, also known as the speckled reedfrog, is characterized by dark speckles on a white and sometimes yellow background with a red mark on the abdomen, but colours may vary within the group. Their distribution is in the same part of the country as that of *H. m. broadleyi* with consequent intergradation (Poynton, 1985).

The subspecies classification of *H. marmoratus* was based on inference as to whether neighbouring taxa interbreed (Poynton, 1964). Suggestions of interbreeding between the *H. marmoratus* groups played a significant role in the subspecies classification of these populations. Poynton and Broadley (1987), however, noted that the delimitation of nearly all subspecies of *H. marmoratus* is still largely a matter that remains open to question. The variation in colour pattern present within *H. marmoratus* does not necessarily represent other genetic differences. Individuals from different populations might therefore interbreed freely if they were not geographically separated. On the other hand, the differences in colour pattern might be an indication that the different populations are genetically so distinct that species status could be assigned to the current subspecies of *H. marmoratus*.

The aim of this study was to determine if: (1) any, and to what extent, genetic differences exist between the subspecies of *H. marmoratus; (2)* the genetic information corresponds to the colour pattern exhibited by the subspecies.

Two subspecies were selected for examining genetic variation; *H. m. broadleyi* and *H. m. verrucosus*. The technique employed to answer these questions was restriction site mapping of ribosomal DNA (rDNA). This technique has been applied in systematics (Allard and Honeycutt, 1991; Hillis and Davis, 1986). The principle and value of the technique is discussed in Chapter 2. The reason for selecting the subspecies *H. m. broadleyi* and *H. m. verrucosus* is that they occur at the extreme ends of the distribution range of the group. *H. m. broadleyi* is

located in the eastern Zimbabwean highveld whereas *H. m. verrucosus* occurs from the Tsitsikamma in South Africa to the Transkei (Figure 1). They exhibit distinct differences in dorsal colour patterns although slight variation from the common colour patterns occurs among individuals within the subspecies. To investigate this variation samples from three different localities within the distribution range of *H. m. verrucosus* were collected to determine the level of intraspecific genetic differences in the rDNA restriction sites. Individuals from *H. angolensis* were included in the study as a reference group. These samples were obtained from Shakawe in Botswana. A map of the collection localities are given in Figure 5.

Status of *H. angolensis*

H. angolensis was initially classified as a separate species of *Hyperolius* (Poynton, 1964), but Poynton and Broadley (1987), referred to it as a subspecies of *H. marmoratus*. Channing and Griffin (1993), favoured specific status for *H. angolensis* (including *H. m. aposematicus*) based on the difference in their mating calls from that of *H. marmoratus*.

CHAPTER 2

MATERIALS AND METHODS

Introduction

Until 1960, systematics was based largely on analysis of morphological and behavioural variation. However, with an increased understanding of bio-molecules researchers have begun to look for variation at the molecular level of organisms. Proteins were the first molecules to be studied for variation between and among species in the 1960's (Hubby and Throckmorton, 1965). Major advances in the manipulation and analysis of nucleic acids (in the past decade) have led to the widespread study of DNA and RNA variation. Approaches used include hybridization and dissociation of DNA, nucleic acid sequencing and restriction endonuclease site mapping of nuclear, mitochondrial and chloroplast DNA (Hillis and Moritz, 1990).

Restriction site mapping

General principle

This approach involves the determination of the localities of various restriction sites through the digestion of DNA with restriction endonucleases. A restriction endonuclease is an enzyme that cleaves DNA at very specific localities which correspond to the recognition sequence, normally 4-6 base pairs long, of the specific enzyme. Restriction enzymes are isolated from different bacteria

(Roberts, 1984) and their natural function is to protect the bacteria from invasion of foreign DNA. Complete digestion of DNA with restriction enzymes will result in a reproducible fragment pattern. The size of these fragments is determined and a map of the relative positions of the various restriction sites can be compiled by analyzing the different fragment lengths. The aim of the technique is to compare the restriction site maps for the different taxa to determine the presence or absence of restriction sites. Identical sites are regarded as being conserved whereas the number of sites that are different is an indication of the evolutionary distance between the taxa. The evolutionary distance is expressed as the sequence divergence between the taxa (Nei and Li, 1979). Formulae for the calculation of sequence divergence are given in Chapter 3.

The probability for the loss of a site is far greater than a gain of a site (Debry and Slade, 1985). Let AAATTC represent a recognition sequence of a restriction enzyme. It requires any point mutation at any one of the six bases in order to lose the site. However, to gain a site eg. GAATTC from the former sequence requires a specific base substitution at a particular base pair. A loss of a site can be achieved by a point mutation (ie., base substitution or deletion) or by sequence rearrangement, within a cleavage site. The search for presence or absence of restriction sites is not directed at the entire genome, but is limited to certain parts, eg. genes or DNA from the mitochondrial and chloroplast organelles. Genes that have been studied previously include: alcohol dehydrogenase (Bishop and Hunt, 1988; Langley et al., 1981), nuclear ribosomal DNA (Appels and Dvorák, 1982; Arnold et al., 1987; Hillis and Davis, 1986; Templeton, 1983) and mitochondrial

ribosomal DNA (Carr et al., 1987, de Villiers et al., 1992). The presence or absence of restriction sites is determined by hybridizing the selected gene with a radioactive labelled complementary strand of DNA, called a "probe".

Restriction site mapping of nuclear ribosomal RNA

In this study, the localities of restriction sites within the gene that codes for nuclear ribosomal RNA were determined and compared. The ribosomal DNA unit consists of coding regions (18s, 5.8s and 28s genes) as well as non-coding regions (internal transcribed spacers (ITS); external transcribed spacers (ETS); non-transcribed spacers (NTS) (Boseley et al., 1979; Salim and Maden, 1981). The coding regions are separated by ITS-1 and ITS-2. The ETS is located between the NTS and the 18s gene. This basic structure of the ribosomal DNA unit is conserved throughout eukaryotes (Goldman et al., 1983) and the structure and processing of a rDNA unit of *Xenopus laevis* is presented in Figure 3. The rDNA gene forms part of the multiple copy nuclear gene family and is therefore repeated in tandem in genomes of eukaryotes (Britten and Kohne, 1968). The ribosomal repeat is shown in Figure 4. A NTS separates the adjacent repeats.

The rapid divergence of mitochondrial DNA (Lansman et al, 1983), coupled with its maternal inheritance - meaning that mitochondrial DNA is inherited only from the female, made it a widely used tool in attempting to solve taxonomic problems for intraspecific and closely related species (Brown et al., 1979; Kessler and Avise, 1984). Interpretation of differences between nuclear ribosomal RNA genes on the other hand provides information on various levels of

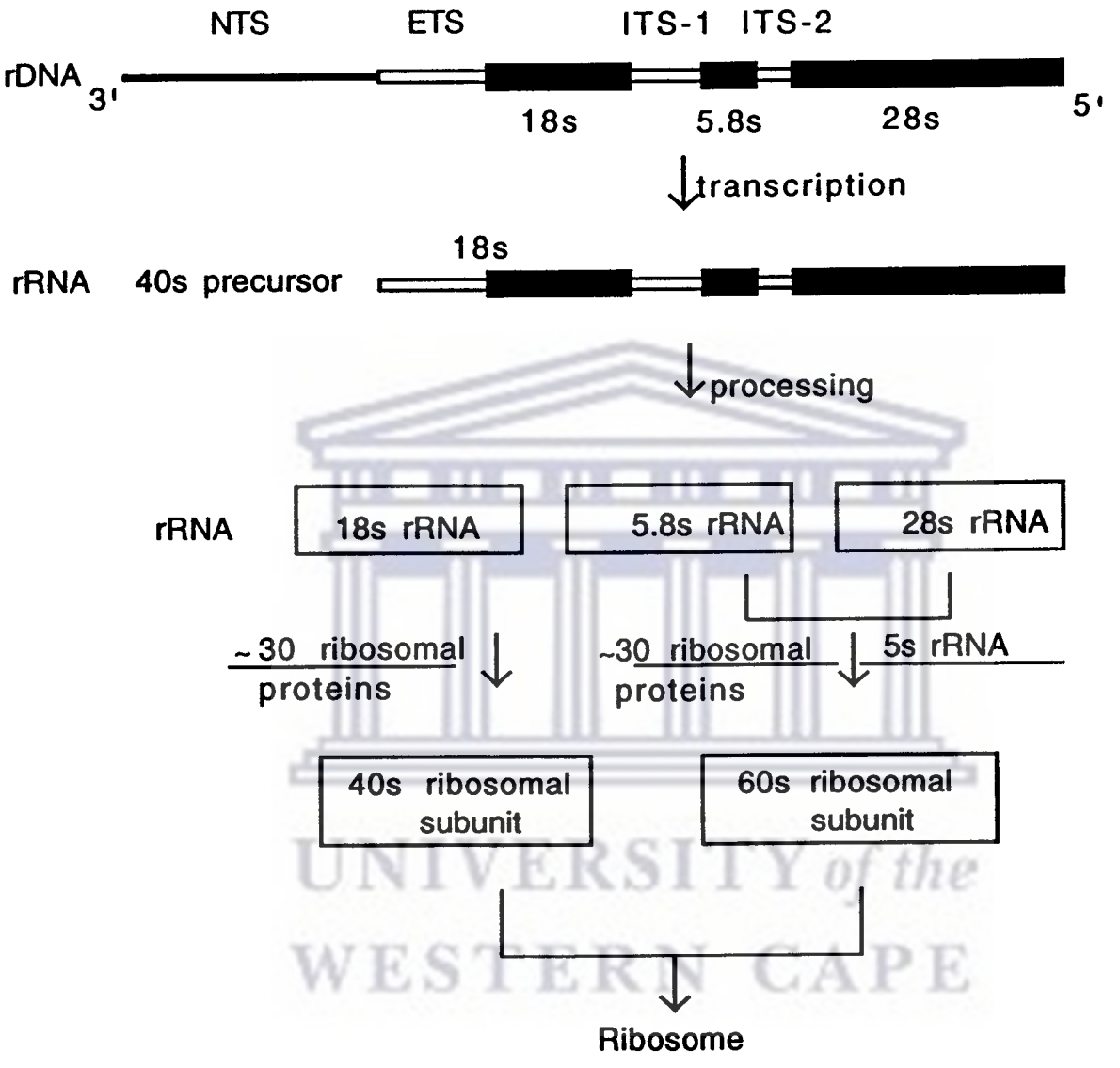

Figure 3. Structure and function of ribosomal DNA repeat.
Abbreviations: NTS- nontranscribed spacer
ETS- external trancribed spacer
ITS- internal transcribed spacer

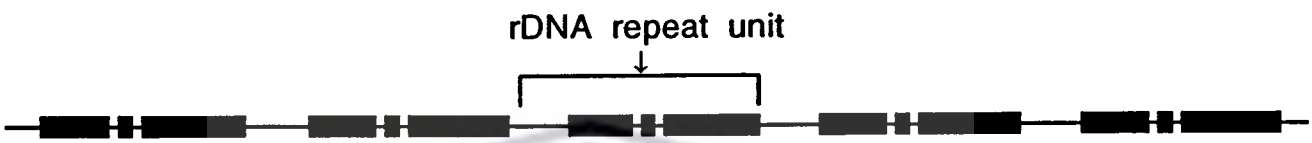

Figure 4. The rDNA unit is repeated in tandem in the genome of eukaryotes.

evolutionary history including distantly related taxa, closely related species and individuals within a species. This is possible due to the presence of both slowly evolving regions (18s, 5.8s and 28s coding regions) as well as regions that evolve more rapidly (transcribed and non-transcribed spacers) (Appels and Dvorák, 1982; Clark, 1987).

The product of the coding regions ie. ribosomal RNA, plays an important role in protein synthesis and hence the low mutational rate within this region (Appels and Dvorák, 1982; Hillis and Davis, 1986; Jorgensen and Cluster, 1988). The conservative nature of the coding regions could therefore be the result of functional constraints. While the coding regions are conserved most of the differences are confined to the external and non-transcribed spacer regions (Hillis and Davis, 1986; Hillis and Davis, 1987; Mindell and Honeycutt, 1990; Suzuki et al., 1987). The presence of both slowly and fast evolving regions within the ribosomal RNA gene makes it an appropriate reporter molecule for studying systematics of frogs which have evolved over millions of years (Hillis and Davis, 1986; Hillis and Dixon, 1991).

When studying closely related species a sequence must be selected that varies among, but not within the species (Hillis and Moritz, 1990). Studies have shown that rDNA displays none or very low intraspecific variation (Jorgensen and Cluster 1988). This is attributed to the fact that rDNA evolves in a concerted fashion (Arnheim, 1979; Coen et al, 1982; Dover and Coen, 1981). During the process of concerted evolution a sequence first becomes homogenous among gene

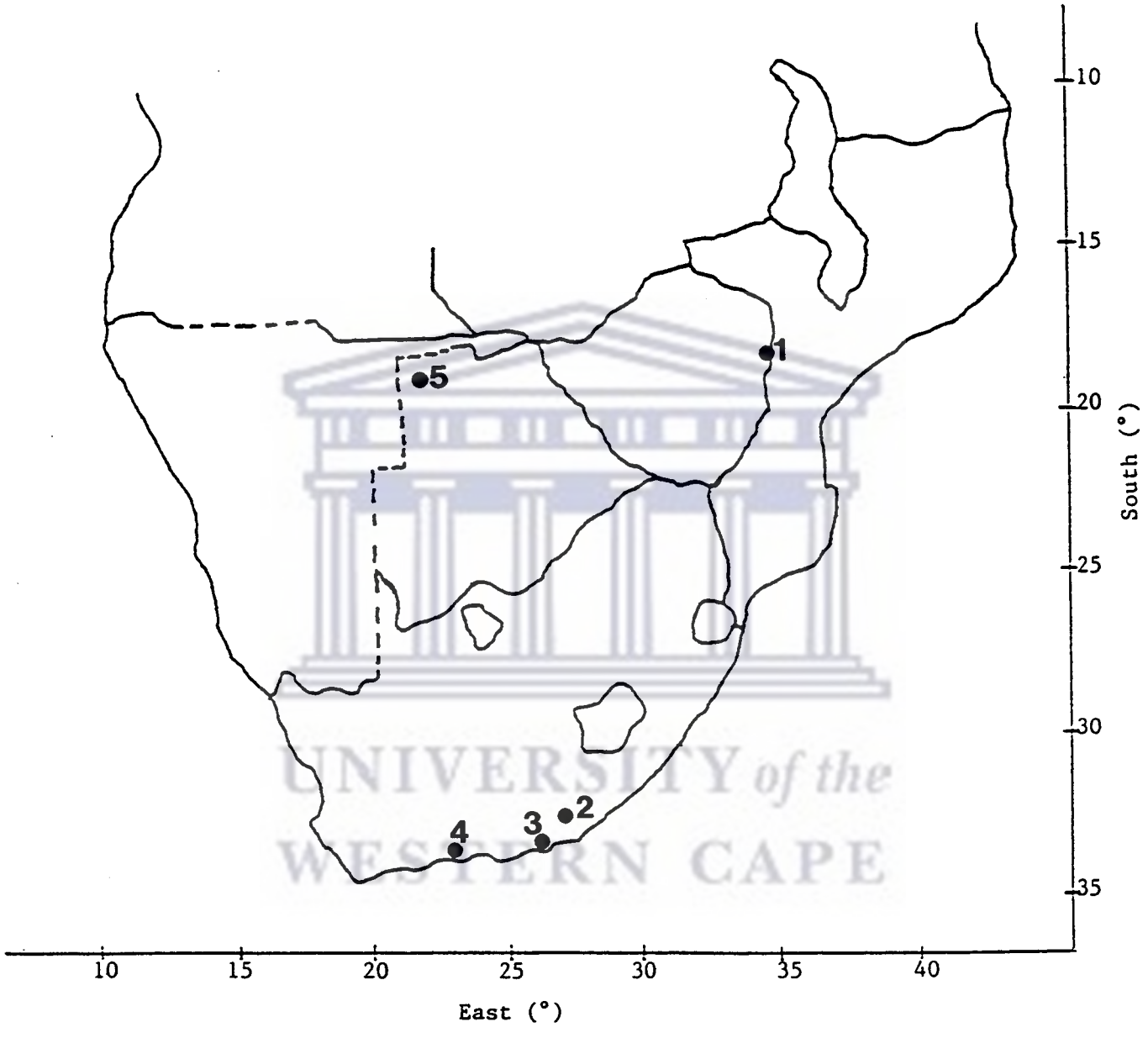

Figure 5. Map indicating the sample collection localities for the three taxa.

1 - Mare Dam - *H. m. broadleyi*

2 - Keiskammahoek
3 - St. Francis Bay — *H. m. verrucosus*
4 - Lake Pleasant

5 - Shakawe - *H. angolensis*

copies within genomes, then among individuals within a population (Dover and Coen, 1981; Long and David, 1980). In other words the tandem repeat copies of the rRNA gene are very similar to each other within individuals and also between individuals within a species. This appears to be the result of unequal crossing-over and/or gene conversion during the process of meiosis (Petes, 1980; Hillis and Davis, 1988).

A limitation of restriction site mapping is the use of restriction sites rather than nucleotide sequences. The restriction sites represent about 230 of the ± 12,000 base pairs of the nuclear ribosomal gene in this study and only the gain and loss of sites and length differences can be detected. On the other hand, because of the size of the gene, sequencing involves only the analysis of a specific part of the gene, whereas restriction site mapping detects differences across the entire spectrum of the gene. Although more expensive than some of the other DNA techniques, restriction site mapping is relatively easy and provides information on the nature as well as the extent of the differences between DNA sequences. The high copy numbers of the rRNA gene in the genome of eukaryotes facilitates the detection of the gene with a radioactive labelled probe. The repeat length is therefore within a range that can easily be examined by restriction site mapping (Hillis and Davis, 1986).

Specimens

The following taxa were examined in this study: *H. m. broadleyi, H. m. verrucosus and H. angolensis*. See Figure 5 for collection localities. Samples

of *H. m. verrucosus* were collected from three different localities within the distribution range of this taxa: Keiskammahoek, Lake Pleasant and St. Francis Bay. Apart from slight differences the above mentioned samples displayed the common colour pattern of *H. m. verrucosus*. The Mare Dam in eastern Zimbabwe, was the only collection locality for *H. m. broadleyi*. These samples also displayed the common colour pattern found within *H. m. broadleyi*. Samples of the reference group *H. angolensis* were collected at Shakawe in Botswana.

The basis of restriction site mapping is as follows: The DNA is digested with restriction endonucleases that have either four base pair, or six base pair recognition sequences. The endonucleases cleave the DNA to produce fragments of different sizes which are then separated on the basis of size on an agarose gel. The fragments are transferred onto special filter paper which is hybridized with radioactive labelled DNA complementary to the 18s and 28s regions of the gene. The filter paper is subsequently exposed to X-ray film. Bands on the X-ray film are fragments generated by restriction enzymes that cut within the ribosomal gene. The sizes of the bands can be determined using a molecular weight marker and restriction site maps are constructed and compared among the taxa.

Isolation of DNA

The method used to isolate DNA from frog tissue is described in Current Protocols in Molecular Biology (Ausubel, et al., 1991). Frogs were anaesthetized with 3-amino benzoic acid ethyl ester. Immediately after removal of the liver and intestines the rest of the frog carcass (usually 1 - 1,5 g) was frozen in liquid

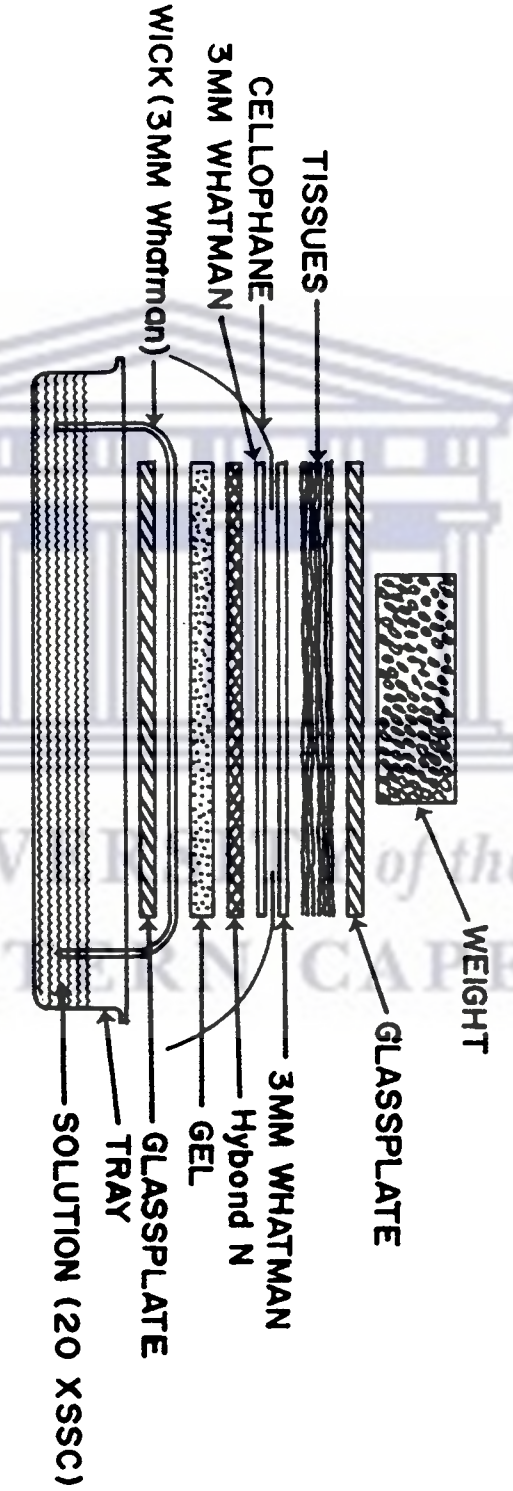

Figure 6. Diagram of Southern Blotting Apparatus

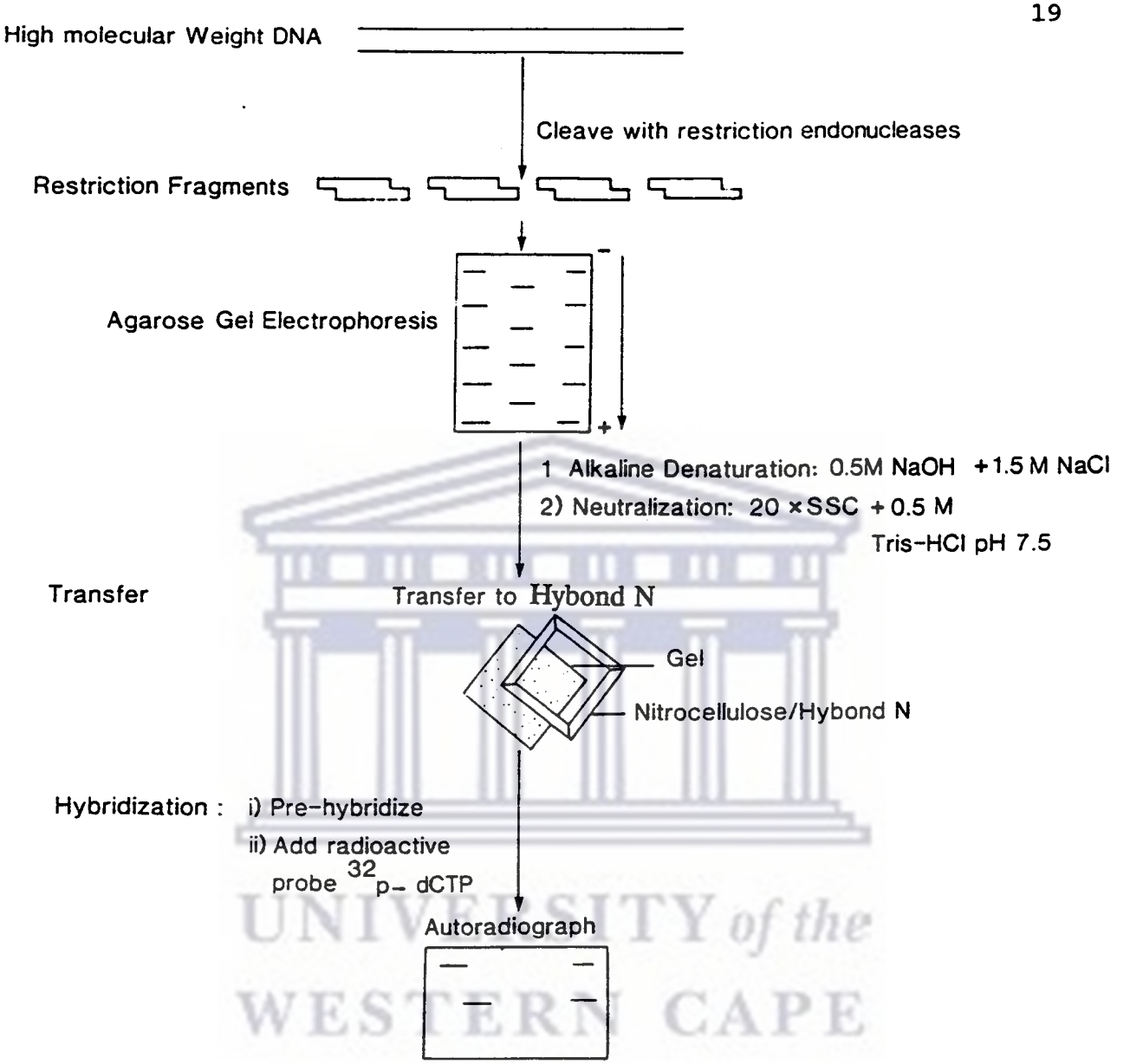

Figure 7. Flow diagram showing Southern Blotting Hybridization.

nitrogen and either stored at -70°C, or processed as follows. The frozen tissue was pulverized to a fine powder with a mortar and pestle that was pre-chilled with liquid nitrogen. The powdered tissue was suspended in digestion buffer in the ratio 100 mg tissue per 1,2 ml digestion buffer and incubated with shaking for 2 h at 55°C. Protocol for the digestion buffer is given in Appendix 1. Then an equal volume of phenol: chloroform: isoamyl alcohol at the ratio of 25: 24: 1 was added, the samples gently shaken and centrifuged at 10 000 rpm for 10 minutes using a Beckman rotor type JA-20. The phenol had previously been buffered to pH 8 and equilibrated with a solution of Tris EDTA (10 mM Tris; 1 mm EDTA; pH 7,4). The aqueous phase was transferred to a new tube, an equal volume of chloroform: isoamyl alcohol at the ratio of 24: 1 was added, mixed gently and the sample centrifuged at 10 000 rpm for 10 minutes. The aqueous phase was collected and 0,5 vol of 7,5 M ammonium acetate and 2 vol of 100% ethanol were added and gently mixed and centrifuged at 10 000 rpm for 1 minute. The ethanol was cooled to -20°C previously. The supernatant was decanted and the pellet washed twice with 3 ml 70% ethanol. The ethanol was decanted and the rest of the ethanol was removed under vacuum. The DNA pellet was dissolved in Tris EDTA and quantified using a spectrophotometer where 50 µg DNA/ml = 1 A_{260} unit. The DNA solution was then stored at -20°C. The quality of the DNA (intact or degraded) was tested by comparing it with uncut Lambda DNA on an agarose gel.

Digestion of DNA with restriction endonucleases

Restriction endonucleases were used to digest 1 - 1,5 µg of DNA in 50 µl reactions as indicated by the manufacturer. The enzymes used and their recognition sequences are given in Table 1. DNA was digested for 2 to 3 h at

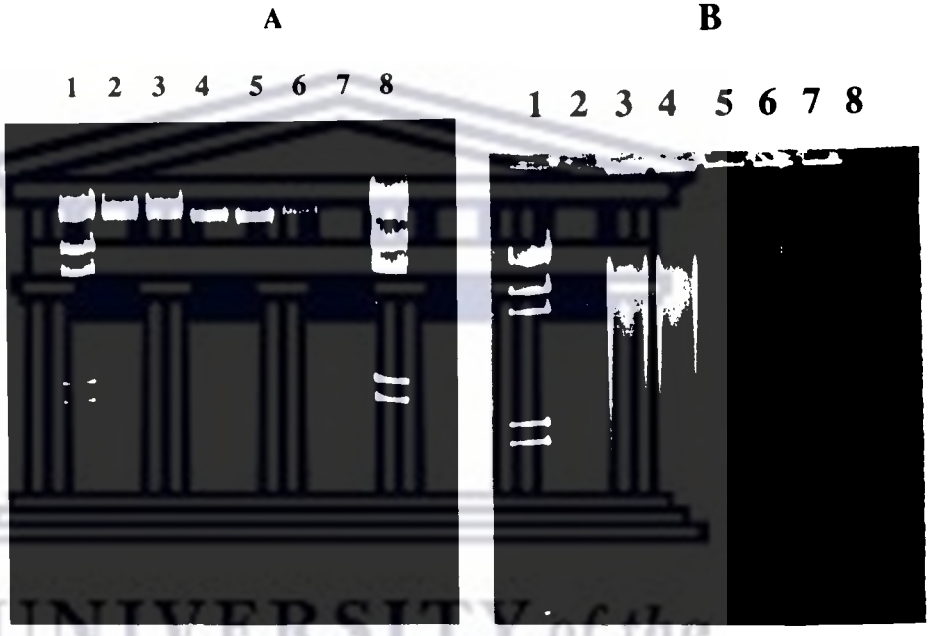

Figure 8. A photograph indicating intact (A) and digested (B) DNA. The molecular weight marker (Lamda/HindIII) was loaded in lanes A1, A8, B1 and B8. Lanes A2 to A6 represents uncut sample DNA: A2, A3 = *H. m. broadleyi*; A4, A5 = *H. m. verrucosus*; A6 = *H. angolensis*. In lanes B3 to B7 sample DNA (loaded in the same sequence as in A3 to A7) was digested with the enzyme Eco RI.

37°C for all the restriction enzymes except Bcl I (50°C) and BstE II (50°C). The tube contents (Appendix 1) were mixed every 30 minutes. At the end of the digestion period, 5 µl of the digestion reaction was separated on an 0,8% agarose mini-gel at 70 V for one hour to assess the extent of digestion, using ethidium bromide (0,1 mg/ml) to visualize the DNA under UV light. For the preparation of the agarose gel see Appendix 1. In Figure 8 a photograph is presented where the extent of digestion was determined. The extent of digestion was determined, because only complete digested DNA was used for further processing. In lanes A3 to A7 uncut sample DNA was loaded - evident as a distinct band corresponding to the 23 130 kb band of the marker (Lambda DNA/Hind III). This band represents uncut/intact DNA. In lanes B3 to B7 this distinct band had disappeared, indicating that all the DNA was digested with the enzyme Eco RI.

Agarose gel electrophoresis

The digested samples were loaded on 0,8% agarose gels and subjected to electrophoresis at 25 V overnight or 20 h. Lambda C1857 phage DNA digested with Hind III was included in the gel as a size standard. After electrophoresis, the gel was stained with ethidium bromide (0,1 mg/ ml) and photographed under UV light.

Southern blotting

The methods used for Southern blotting were those described by Ausebel et al., (1991). Protocols of all solutions utilized for southern blotting are presented in Appendix 1. The gel was soaked in denaturing solution for 30 minutes with

shaking, and rinsed with distilled water. The gel was then placed in neutralization solution for 30 minutes. Hybond N filter paper, the same size as the gel, and two pieces 3MM Whatman paper also the same size as the gel, were

Table 1. Enzymes and their recognition sequences utilized in this study. N = unspecified nucleotides.

Enzyme	Recognition sequence
Eco RI	G/AATTC
Pst I	CTGCA/G
Bst EII	G/GTNACC
Bcl I	T/GATCA
Xmn I	GAANN/NNTTC
Sac I	GAGCT/C
Pvu II	CAG/CTG
Bgl II	A/GATCT
Nco I	C/CATGG
Bam HI	G/GATCC
Dra I	TTT/AAA

soaked in 2x SSC. A wick (3MM Whatman paper) was soaked in 20x SSC. 10x SSC was placed into a container and the wick placed on a glass plate in such a manner that it overhung into the 10x SSC. On the wick the following were layered in sequence: gel, Hybond N; two pieces 3MM Whatman paper; tissues.

A weight was placed on top of tissues and transfer took place overnight. Afterward the tissues and Whatman paper were removed and the position of the wells were marked onto the filter paper. The filter was placed between Whatman paper and baked at 80°C for 30 minutes. An illustration of the Southern blotting apparatus is given in Figure 6.

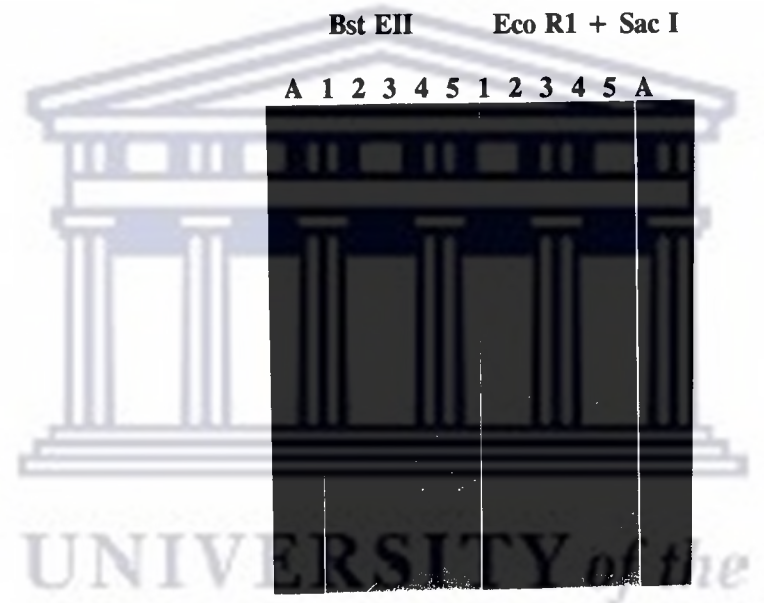

Figure 9. An autoradiograph illustrating the fragments generated by restriction enzymes Bst EII (single digests) and Eco RI + Sac I (double digests). The molecular weight marker (Lambda/Hind III) is present in the lanes indicated by the letter A. DNA from two individuals (one in each lane) of the same population is presented in lanes 1, 2 (*H. m. broadleyi*); 3, 4 (*H. m. verrucosus*) with a single sample representing the reference group, *H. angolensis* in lane 5. This loading sequence is common for all the enzymes.

Hybridization of DNA

The methods are those described in Hillis and Moritz, (1990). Hybridization of the filter paper took place in perspex chambers. The filter paper was cut into 8 pieces and prehybridized with 25 ml hybridization solution at 64°C for 2 h. The protocol for the hybridization solution is given in Appendix 1. The 28s and 18s probes (0,2 μg) were separately labelled radioactive with ^{32}P by making use of a Random Prime Labelling kit. The labelled probe was mixed with 10 ml hybridization solution and boiled for 10 minutes then immediately cooled on ice for 5 minutes. The newly prepared probe was preincubated for 2 h with filter paper which had previously been incubated with hybridization solution for 2 h at 64°C, to reduce non-specific binding. Before filters were probed the 15 ml prehybridization solution was removed and hybridization with the 28s and 18s probes was performed separately. Hybridization took place overnight at 64°C. A flow diagram of the southern blotting and hybridization process is presented in Figure 7.

Post hybridization washes

The probed filters were soaked twice for 15 minutes at room temperature in 500 ml Solution I and rinsed with 100 ml Solution II followed by gentle shaking in 500 ml Solution II at 37°C for 1 h. Protocols for the solutions are given in Appendix 1.

Autoradiography

The hybridized rinsed filters were exposed to X-ray film for seven to ten days at

- 70°C and developed normally: 5 min developer; 1 min stop: 5 minutes fix (Appendix 1). After developing, the autoradiographs were washed in water. Exposure of the filter to X-ray film produced bands on an autoradiograph as shown in Figure 9. The migration distances and lengths of the bands generated with the various restriction enzyme digests are given in Appendix 2.

Construction of restriction site maps

The sizes of the fragments were determined by using the computer programme FRITENSKY (Schaffer and Sederoff, 1981) and was estimated within an error of ±100 base pairs. The relative positions of the restriction sites were determined by single and double digestions, using as reference points the Eco RI sites that are uniformly present in vertebrates near the 5' ends of the 18s and 28s genes (Cortadas and Pavon, 1982).

CHAPTER 3

RESULTS

The localities of the restriction endonuclease sites were mapped manually and final maps were constructed for *H. m. broadleyi, H. m. verrucosus* and *H. angolensis*. Based on the presence and absence of sites for the three taxa combined, a total of 37 restriction sites were mapped. These sites are presented in Table 2. Of the 37 sites that were mapped 15 were in the conserved regions. These sites were identical to the sites mapped in the conserved regions throughout *Rana* (Hillis and Davis, 1986). 20 sites were informative ie., not identical in all taxa and were present in the NTS region except for Sac I (site no. 27) and Bam HI (site no. 28) present in the internal spacer region-1. The total of sites mapped individually are as follows: *H. m. broadleyi*, 30 present, 7 absent; *H. m. verrucosus*, 23 present, 14 absent; *H. angolensis*, 26 present, 11 absent. Maps of the relative position of the restriction sites are given in Figure 10. The repeat length of *H. angolensis* was calculated as 12 600 ± 200 base pairs, *H. m. broadleyi* 12 800 ± 200 and *H. m. verrucosus* 12 400 ± 200 base pairs. As shown in Figure 10 the variation in restriction site maps was confined to the non coding regions.

Interspecific variation

Variation between the subspecies

Between the subspecies *H. m. broadleyi* and *H. m. verrucosus* a total of 33 sites

were mapped of which 20 were shared including the sites in the coding regions. Of these sites 5 out of 18 were shared in the variable regions only. The repeat length of *H. m. verrucosus* was 400 base pairs smaller than *H. m. broadleyi*. A deletion was detected between the Pst I and Dra I sites of *H. m. verrucosus*.

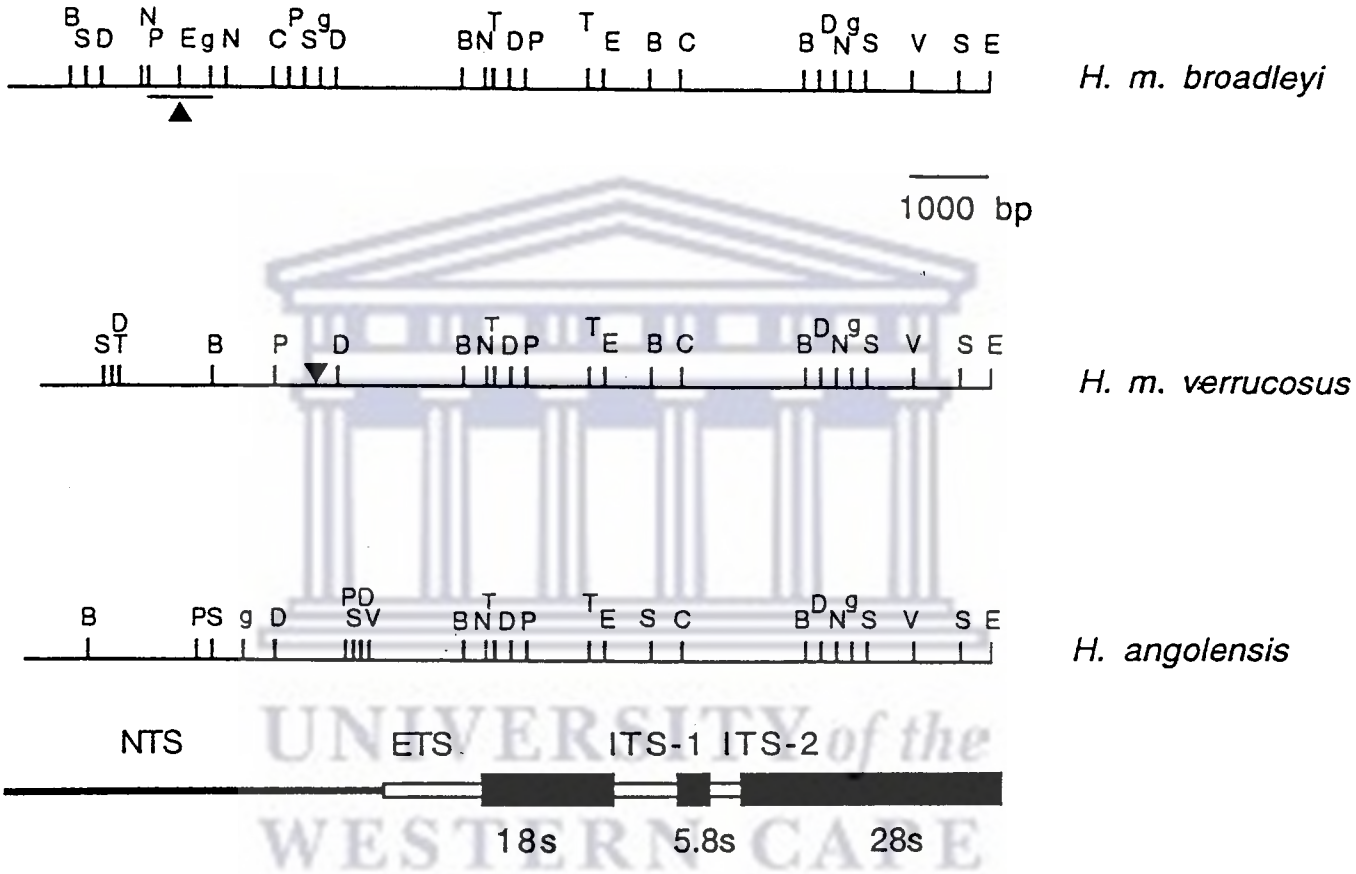

Figure 10. Restriction site maps of *H. m. broadleyi, H. m. verrucosus* and *H. angolensis* indicating the relative locations of the restriction sites produced by ten different restriction enzymes. For abbreviations of enzymes, see Appendix 3. The position of the deletion detected in *H. m. verrucosus* is indicated by a triangle. The maps were aligned relative to the conserved Eco RI sites present in the 28s and 18s coding regions. The triangle on the *H. m. broadleyi* map indicates the transversion involving Pst I, Eco RI and Bgl II. See Chapter 4 for discussion.

Table 2. Restriction sites present (+) and sites absent (-) in *H. m. broadleyi*, *H. m. verrucosus* and *H. angolensis*. Sites are numbered from the left starting with Bam HI as site no. 1.

Restriction sites	*H. ang*	*H. m. bro*	*H. m. ver*
1. Bam HI	+	+	-
2. Sac I	-	+	+
3. Dra I	-	+	+
4. Bst EII	-	-	+
5. Nco I	-	+	-
6. Pst I	+	+	-
7. Eco RI	-	+	-
8. Bam HI	-	-	+
9. Sac I	+	-	-
10. Bgl II	+	+	-
11. Nco I	-	+	-
12. Dra I	+	-	-
13. Pst I	-	-	+
14. Bcl I	-	+	-
15. Pst I	+	+	-
16. Sac I	+	+	-
17. Bgl II	-	+	-
18. Dra I	+	+	+
19. Pvu II	+	-	-
20. Bam HI	+	+	+
21. Nco I	+	+	+
22. Bst EII	+	+	+
23. Dra I	+	+	+
24. Pst I	+	+	+
25. Bst EII	+	+	+
26. Eco RI	+	+	+
27. Sac I	+	-	-
28. Bam HI	-	+	+
29. Bcl I	+	+	+
30. Bam HI	+	+	+
31. Dra I	+	+	+
32. Nco I	+	+	+
33. Bgl II	+	+	+
34. Sac I	+	+	+
35. Pvu II	+	+	+
36. Sac I	+	+	+
37. Eco RI	+	+	+

H. ang = *Hyperolius angolensis*

H. m. bro = *Hyperolius marmoratus broadleyi*

H. m. ver = *Hyperolius marmoratus verrucosus*

This was inferred based on a shorter size fragment in *H. m. verrucosus* compared to *H. m. broadleyi* which existed between the two Dra I sites in the NTS region of the gene. The deletion is indicated by a triangle on the *H. m. verrucosus* map in Figure 10. The deletion has led to the absence of the Sac I site (no. 16) and Pst I site (no. 15) which are present in *H. m. broadleyi* and *H. angolensis*.

Variation between the subspecies and reference group

Between *H. m. broadleyi* and *H. angolensis* a total of 34 sites were mapped of which 22 were shared. The number of sites shared in the variable regions were 7 out of a total of 19. The repeat length of *H. angolensis* was 200 base pairs smaller than *H. m. broadleyi*. *H. angolensis* and *H. m. verrucosus* produced 32 sites between them of which 17 were shared overall whereas 2 out of a total of 17 sites were shared in the variable regions. The sites present and absent compared amongst all three taxa is given in Table 2.

Analysis

Informative sites

Sites present in all three taxa were regarded as uninformative. Except for the Dra I site (no. 18) and the Bam HI site (no. 20) all these sites were present in the conserved regions. Inclusion of these sites when determining the percentage sites shared gave a different perspective of the relationship between the taxa. This is evident in Figure 11 which indicates the difference between the percentage sites

shared, including all restriction sites and the percentage shared informative sites between the taxa. All three taxa exhibited unique sites ie., sites present in the specific taxa only. *H. angolensis* displayed 4, *H. m. broadleyi* 5 and *H. m. verrucosus* 3. The number of shared informative sites between *H. angolensis* and

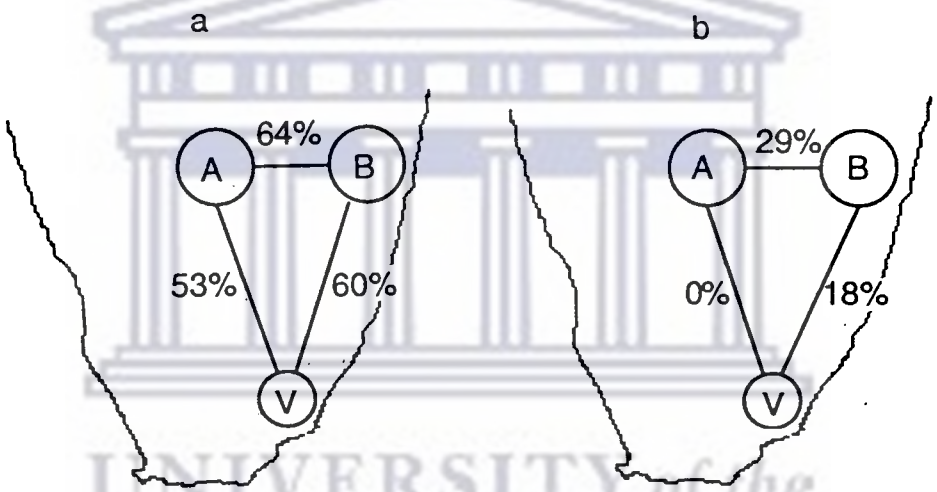

Figure 11. Percentage sites shared including all restriction sites (a) and percentage informative sites shared (b). Circles indicate the relative localities of the different taxa. Abbreviations: A = *H. angolensis*; B = *H. m. broadleyi*; V = *H. m. verrucosus*. See following chapter for further discussion.

H. m. broadleyi are 5, *H. angolensis* and *H. m. verrucosus* 0 and *H. m. broadleyi* and *H. m. verrucosus* 3. The informative sites for the three taxa are *H. m. broadleyi* 13, *H. m. verrucosus* 6 and *H. angolensis* 9. This information resulted

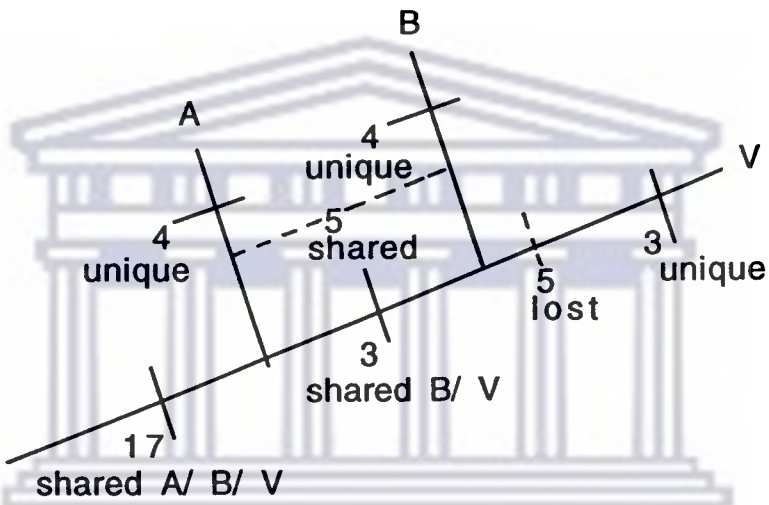

FIGURE 12. A tree constructed from the information in Table 2 indicating the relationships in terms of presence and absence of restriction sites between the three taxa. Abbreviations: A = *H. angolensis*; B = *H. m. broadleyi*; V = *H. m. verrucosus*

in a tree presented in Figure 12 which shows the relationship between the three taxa. An alternative way of determining the genetic difference between the taxa is the calculation of sequence divergence values.

Sequence divergences

The sequence divergence (δ) of the three taxa were determined by using the formula of Nei and Li (1979), where $\delta = - \ln S/r$. The symbol "r" represents the number of bases in the recognition sequence of the restriction enzymes used

Table 3. Similarities and differences among the rDNA maps of the three taxa shown in Figure 10. This table gives the total number of sites examined per taxon (diagonal, underlined), the number of sites shared between each pair of taxa (upper right matrix), and the fraction of sites shared (S) by each pair (lower left matrix). This fraction is calculated as 2 Nxy/(Nx + Ny), where Nxy is the number of sites shared between the two taxa, and Nx, Ny are the total number of sites examined in X and Y respectively. Sites in the conserved regions are excluded from the above calculations. Sites present in the conserved regions are given in brackets.

rDNAs compared	*H. ang*	*H. m. bro*	*H. m. ver*
H. ang	11 (15)	7	2
H. m. bro	0,461	15 (15)	5
H. m. ver	0,210	0,434	8 (15)

H. ang* = *Hyperolius angolensis

H. m. bro* = *Hyperolius marmoratus broadleyi

H. m. ver* = *Hyperolius marmoratus verrucosus

for the particular study. An estimate of the proportion of restriction sites shared (S) is given by the equation: S = 2 Nxy/ (Nx + Ny), where Nxy is the number

Table 4. Estimates of sequence divergence calculated from the data in Table 2 and Figure 10. The formulas for the calculation of the sequence divergence (δ) were obtained from Nei and Li (1979) where δ = -ln S/r. The value for "r" is always equal to 6 because the restriction endonucleases used in this study have all 6 base pair recognition sequences. The percentage sequence divergence is given in brackets.

rDNA compared	Sequence divergence
H. ang x *H. m. bro*	0,103 (10,3%)
H. ang x *H. m. ver*	0,260 (26%)
H. m. bro x *H. m. ver*	0,138 (13,8%)

of sites shared between the two taxa and Nx, Ny are the total number of sites examined in x and y respectively. Because of the conservative nature of the coding regions, sequence divergence values were calculated only for sites in the

variable regions. Table 3 gives the total number of sites examined per taxon, the sites shared as well as the proportion of sites shared. The "S" values were recorded as follows: *H. angolensis* and *H. m. broadleyi* (0,538); *H. angolensis* and *H. m. verrucosus* (0,210); *H. m. broadleyi* and *H. m. verrucosus* (0,437). The sequence divergences between the taxa are given in Table 4. The sequence divergence between the reference group, *H. angolensis* and *H. m. broadleyi* is recorded as 0,103. Likewise between *H. angolensis* and *H. m. verrucosus* 0,260 is recorded. The sequence divergence between the subspecies *H. m. broadleyi* and *H. m. verrucosus* was calculated as 0,138. The percentage sequence divergence is presented in brackets in Table 4.

CHAPTER 4

DISCUSSION

The average of 26 sites that were mapped per taxon in this study falls within the same range as that recorded in other studies (Carr et al., 1987; Hillis and Davis, 1986). Similarities in the maps are mostly confined to the conserved regions of the gene whereas most differences are located in the NTS regions as was previously observed in *Rana* by Hillis and Davis (1986). The rDNA restriction site maps (Figure 10) indicate substantial differences between the subspecies *H. m. broadleyi* and *H. m. verrucosus* as well as between the subspecies and the reference species *H. angolensis*. Very significant is the difference in percentage sites shared between the taxa when all sites are considered compared to percentage informative sites shared (Figure 11). Inclusion of the sites present in the coding regions when determining the difference of sites shared, will not give a true reflection of the relationships between the various taxa because of the conservative nature of these regions. This is evident in Figure 11(b) which indicates no gene flow between *H. angolensis* and *H. m. verrucosus* opposed to Figure 11(a) which suggests an equal distribution of gene flow between the different taxa. Out of a total of 18 sites that were mapped in the variable regions between the subspecies, a difference of 72% was recorded. Hillis and Davis (1986), determined restriction site maps of rDNA between different species of the genus *Rana* and recorded a site difference of 22% between *Rana pustulosa* and *Rana tarahumarae* as well as a value of 65% between *Rana pustulosa* and *Rana*

pipiens. The value of 72% between *H. m. broadleyi* and *H. m. verrucosus* therefore seems to correspond to differences found between species. The deletion detected in *H. m. verrucosus* (Figure 10) contributed to the difference in the repeat length between it and *H. m. broadleyi*. The restriction site map of the reference group, *H. angolensis* also shows significant differences with the subspecies. The variable region site difference of 72% recorded between the subspecies *H. m. broadleyi* and *H. m. verrucosus* falls within the range 63-89% recorded between *H. angolensis* + *H. m. broadleyi* (63%) and *H. angolensis* + *H. m. verrucosus* (89%). This is significant, because if *H. m. broadleyi* and *H. m. verrucosus* were closely related a much bigger difference would be expected in sites difference between the subspecies on the one hand and the subspecies compared with the reference group on the other hand.

Based on the analysis of the informative sites present in the three taxa a tree can be constructed to illustrate the relationships between them (Figure 12). All three taxa exhibited unique sites: *H. m. broadleyi* possessed 4, *H. angolensis* possessed 4 and *H. m. verrucosus* possessed 3. Five informative sites are shared between *H. angolensis* and *H. m. broadleyi* whereas the same sites are lost in *H. m. verrucosus*. The fact that *H. m. broadleyi* and *H. m. verrucosus* have unique restriction sites and the fact that *H. m. broadleyi* and *H. angolensis* shared informative sites which are not shared by *H. m. verrucosus* and *H. angolensis*, is an indication that the subspecies have diverged genetically into separate, isolated populations. This is important in the debate of whether *H. m. broadleyi* and *H. m. verrucosus* should be regarded as subspecies or separate species. The

informative sites suggests that *H. m. broadleyi* and *H. m. verrucosus* can be regarded as separate species.

Intraspecific variation

Samples from three different localities within the distribution range of *H. m. verrucosus* were examined for intraspecific variation (Figure 5). DNA of five individuals from Keiskammahoek and two each from St. Francis Bay and Lake Pleasant were digested with six different enzymes: Bst EII; Nco I; Pst I; Bam HI; Eco RI; Xmn I. The selection of these enzymes was based on the observation that *H. m. broadleyi* and *H. m. verrucosus* displayed different fragment patterns and hence restriction site maps for these enzymes. The restriction site maps for these enzymes were identical for all the individuals of *H. m. verrucosus*. The fragment patterns of these samples are presented in Figure 13. The absence of intraspecific variation is an indication of the concordance of overall geographical colour pattern and the genetic composition of *H. m. verrucosus*.

The same study was not performed on *H. m. broadleyi* and *H. angolensis* due to unavailability of samples from different areas. However, five individuals of *H. m. broadleyi* from the same locality (Mare Dam) were examined for intraspecific variation. All five samples were identical in their fragment length pattern except for an extra fragment that was recorded for two individuals with the digestion of the DNA with the enzymes Pst I, Eco RI and Bgl II. The bands for Pst I and Bgl II are marked by arrows in Figure 14. The size of the extra band generated with Pst I was calculated as 8 397 $\pm$ 200 base pairs, 2 153 $\pm$ 200 for Eco RI and 4

318 ± 200 in the case of the enzyme Bgl II. The size of the "common"/"normal" fragment for all individuals was calculated as 7 890 ± 200, 2 153 ± 200 and 4 506 ± 200 for Pst I, Eco RI and Bgl II respectively. The rDNA repeat length of the two individuals, with the

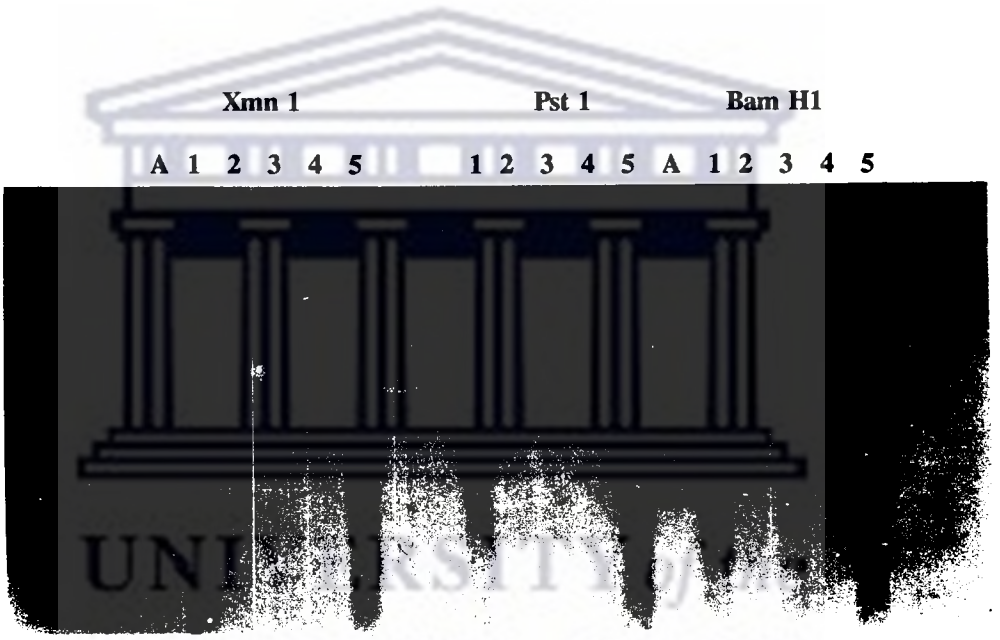

Figure 13. A picture of an autoradiograph indicating the identical fragment pattern for the *H. m. verrucosus* samples from three different localities within the distribution range. Lanes 2, 3, 4, represent the *H. m. verrucosus* samples (specific localities) whereas lanes 1 and 5 are samples from *H. m. broadleyi* and *H. angolensis* respectively. The molecular marker is present in lanes marked by the letter A. See text for further explanation.

addition of the sizes of the extra fragments, did not correspond to the repeat length found when the same DNA was cleaved with the other enzymes. The rDNA repeat length of the two individuals of *H. m. broadleyi* were therefore longer than other individuals, when their DNA was digested with Pst I and Bgl II. A strange phenomenon however was that the difference in repeat length was not observed when the same (two) individuals were digested with other enzymes.

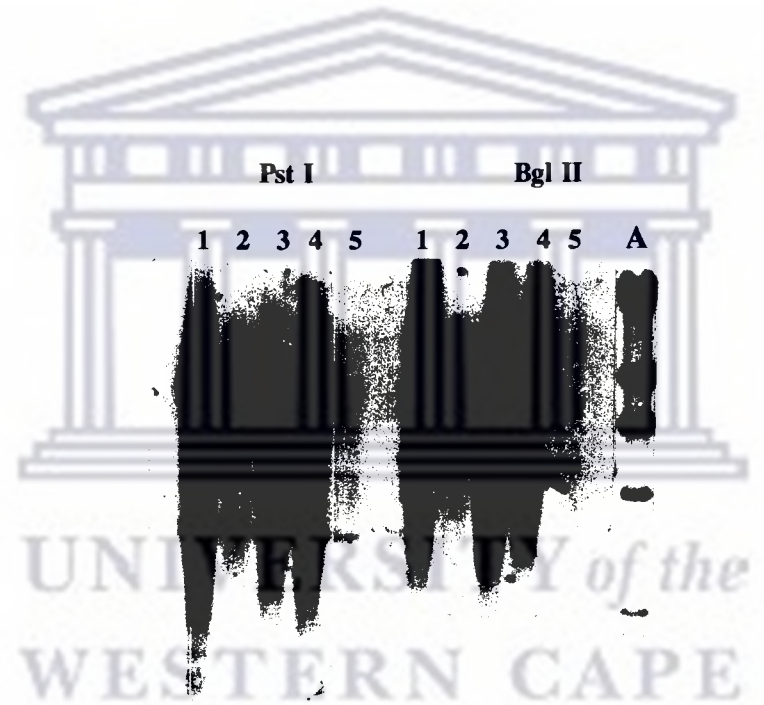

Figure 14. A picture of an autoradiograph showing the fragment patterns produced by the enzymes Pst I and Bgl II for five individuals of *H. m. broadleyi*. The fragment patterns are identical except for an extra band (indicated by an arrow) present in samples 3 and 4 for the two enzymes. The molecular marker is present in lane A. Further discussion follows in the text.

Differences in length of the rDNA repeat spacers were detected in the same genome of a relatively small number of individuals of the tailed frog, *Ascaphus truei* (Morgan and Middleton, 1992). They recorded two spacer size classes per genome with the maximum difference in length measured between the classes only 120 base pairs. Frogs are not the only organisms in which intra-individual length variation was recorded. Intra-individual length variation was also recorded among the multiple copies of the human rRNA gene (Gonzales et al., 1985). The intra-individual variation was, however, minimal compared to overall similarity of the multiple copies of the rRNA gene within and among individuals. The possibility exists that the differences observed in the two individuals of *H. m. broadleyi* were also due to length heterogeneity of the non-transcribed spacers where most of the differences between the various taxa occurred. On the other hand if length heterogeneity exists, why then is the new fragment generated with Bgl II and Eco RI $\pm$200 base pairs smaller than the "normal"/"common" fragment and $\pm$500 base pairs longer for Pst I? There is also no explanation why the difference in length was not detected with the other enzymes. The most likely explanation for the extra fragment is a transversion in one of the alleles of the rDNA repeat in the vicinity of the Pst I, Eco RI and Bgl II sites. The locality of the suggested transversion in the two individuals of *H. m. broadleyi* is indicated by a triangle on the *H. m. broadleyi* map in Figure 10. The transversion was located in this region based on analysis of the fragments generated with the single digests Pst I, Eco RI and Bgl II and double digests of Eco RI with the forementioned enzymes. The sizes of these fragments are given in Appendix 2. The transversion theory explains why no difference in repeat length was detected

with the other enzymes and also why the extra fragment is larger for Pst I and smaller for Bgl II and Eco RI. Extra fragments only appeared with the digestion of the enzymes Pst I, Eco RI and Bgl II and it was concluded that the transversion only involves the restriction sites identified in the restriction map - figure 10.

Sequence divergence versus subspecific classification

A sequence divergence value of 13,8% was calculated between the subspecies *H. m. broadleyi* and *H. m. verrucosus*, 10,3% between *H. angolensis* and *H. m. broadleyi* and 26% between *H. angolensis* and *H. m. verrucosus*. These values were in the same range as that recorded by Hillis and Davis, (1986) for separate species. A rDNA sequence divergence value as low 2,2% was recorded between *Rana pustulosa* and *Rana tarahumarae* and a value of 10,1% between *Rana pustulosa* and *Rana pipiens*. Allard and Honeycutt (1991), found rDNA sequence divergences of 3% between species of rodents in the genus *Onychomys*. Sequence divergences for mitochondrial DNA were determined for a wide range of organisms including frogs. Examples are discussed below.

Sequence divergence differences have been determined between species and subspecies of the frog genus *Xenopus* (Carr et al., 1987). They recorded mtDNA sequence divergence values that range from 3 -7% for subspecies of *Xenopus laevis* and 11 - 39% for different species of *Xenopus*. These high sequence divergence values for mitochondrial DNA can be explained by the observation of Brown et al. (1979), that mtDNA has a higher sequence divergence rate than

nuclear DNA. Honeycutt et al. (1987), examined genetic variation within the genus *Cryptomys* (African mole rats). External and cranial characters as well as presence or absence of head spots are extremely variable both within and among populations. The mtDNA sequence divergence values recorded for three subspecies of *Cryptomys hottentotus (C. h. hottentotus, C. h. natalensis* and *C. h. damarensis*) were higher than that recorded for separate species in this genus and specific ranking was suggested by the authors. An average sequence divergence value of 15,8% was recorded for the subspecies compared to an average value of 7,5% for separate species of the genus. Restriction site mapping of mtDNA for species of the genus *Equus* revealed sequence divergence values that range between 3,3-7,8% (George and Rider, 1986). Subspecies of the Canada geese display mtDNA sequence divergence values of 0,04 - 2,54% whereas a value of 8% was recorded between two separate species, *Branta canadensis* and *Branta bernicla* (Van Wagner and Baker, 1990).

Compared to the sequence divergence value found between species in these studies, the value of 13,8% between the subspecies *H. m. broadleyi* and *H. m. verrucosus* indicates that they are genetically enough diverged to be classified as separate species. The sequence divergence values of the reference group *H. angolensis*, relative to the subspecies were also in the same range of the value found between the subspecies.

If the restriction site maps of the two subspecies were similar with a low sequence divergence value, then it could be argued that they belong to the same species.

In such a case the difference in colour and colour pattern observed within the southern African *H. marmoratus* will be regarded as a polymorphism of the species. However, the sequence divergence values found between the subspecies *H. m. broadleyi* and *H. m. verrucosus* are in the same range or higher when compared to separate species in other studies. Another significant factor is the good correlation between the sequence divergence values of the subspecies when it is compared with the values found between the reference group and the subspecies. The tree presented in Figure 12 also clearly suggests that *H. m. broadleyi* and *H. m. verrucosus* are genetically separate. The genetic data on *H. m. verrucosus* and *H. m. broadleyi* complemented by their difference in colour pattern suggest specific ranking for these taxa.

The subspecies classification had been based mainly on the fact that neighbouring subspecies of *H. marmoratus,* which form the gradient along the east coast of southern Africa, interbreed at the overlap zones (Poynton and Broadley, 1987). The only evidence for the suggested interbreeding was the combination of two colour patterns displayed by individuals from the overlap zones, one each from the neighbouring taxa. Poynton (1985), discussed interbreeding in the overlap zone between *H. m. taeniatus* and *H. m. broadleyi*. Examples are shown of individuals exhibiting a combination of *H. m. taeniatus* and *H. m. broadleyi* colour patterns. These individuals were regarded as hybrids between *H. m. taeniatus* and *H. m. broadleyi* by Poynton (1985). These intermediate colour patterns are the only evidence for interbreeding. Whether the colour patterns are a true reflection of interbreeding can only be concluded if individuals from the

overlap zone which display colour patterns from both sides of the zone as recorded, display any heterozygosity. For this purpose a large sample size from both the overlap and flanking zones must be examined to determine the extent of heterozygosity. Species can interbreed, especially if their ranges are increasing, and they come into contact with each other. Interbreeding could then be regarded as plesiomorphic (or a "primitive" character shared by both species through a common ancestor). Restriction site mapping of ribosomal DNA in combination with another molecular biology technique eg. protein electrophoresis can be utilized to determine whether interbreeding is occurring.

The distinct dorsal colour patterns in combination with the genetic data seem to support species status for *H. m. broadley*i and *H. m. verrucosus*. However, this study has examined only two subspecies which occur at the extreme ends of the distribution range of the *H. marmoratus* gradient and, therefore, studies will have to be conducted on all the subspecies and their overlap zones to determine if similar genetic differences exist. Such a study will contribute to the debate whether the subspecific status currently given to the group, is justified or not.

CHAPTER 5

SUMMARY

H. m. broadleyi and *H. m. verrucosus* are not only different in terms of colour pattern, but distinct genetic differences were detected in restriction site maps of their ribosomal DNA. A sequence divergence value of 13,8% was found between the subspecies. This value exceeds the range recorded between separate species of the genus *Rana*, ie. 2,2% between *Rana pustulosa* and *Rana tarahumarae*, and 10,1% between *Rana pustulosa* and *Rana pipiens*. The value of 13,8% between the subspecies is also in the same range as that found between the subspecies and the reference group, *H. angolensis*. No intraspecific variation was detected between samples from three different localities within the distribution range of *H. m. verrucosus*. The genetic data associated with the different colour patterns, suggests that *H. m. broadleyi* and *H. m. verrucosus* can be regarded as distinct species. However, a similar study should be performed to examine the genetic status of the subspecies forming the gradient along the east coast of southern Africa. Although the effect of concerted evolution (as discussed in Chapter 2) allows for small sample sizes it would be useful to examine a large number of individuals especially from the overlap zones to determine the extent of genetic heterozygosity and to determine if similar genetic differences (found between *H. m. broadleyi* and *H. m. verrucosus*) exist between the rest of the subspecies. The current study has shown that genetic evaluation of all the southern African *H. marmoratus* subspecies could have a positive impact on the taxonomy of this group of frogs which is still unresolved. This study has identified at least two species within the *H. marmoratus* complex and it is possible

that more species exist within the group. Because of morphological homogeneity it will be difficult to use morphological characters, but more than one molecular technique can be utilized to verify results obtained with one technique.

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APPENDIX 1

ISOLATION OF DNA

Digestion buffer

100 mM NaCl

10 mM Tris-Cl, pH 8

25 mM EDTA, pH 8

0,5% SDS (sodium dodecyl sulfate)

0,1 mg/ml proteinase K (10 mg/ml stock was added to digestion buffer)

DIGESTION OF DNA WITH RESTRICTION ENZYMES

Digestion mix

1-2 µg DNA sample

1 µl enzyme buffer [or Voodoo buffer, 5 µl of A, B and C in case of double digest]

2 µl enzyme

H_2O to final volume of 50 µl

Voodoo buffer

A 0,1 M 1,4 Dithio-DL- threitol

B 1% Digitonin

C 0,2 M Tris-Cl pH 7,5

0,7 M NaCl

0,2 M KCl

0,1 M $MgCl_2$

0,5 mM Spermine

0,125 mM Spermidine

1:1000 diluted Aprotinin

AGAROSE GEL ELECTROPHORESIS

0,8% agarose

0,8 g agarose in 100 ml TEA (10 mM Tris, 25 mM EDTA, pH 8 with acetic acid)

SOUTHERN BLOTTING

20x SSC

3 M NaCl

0,3 M Na Citrate pH 7 with HCl

Denaturing Solution

0,5 N NaOH

1,5 M NaCl

Neutralizing Solution

0,5 M Tris

1,5 M NaCl, pH 7,5 with HCl

HYBRIDIZATION OF FILTERS

Hybrization solution

6x SSC

5x Denhart's (see below)

0,5% SDS

sterile H_2O to 25 ml

100x Denhart's Solution

2% Ficoll 400

2% PVP (polyvinyl pyrrolidone)

2% Bovine Serum Albumin.

dissolve in H_2O

POST HYBRIDIZATION WASHES

Wash I (1L)

100 ml 20x SSC

10 ml 20% SDS

890 ml H_20

Wash II (1L)

945 ml H_20

5 ml 20% SDS

50 ml 20x SSC

SOLUTIONS FOR DEVELOPING X-RAY FILMS

Developer

Ilford Phenisol (Dilution: 1 + 4)

Supplier: Trimark

Stop buffer

glacial acetic acid (Dilution for 1L: 60 ml 50% glacial acetic acid + 940 ml H_2O)

Fixer

Amphix (Dilute: 1 + 4)

Supplier: Trimark

APPENDIX 2

Fragment sizes (base pairs) produced by single and double digests with different enzymes. "Extra" referred to the extra fragment present in two individuals of *H. m. broadleyi* as described in the text. (Fragments that hybridized to the 18s and 28s probes are given).

Enzyme: Eco RI

Taxon	Distance (mm)	Length	
	18s		
H. m. bro	71	5 346	
	110	2 212	
	114	2 153	extra
H. m. ver	56	7 362	
H. ang	54	7 423	
	28s		
H. m. bro	72	5 162	
H. m. ver	72	5 162	
H. ang	72	5 162	

Enzyme: Sac I

18s

H. m. bro	56	7 034
	146	1 073
H. m. ver	43	10 090
	146	1 073
H. ang	86	3 545
	95	2 772

28s

H. m. bro	56	7 034
	138	1 192
	146	1 073
H. m. ver	43	10 090
	138	1 192
	146	1 073
H. ang	90	3 067
	139	1 161

Enzyme: Eco RI + Sac I

18s

H. m. bro	93	3 166
	151	771
H. m. ver	65	6 348
	151	771
H. ang	92	3 238
	105	2 416

28s

H. m. bro	88	3 545
	136	1 232
H. m. ver	88	3 545
	136	1 232
H. ang	94	3 095
	139	1 161

Enzyme: Pvu II

18s

H. m. bro	35	12 940
H. m. ver	39	12 574
H. ang	55	6 648
	66	5 994

28s

H. m. bro	35	12 940
H. m. ver	39	12 574
H. ang	55	6 648
	66	5 994

Enzyme: Eco RI + Pvu II

18s

H. m. bro	73	5 024
	111	2 234
H. m. ver	61	6 835
H. ang	79	4 372
	101	2 706

28s

H. m. bro	84	4 112
	149	809
H. m. ver	84	4 112
	149	809
H. ang	84	4 112
	149	809

Enzyme: Bgl II

18s

H. m. bro	63	6 944
	81	4 506
	83	4 318 extra
H. m. ver	46	11 407
H. ang	62	7 024
	73	5 598

28s

H. m. bro	63	6 944
	81	4 506
	83	4 318
H. m. ver	46	11 407
H. ang	62	7 024
	73	5 598

Enzyme: Eco RI + Bgl II

18s

H. m. bro	84	3 582	
	110	2 110	
	116	1 950	extra
H. m. ver	56	6 820	
H. ang	69	4 969	
	88	3 296	

28s

H. m. bro	93	3 235
	123	1 838
H. m. ver	93	3 235
	123	1 838
H. ang	93	3 235
	123	1 838

Enzyme: Nco I

18s

H. m. bro	67	4 378
	74	3 883
	136	1 023
H. m. ver	42	8 102
	68	4 378
H. ang	41	8 230
	67	4 378

28s

H. m. bro	67	4 378
	74	3 883
H. m. ver	42	8 102
	68	4 378
H. ang	41	8 230
	67	4 378

Enzyme: Eco RI + Nco I

18s

H. m. bro	88	3 441
	127	1 545
	137	1 257
H. m. ver	68	5 827
	137	1 257
H. ang	66	6 128
	137	1 257

28s

H. m. bro	98	3 191
	107	1 982
H. m. ver	98	3 191
	107	1 982
H. ang	98	3 191
	107	1 982

Enzyme: Dra I

18s

H. m. bro	82	3 875
	93	3 108
	104	2 515
H. m. ver	82	3 875
	99	2 876
	104	2 515
H. ang	68	5 252
	82	3 875
	110	2 245

28s

H. m. bro	82	3 875
	93	3 108
H. m. ver	82	3 875
	99	2 876
H. ang	68	5 252
	82	3 875

Enzyme: Eco RI + Dra I

18s

H. m. bro	93	3 166
	137	1 101
	142	952
H. m. ver	103	2 528
	137	1 101
H. ang	96	2 959
	107	2 309
	137	1 101

28s

H. m. bro	99	2 866
	110	2 196
H. m. ver	99	2 866
	110	2 196
H. ang	99	2 866
	110	2 196

Enzyme: Bam HI

18s

H. m. bro	93	3 088
	107	2 406
H. m. ver	76	4 378
	107	2 406
H. ang	77	4 308
	92	3 128

28s

H. m. bro	93	3 088
	107	2 406
	116	1 995
H. m. ver	76	4 378
	107	2 406
H. ang	77	4 308
	92	3 128
	107	1 995

Enzyme: Eco RI + Bam HI

18s

H. m. bro	113	2 174
H. m. ver	113	2 174
H. ang	113	2 174

28s

H. m. bro	105	2 494
	115	2 071
H. m. ver	105	2 494
	115	2 071
H. ang	105	2 494
	107	2 472

Enzyme: Bcl I

18s

H. m. bro	62	7 343
	74	5 518
H. m. ver	33	13 040
H. ang	32	13 261

28s

H. m. bro	62	7 343
	74	5 518
H. m. ver	33	13 040
H. ang	32	13 261

Enzyme: Eco RI + Bcl I

18s

H. m. bro	74	3 979
	113	2 107
H. m. ver	59	7 194
H. ang	57	7 430

28s

H. m. bro	83	4 066
H. m. ver	83	4 066
H. ang	83	4 066

Enzyme: Bst EII

18s

H. m. bro	41	12 247
	140	1 226
H. m. ver	68	5 786
	73	5 153
	140	1 226
H. ang	44	11086
	140	1 226

28s

H. m. bro	41	12 247
H. m. ver	68	5 786
H. ang	44	11 086

Enzyme: Eco RI + Bst EII

18s

H. m. bro	110	2 269
	138	1 257
H. m. ver	70	5 207
	138	1 257
H. ang	63	6 294
	138	1 257

28s

H. m. bro	71	5 183
H. m. ver	71	5 183
H. ang	71	5 183

Enzyme: Pst I

18s

H. m. bro	55	8 113
	57	7 890
	103	2 727
H. m. ver	52	8 825
	91	3 484
H. ang	56	8 012
	106	2 137

28s

H. m. bro	55	8 113
	57	7 890
H. m. ver	52	8 825
H. ang	56	2 137

Enzyme: Eco RI + Pst I

18s

H. m. bro	100	2 824
	130	1 811
	146	903
H. m. ver	873	3 454
	117	2 765
	146	903
H. ang	104	2 558
	126	1 981
	146	903

28s

H. m. bro	71	5 123
H. m. ver	71	5 123
H. ang	71	5 123

H. m. bro = *Hyperolius marmoratus broadleyi*

H. m. ver = *Hyperolius marmoratus verrucosus*

H. ang = *Hyperolius angolensis*

APPENDIX 3

Abbreviations for enzymes

Bam HI	B
Bcl I	C
Bgl II	g
Bst EII	T
Eco RI	E
Dra I	D
Nco I	N
Pst I	P
Sac I	S
Pvu II	V