Description of electrophoretic loci and tissue specific gene expression in the horseshoe bat genus *Rhinolophus* (Rhinolophidae)

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Received 9 February 1994; accepted 19 October 1995

The number of loci encoding an enzyme system, tissue specificity of gene expression and the degree of gene expression in various tissues are often not mentioned in evolutionary studies, but could indirectly provide evidence of evolutionary relationships. Protein electrophoresis was used to study the distributions and tissue specificity of gene expression of enzymes encoded by 42 loci in *Rhinolophus clivosus* and *R. landeri*, the genetically most divergent of the ten species of southern African horseshoe bats. No differences in gene expression were found between *R. clivosus* and *R. landeri* and isozyme patterns may be compared with other bat species to derive possible phylogenetic relationships.

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Different categories of biochemical characters can be gathered from electrophoretic data and used in evolutionary studies (Buth 1984; Murphy & Crabtree 1985). In addition to allele frequency data, gene duplication, regulation of gene expression in different tissues and heteropolymer assembly of multimeric enzymes can be used to make phylogenetic inferences. Gene duplication data consist of the number of homologous genes present in an evolutionary lineage. The appearance of a new locus through gene duplication in a lineage can serve as a synapomorphy to identify taxa belonging to that lineage. Secondly, the distribution among taxa of tissue specific gene expression can also be used to infer phylogenetic relationships. The expression of some genes becomes tissue specific during development as embryonic cells differentiate into tissues, and tissue specific expression often characterizes a lineage. Such data have been used in the systematics of fishes (Shaklee & Whitt 1981), amphibians and reptiles (Buth, Murphy, Miyamoto & Wier 1985). Thirdly, after evolutionary recent gene duplication, the interlocus subunits of multimeric enzymes encoded by different loci may interact to form heteropolymers, because of the nucleotide sequence similarity between the loci. The ability to form heteropolymers is lost as the sequences diverge from one another. The electrophoretic mobilities of the heteropolymers are intermediate to the products of the two loci and depend on the number of each kind of subunit in the heteropolymer. The absence of interlocus heteropolymers is considered to be a derived condition (Murphy & Crabtree 1985). However, patterns of heteropolymer assembly may be difficult to assess in cases where the products of different loci are expressed in different tissues.

Patterns of gene expression vary in early development but are stable in adults. Therefore investigations within and between taxa should use only individuals belonging to the same developmental stage (Shaklee & Whitt 1981). In this study, we investigated the expression of loci encoding isozymes in adult representatives of *Rhinolophus clivosus* and *R. landeri*, the species which appear to be the most distantly related of the ten southern African species of *Rhinolophus* bats (Maree & Grant in preparation). We specifically investigated (i) the number of loci encoding a protein system, (ii) gene expression among tissues and (iii) patterns of interlocus heteropolymer assembly.

Materials and Methods

We inferred the degree and tissue specificity of gene expression after the electrophoresis of extracts from tissues representing each embryonic germ layer. Brain represented ectoderm, heart, skeletal muscle and kidney represented mesoderm, and lung, liver and spleen represented endoderm. Tissue samples of *R. clivosus* (20 bats) and *R. landeri* (2 bats) were analysed. Enzyme systems encoded by 42 presumptive loci, Enzyme Commission numbers (E.C.), locus abbreviations and appropriate buffers are presented in Table 1.

Preparation, electrophoresis and staining of tissues and gels

Bats were collected from natural populations in caves by mistnets or by hand, or by covering the entrances to caves with nets. Outside, mistnets hoisted on catamaran masts were used. Tissues were taken soon after sacrificing and were quick frozen in liquid nitrogen and stored at –70°C. A comparison of electrophoretic banding patterns in fresh tissues and in tissues stored with ultra cold storage for several months revealed no substantial differences.

Horizontal starch gel electrophoresis was used (Selander, Smith, Yang, Johnson & Gentry 1971). Tissues were homogenized with a metal rod in equal volumes of 0,lM tris-HCL pH 8,0; and 0,001M pyridoxal-5-phosphate and centrifuged at 10000*g* for 3 min. A 50/50 mixture of Sigma (St. Louis, Missouri, USA) and StarchArt (Smithville, Texas, USA) hydrolysed potato starch and an appropriate gel buffer were used to make 13% gels (w:vol). One discontinuous electrophoretic buffer system (Ridgway, Sherburne & Lewis 1970, TCL–Tris-citrate/Lithium hydroxide-borate) and three continuous buffer systems (Whitt 1970, TC–Tris-citrate; Markert & Faulhaber 1965, TBE–Tris-borate-EDTA; Whitt 1970, TC/P–Tris-citrate-phosphate) were used. Extracts of the enzymes were applied to the gel with wicks of filterpaper. Proteins were separated by electrophoresis in a cold room (± 4°C) at 6–8 V/cm.

After electrophoresis, gels were sliced horizontally and stained. Stain solutions were applied to the gel mixed 1:1 with

boiling 2% (W/V) solution of technical grade, granular agar dissolved in distilled water. Gels were placed in a dark incubator at ± 45°C. Specific histochemical protein stains followed recipes in Selander *et al.* (1971), Harris & Hopkinson (1976) and Grant (1990).

General nomenclature

Enzyme nomenclature followed the recommendations of the Nomenclature Committee of the International Union of Biochemistry (1984). Upper case letters designated an enzyme system in a general sense: e.g. malate dehydrogenase – MDH. Loci were designated on the basis of evolutionary nomenclature inferred from tissue distributions of gene expression. When homologies with other organisms were known, an enzyme locus was designated with evolutionary nomenclature; e.g. *Ck-A* is the locus of the CK enzyme system expressed predominantly in skeletal muscle. When locus homologies with other bats or related organisms were unknown, loci were numbered according to the mobilities of their products starting at the anodal end of the gel. The supernatant form of a duplicated locus was indicated by prefixing an *S* to the abbreviation and the mitochondrial form by prefixing an *M* (Engel, Schmidtke, Vogel & Wolf 1973). Peptidase loci were given the letter designations of Harris & Hopkinson (1976) corresponding to their substrate specificities. Peptidase-A (substrate: leucyl-tyrosine-2 (*Pep-A*)), Peptidase-B (substrate: DL-leucyl-glycyl-glycine (*Pep-B*)) Peptidase-C (substrate: leucyl-tyrosine-1 (*Pep-C*)) and Peptidase-D (substrate: phenylalanyl-proline (*Pep-D*)). Alleles encoding allozymes were designated as with mobilities relative to the most common allele which was designated 100.

Results and Discussion

Tissue distributions of gene expression and relative banding intensities of enzymes encoded by 42 loci are shown in Table 1. The tissues have been grouped according to embryonic origins from the three germ layers.

Two loci, *Gpd-1* and *Pgd-3*, encoded enzymes found in only mesodermal tissues and were restricted to skeletal and heart muscles. The expression of one locus (*L-Idh-1*) was restricted to endodermal tissue, specifically to liver, and no locus was expressed exclusively in tissues of ectodermal origin. Of the remaining 39 loci, 23 were expressed in one or more tissues derived from each germ layer. The products of 13 loci were found in only endodermal and mesodermal tissues. One locus, *Ck-A*, was expressed in only ectoderm and mesoderm, and *Gdh-2* was expressed in only ectoderm and endoderm. One locus, *Gap-B*, was found in only ectoderm. The numbers of loci expressed in each tissue were as follows: brain (26), skeletal muscle (35), heart (34), kidney (35), lung (24), liver (36) and spleen (27). Where possible, patterns of tissue expression were compared with those of *Rhinolophus* from Thailand, Jordan and Japan (Qumsiyeh, Owen & Chesser 1988), New World bats (superfamily Phyllostomoidea) (Arnold, Honeycutt, Baker, Sarich & Knox-Jones 1982), seven species of Antillean bats, of the subfamily Brachyphyllinae (Phyllostomidae) (Baker, Honeycutt, Arnold, Sarich & Genoways 1981) and species of *Lasiurus* (Phyllostomidae) (Baker, Patton, Genoways & Bickham 1988). Additional comparisons were made with rattlesnakes (*Crotalus viridis*) (Murphy & Crabtree 1985), tuatara (*Sphenodon punctatus*) (Murphy & Matson 1986), alligators (*Alligator mississippiensis*) (Dessauer & Densmore 1983), lizards of the families Xantusiidae (Bezy & Sites 1987) and Cordylidae (Brody 1991), the old-field mouse (*Peromyscus polionotus*) (Selander *et al.* 1971), birds, humans and other chordates (Harris & Hopkinson 1976). These comparisons may indicate evolutionary polarity of the gene expression and may give a perspective on the evolution of isozyme loci in *Rhinolophus*.

Aspartate aminotransferase (AAT 2.6.1.1) catalyses the conversion of 2-oxaloglutarate to L-glutamate (Harris & Hopkinson 1976). In *Rhinolophus* a cathodal, monomorphic enzyme (*M-Aat-1*) was strongly expressed in skeletal muscle and was presumably the mitochondrial form of AAT. A second anodal enzyme (*S-Aat-1*) was polymorphic and most prominent in skeletal muscle and liver and presumably represented the supernatant form of AAT. Two apparently homologous polymorphic loci occur in phyllostomoid bats (Arnold *et al.* 1982), and bats of the Brachyphyllinae (Baker *et al.* 1981). Supernatant and mitochondrial forms of AAT are found in alligators (Dessauer & Densmore 1983), lizards of the Xantusiidae (Bezy & Sites 1987) and *Cordylus* (Brody 1991). Two autosomal loci are reported in humans: a mitochondrial enzyme and a supernatant enzyme of AAT which are both dimeric (Harris & Hopkinson 1976).

Adenylate kinase (AK 2.7.4.3) catalyses the reversible conversion of adenosine diphosphate (ADP) to adenosine triphosphate (ATP) and adenosine monophosphate (AMP) (Harris & Hopkinson 1976). In *Rhinolophus* the products of two anodal loci were detected. A fast polymorphic enzyme (*Ak-1*), was most active in skeletal muscle and moderately active in heart and kidney. A second polymorphic enzyme (*Ak-2*) was most prominent in skeletal muscle. Satellite bands were observed for both enzymes, but specifically reflected variation at *Ak-2*. One *Ak* locus is expressed in all tissues of *Cordylus* (Brody 1991), and alligators (Dessauer & Densmore 1983) and may represent a derived condition which probably resulted from the loss or silencing of one locus. Two autosomal loci encoding different sets of isozymes are found in most tissues of humans (Harris & Hopkinson 1976).

Creatine kinase (CK 2.7.3.2) is a dimer and catalyses the reversible conversion of creatine to phosphocreatine. In humans, two loci produce three main forms: a cathodal homodimer expressed in muscle (*Ck-MM*), an intermediate heterodimer expressed in heart (*Ck-MB*) and an anodal homodimer expressed in brain (*Ck-BB*) (Harris & Hopkinson 1976). In *Rhinolophus* two monomorphic loci encoded CK (Figure 1). The upper anodal enzyme (*Ck-B*) is most active in brain and moderately active in skeletal muscle, kidney and spleen. The second anodal enzyme (*Ck-A*) is almost exclusively expressed in skeletal muscle, and only slightly active in brain and heart. Extra bands above the banding zone of *Ck-A* may be satellite bands (Harris & Hopkinson 1976). Faint bands below the bands of *Ck-B* may have been satellite bands, or the intermediate heterodimeric form of CK similar to that observed in heart muscle of humans (*Ck-MB*), as both loci were expressed in brain and skeletal muscle. One anodal, polymorphic enzyme is active in heart and kidney muscles and liver of *Rhinolophus* from Thailand, Jordan and Japan (Qumsiyeh *et al.* 1988) and may represent *Ck-B*.

Table 1 Tissue distribution and relative intensity of enzyme activity in 7 tissues of *Rhinolophus clivosus* and *R. landeri* as representatives of the 10 southern African *Rhinolophus* species. Relative enzyme activities compared as follows: 0 = no activity; – = slight activity, not scorable; + = slight activity, scorable; + + = moderate activity; +++ = strong activity; ++++ = very strong activity. Enzyme Commission Numbers, locus abbreviations and buffer systems used are given. * = Polymorphic loci

	Enzyme			Ectoderm	Mesoderm			Endoderm		
Enzyme	number	Locus	Buffer	Brain	Muscle	Heart	Kidney	Lung	Liver	Spleen
Aspartate	2.6.1.1	*S-Aat-1	TC	–	+++	+	+	–	+++	–
aminotransferase		M-Aat-1	TC	–	++++	–	+	0	–	–
Adenylate kinase	2.7.4.3	*Ak-1	TC	0	+++	++	++	0	–	0
		*Ak-2	TC	–	++++	–	–	–	++	0
Creatine kinase	2.7.3.2	Ck-A	TCL	–	++++	–	0	0	0	0
		Ck-B	TCL	++++	++	–	++	–	+	++
Esterase	3.1.1.1	*Est-1	TCL	+++	0	+	++	++	–	–
		*Est-2	TCL	0	+++	0	++	0	++	+
		*Est-3	TCL	0	++++	+	++	+	+++	+
Glucose dehydrogenase	1.1.1.47	Gdh-1	TBE	0	–	+++	–	0	0	–
		Gdh-2	TBE	++	0	0	0	0	+	0
Glucose phosphate isomerase	5.3.1.9	Gpi-1	TCL	+++	++++	0	++++	+++	++++	++
Glutamate-pyruvate transaminase	2.6.1.2	*Gpt-1	TCL	0	+++	–	+	0	++++	0
Glyceraldehyde-	1.2.1.12	Gap-A	TC/P	–	++++	–	+++	–	+++	++
phosphate dehydrogenase		Gap-B	TC/P	+	0	0	0	0	0	0
Glycerol-3-phosphate	1.1.1.8	Gpd-1	TC	0	++	+	0	0	0	0
dehydrogenase		*Gpd-2	TC	–	++++	–	+++	++	+++	–
Isocitrate dehydrogenase	1.1.1.42	*Icd-A	TC	0	++++	+++	–	–	++	–
		*Icd-B	TC	–	+	0	+++	–	+++	+
L-Iditol dehydrogenase	1.1.1.14	*L-Idh-1	TCL	0	0	0	0	0	+++	0
Lactate dehydrogenase	1.1.1.27	*Ldh-A	TCL	–	++	0	++	–	–	–
		*Ldh-B	TCL	+++	++++	++++	+++	++	++	+
Malate dehydrogenase	1.1.1.37	M-Mdh-1	TC	+	+++	++	+	–	++++	++
		S-Mdh-1	TC	++	++++	++++	+++	++	++++	++
Malic enzyme	1.1.1.40	*S-Me-1	TC/P	+++	++++	++++	+++	–	–	0
		*S-Me-2	TC/P	++	++	–	++	+	–	++
		M-Me-1	TC/P	0	+++	+	+	0	–	0
Mannose phosphate isomerase	5.3.1.8	*Mpi-1	TBE	++++	++++	++	++++	++	++++	+++
'Nothing' dehydrogenase		Ndh-1	TBE	0	–	0	–	–	++	+
Nucleoside phosphorylase	2.4.2.1	Np-1	TBE	+++	0	+++	0	++	+++	0
		*Np-2	TBE	0	++++	0	+++	0	–	–
		Np-3	TBE	0	0	+++	+++	+++	0	0
Peptidase A	3.4.11/13	*Pep-A	TBE	++++	+++	+++	+	0	++	–
Peptidase B		*Pep-B	TBE	0	++	++	+	0	+++	0
Peptidase C		*Pep-C	TBE	+++	++	–	+++	0	++	–
Peptidase D	3.4.13.9	*Pep-D	TBE	++	+++	++	++++	–	+++	++
Phosphoglucomutase	2.7.5.1	*Pgm-1	TCL	+	+	++	+	++	++	++
		*Pgm-2	TCL	0	++++	–	–	0	++	+
6-Phosphogluconate	1.1.1.44	*Pgd-1	TC/P	0	0	++	++	0	++	0
dehydrogenase		*Pgd-2	TC/P	+++	+	+	+++	++	++++	++++
		*Pgd-3	TC/P	0	++++	++	0	0	0	0
Superoxide dismutase	1.15.1.1	Sod-1	TBE	+++	++++	++	++++	++	++++	+++

The transition from a single *Ck* locus in invertebrates to two *Ck* loci (*Ck-A* and *Ck-C*), which are orthologous to those present in all vertebrates, probably occurred early in the chordate line. Two loci are expressed in a generalized fashion in

Figure 1 Creatine kinase (CK) isozyme patterns resolved from brain (B), skeletal muscle (M), heart (H), kidney (K), lung (Lu), liver (Li) and spleen (S) tissue extracts of *R. clivosus* and *R. landeri* from southern Africa.

all tissues of mammals (Fisher & Whitt 1978). Four *Ck* loci occur in advanced bony fishes (Teleostei) (*Ck-A, Ck-B, Ck-C* and *Ck-D*) with an increase in tissue specificity of expression. In birds (Dawson, Eppenberger & Kaplan 1967), rattlesnakes (Murphy & Crabtree 1985), the tuatara (Murphy & Matson 1986) and *Cordylus* (Brody 1991), two *Ck* loci (*Ck-A* and *Ck-C*) are expressed in skeletal muscle, brain and stomach tissue, respectively.

Esterases (EST 3.1.1.1) catalyses the conversion of 4-methyl umbelliferyl ester to a carboxylic acid anion and 4-methyl umbelliferone, or α-naphthyl ester to a carboxylic acid anion and α-naphthol. In humans, nine sets of acetylesterases and four sets of butylesterases are encoded by six loci and three loci, respectively (Harris & Hopkinson 1976). In *Rhinolophus* of southern Africa, three anodal acetylesterases (*Est-1, Est-2* and *Est-3*) were found using an acetate substrate. Double-banded heterozygotes in *R. clivosus* suggest that *Est-1* encodes a monomeric enzyme. In humans, *Esa-1* is expressed in red blood cells, and *Esa-5* in brain (Harris & Hopkinson 1976) which correspond with the tissue expression in *Rhinolophus* where *Est-1* was strongly expressed in brain and to a lesser extent in all other tissues except skeletal muscle. A second *Est-2* locus was strongly expressed in skeletal muscle with lesser activity in kidney and liver. The subunit structure of this polymorphic enzyme is probably dimeric as indicated by a triple-banded heterozygote in *R. fumigatus* (Maree & Grant in preparation). The *Est-2* locus is probably homologous to the *Esd* locus of humans judged by their overlapping tissue expression. The *Est-3* locus probably encodes a monomeric enzyme as was indicated by double-banded heterozygotes in *R. clivosus*. Aside from brain, *Est-3* was expressed in all tissues and was strongest in skeletal muscle. This locus may be homologous with *Esa-3* expressed in red blood cells of humans. The similar patterns of tissue expression of the *Est* locus in *Rhinolophus* from Thailand, Jordan and Japan (Qumsiyeh *et al.* 1988) and *Est-2* of southern African rhinolophids suggest that they are homologous loci. Three loci encode esterases in the tuatara (Murphy & Matson 1986) and rattlesnakes (Murphy & Crabtree 1985) and seven loci encode esterases in *Cordylus* (Brody 1991).

Glucose dehydrogenase (GDH 1.1.1.47) is a dimer and catalyses the conversion of glucose to gluconic acid (Harris & Hopkinson 1976). Two *Gdh* loci encode anodal enzymes in *Rhinolophus*. The fast anodal enzyme (*Gdh-1*) was monomorphic and strongly expressed in heart muscle. *Gdh-2* was moderately expressed in brain, but was not resolved well enough to distinguish genotypes. This restriction of tissue expression may be a derived condition in bats. In *Cordylus*, one monomorphic enzyme is found in tissues of endodermal origin, and there is a possibility of a second fast anodal enzyme expressed in the femoral gland (ectoderm) (Brody 1991). Similar patterns of tissue expression suggest that these *Gdh* loci may be homologous with the loci encoding GDH in *Rhinolophus*. In the tuatara (Murphy & Matson 1986) and rattlesnakes (Murphy & Crabtree 1985), one locus (*Gdh-A*) is expressed in tissues of endodermal and mesodermal origin. In humans, one *Gdh* locus encodes a monomorphic enzyme which is distributed in all tissues except red cells. One polymorphic *Gdh* locus encodes GDH in the deer mouse (*Peromyscus maniculatus*) and the house mouse (*Mus musculus*) (Harris & Hopkinson 1976).

Glucose phosphate isomerase (GPI 5.3.1.9) catalyses the conversion of fructose-6-phosphate to glucose-6-phosphate in glycolysis (Harris & Hopkinson 1976). In southern African rhinolophids, one locus (*Gpi-1*) encoded a monomorphic, cathodal enzyme which was very active in all tissues examined, except for heart (Figure 2). Qumsiyeh *et al.* (1988) reported that a polymorphic *Gpi-1* locus encoded anodal isozymes, and *Gpi-2* encoded cathodal isozymes of GPI in *Rhinolophus* species from Thailand, Jordan and Japan. However, only a single *Gpi* locus is expressed in mammals (Fisher, Shaklee, Ferris & Whitt 1980), and therefore the second set of bands observed by Qumsiyeh *et al.* (1988) are most probably satellite bands of *Gpi-1* and not a second locus encoding GPI. One polymorphic *Gpi* locus encodes GPI in *Lasiurus* (Baker *et al.* 1988). A single *Gpi* locus is expressed in tissues derived from all three germ layers in rattlesnakes (Murphy & Crabtree 1985) and alligators (Dessauer & Densmore 1983). In humans, autosomal isozymes of GPI migrate cathodally in all tissues (Harris & Hopkinson 1976).

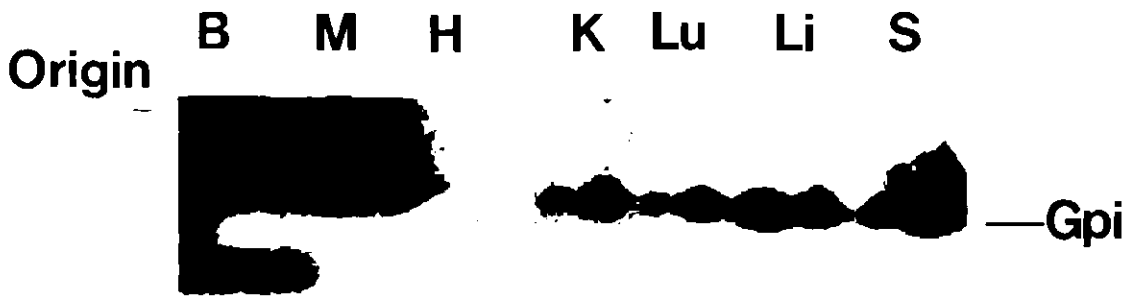

Figure 2 Glucose phosphate isomerase (GPI) isozyme patterns resolved from brain (B), skeletal muscle (M), heart (H), kidney (K), lung (Lu), liver (Li) and spleen (S) tissue extracts of *R. clivosus* and *R. landeri* from southern Africa.

Glutamate pyruvate transaminase (GPT 2.6.1.2) is a dimeric enzyme and catalyses the conversion of 2-oxoglutarate to glutamate. In humans, autosomal isozymes of one locus migrate anodally and appear in all tissues (Harris & Hopkinson 1976). In *Rhinolophus* of southern Africa, one polymorphic *Gpt-1* locus encoded an anodal enzyme predominantly expressed in liver and skeletal muscle. The more specific tissue expressions of *Gpt-1* in bats may be a derived condition.

Glyceraldehyde-phosphate dehydrogenase (GAP 1.2.1.12) is a tetramer and catalyses the conversion of D-glyceraldehyde-3-phosphate and orthophosphate to 3-phospho-D-glycerol phosphate (Harris & Hopkinson 1976). In *Rhinolophus* of southern Africa, one locus (*Gap-B*) encoded an anodal enzyme with multiple satellite bands and was restricted to expression in brain, but was not resolved well enough to distinguish genotypes. A second cathodal monomorphic enzyme was encoded by *Gap-A* and was most prominent in skeletal muscle, kidney and liver. In *Cordylus*, *Gap-A*, encodes an anodal polymorphic enzyme which was strongly expressed in skeletal and heart muscles (Brody 1991). In humans, an anodal, almost exclusively monomorphic enzyme is expressed in all tissues (Harris & Hopkinson 1976).

Glycerol-3-phosphate dehydrogenase (GPD 1.1.1.8) is a dimer and catalyses the conversion of glycerol-3-phosphate to dihydroxyacetone phosphate. Harris & Hopkinson (1976) report that two autosomal loci (*Gpd-1* and *Gpd-2*), strongly expressed in skeletal muscle, encode GPD in humans. GPD is however not consistently encoded by two loci in all mammals (Hopkinson, Peters & Harris 1974). Two loci encoded GPD in *Rhinolophus* of southern Africa. A monomorphic locus (*Gpd-1*) encoded a fast anodal enzyme which was moderately active in skeletal muscle and slightly active in heart. A polymorphic locus (*Gpd-2*) encoded an anodal enzyme which was most prominent in skeletal muscle, kidney and liver. Interlocus heteropolymers were observed in especially muscle tissue. One polymorphic locus (*Gpd-1*) encodes GPD in phyllostomoid bats (Arnold *et al.* 1982), *Lasiurus* (Baker *et al.* 1988), bats of the Brachyphyllinae (Baker *et al.* 1981) and *Rhinolophus* of Thailand, Jordan and Japan (Qumsiyeh *et al.* 1988). The silencing of one locus may be a derived condition in some bat species. *Gpd-A* expressed in skeletal muscle, and *Gpd-B* expressed in liver encode GPD in fishes (Fisher *et al.* 1980), primitive reptiles and rattlesnakes (Murphy & Crabtree 1985). These loci are presumably homologous to *Gpd-1* and *Gpd-2* of *Rhinolophus* and humans judged by their patterns of tissue expression. One locus encodes GPD in the whiptail lizard (*Cnemidophorus tigris*) (Dessauer & Cole 1984), and *Cordylus* (Brody 1991).

Isocitrate dehydrogenase (ICD 1.1.1.42) is a dimer and catalyses the conversion of isocitrate to 2-oxoglutarate. Harris & Hopkinson (1976) report two *Icd* loci in adult tissue of humans: an anodal, supernatant enzyme (*S-Icd*) and a mitochondrial enzyme (*M-Icd*) close to the origin. A similar pattern was found in *Rhinolophus*, from southern Africa, except for the cathodal migration of the slow electromorph (*Icd-A*) to a position just beneath the origin (Figure 3). *Icd-A* is probably the mitochondrial form of ICD and was strongly expressed in muscle and heart. *Icd-B* encodes an anodal polymorphic enzyme which is probably the soluble form of ICD and is expressed in all tissues except heart. Two polymorphic loci with similar migratory patterns are reported for *Lasiurus* (Baker *et al.* 1988) and phyllostomoid bats (Arnold *et al.* 1982). These loci may be homologous with the two *Icd* loci in *Rhinolophus*. ICD is encoded by one locus in primitive reptiles, but the general rule for advanced reptiles is two loci (Brody 1991). Two loci both restricted to expression in liver encode ICD in rattlesnakes. Following general trends of mitochondrial products being more slowly migrating than soluble forms, *M-Icdh-A* of rattlesnakes is probably homologous with *Icd-A* and *S-Icdh-A* homologous with *Icd-B* of bats.

L-Iditol dehydrogenase (L-IDH 1.1.1.14) is a tetramer and catalyses the conversion of sorbitol to sorbose in the presence of NAD (Harris & Hopkinson 1976). One autosomal *L-Idh-1* locus encoded an anodal polymorphic enzyme of L-IDH in southern African *Rhinolophus*. This locus is probably homologous to the locus in bats of the Brachyphyllinae (Baker *et al.* 1981), rattlesnakes (Murphy & Crabtree 1985), *Cordylus* (Brody 1991), *Anura*, other higher land vertebrates (Duellman & Hillis 1987) and humans (Harris & Hopkinson 1976). Qumsiyeh *et al.* (1988) report one cathodal, polymorphic locus encoding L-IDH in *Rhinolophus* from Thailand, Jordan and Japan, and *R. clivosus* and *R. darlingi* from southern Africa. The difference in migration of the enzyme may be the consequence of using a different electrophoretic buffer system. In *Rhinolophus* and rattlesnakes (Murphy & Crabtree 1985) expression of *L-Idh* was restricted to liver tissue and may be a derived condition. The enzyme was active in kidney, duodenum and liver in *Cordylus* (Brody 1991) and in most tissues of humans (Harris & Hopkinson 1976).

Lactate dehydrogenase (LDH 1.1.1.27). This tetrameric enzyme has five sets of isozymes encoded by two loci in

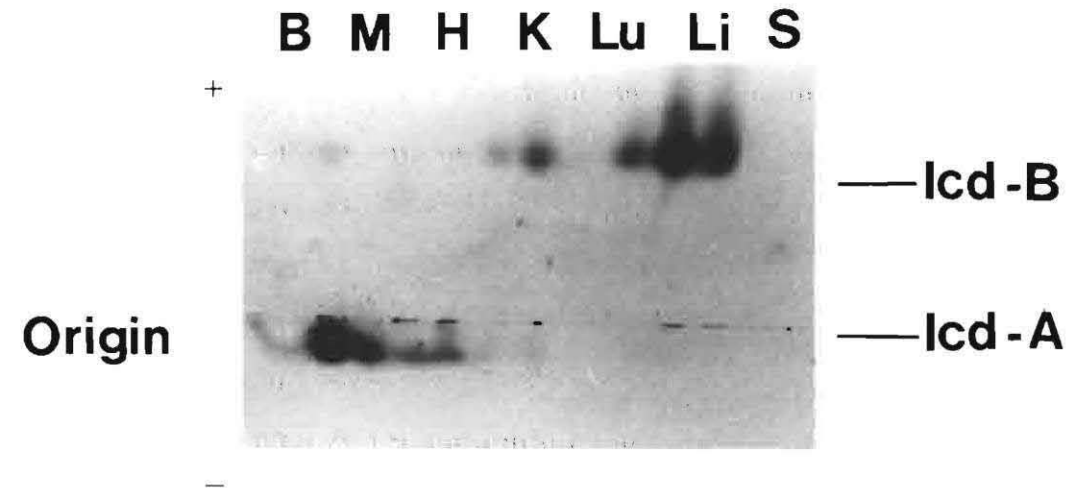

Figure 3 Isocitrate dehydrogenase (ICD) isozyme patterns resolved from brain (B), skeletal muscle (M), heart (H), kidney (K), lung (Lu), liver (Li) and spleen (S) tissue extracts of *R. clivosus* and *R. landeri* from southern Africa.

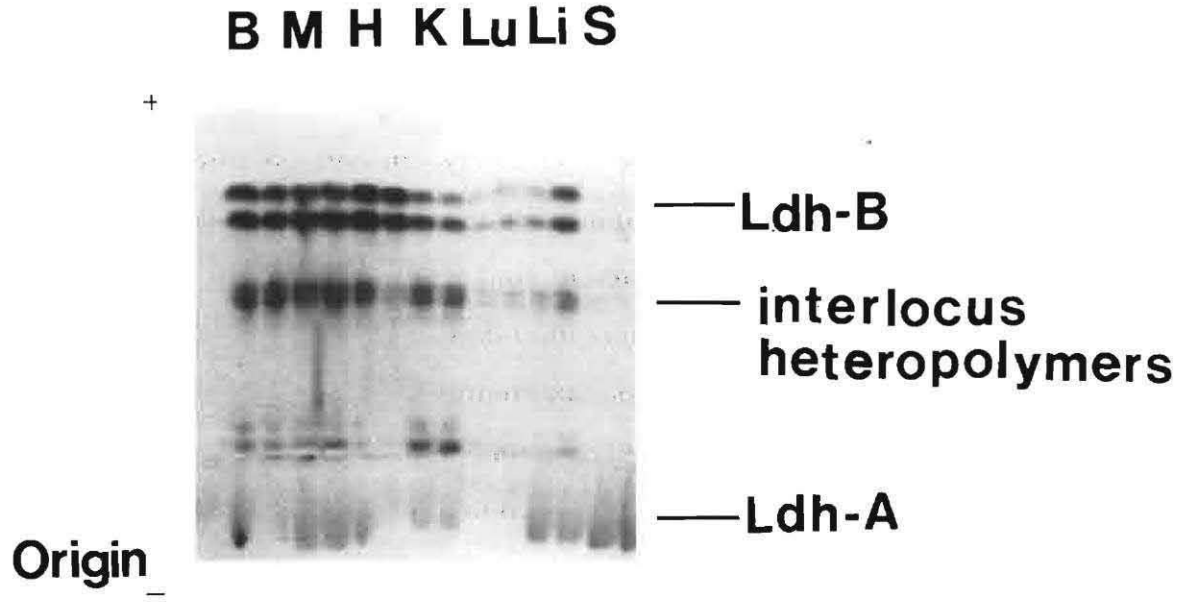

Figure 4 Lactate dehydrogenase (LDH) isozyme patterns resolved from brain (B), skeletal muscle (M), heart (H), kidney (K), lung (Lu), liver (Li) and spleen (S) tissue extracts of *R. clivosus* and *R. landeri* from southern Africa.

humans. In later fetal life, *Ldh-A* polypeptides increase in heart muscle and *Ldh-B* in liver (Harris & Hopkinson 1976). A third *Ldh* locus is expressed in some mammals and birds (Fisher *et al.* 1980). Two polymorphic loci encoded LDH in *Rhinolophus* and are probably homologous to the two loci in humans (Figure 4). The anodal enzyme, *Ldh-B*, is probably the same enzyme designated *Ldh-1* by Qumsiyeh *et al.* (1988) in *Rhinolophus* from Thailand, Jordan and Japan. Isozymes of a second locus (*Ldh-A*) migrated both cathodally and anodally, and were expressed in all tissue types except heart and was most active in skeletal muscle and kidney. *Ldh-B* was strongly expressed in all tissue types but was less active in tissues of endodermal origin. Interlocus heteropolymers with intermediate electrophoretic mobility appeared. In phyllostomoid bats (Arnold *et al.* 1982) and bats of the Brachyphyllinae (Baker *et al.* 1981), two loci (*Ldh-1*) and (*Ldh-2*) encode LDH. These loci are probably homologous with *Ldh-A* and *Ldh-B* of *Rhinolophus* of southern Africa. *Ldh-A* is most prominently expressed in skeletal muscle and liver tissue of *Cordylus* and rattlesnakes. In *Cordylus*, *Ldh-B* is strongly expressed in all tissues but strongest in heart (Brody 1991), and in rattlesnakes, the enzyme is most active in heart and kidney tissues (Murphy & Crabtree 1985). Tissue expression of LDH in reptiles is similar to that in bats and humans and may indicate homologous loci.

Malate dehydrogenase (MDH 1.1.1.37) is a dimer and catalyses the conversion of L-malate to oxaloacetate (Harris & Hopkinson 1976). MDH is encoded by two loci in most higher vertebrates (Fisher *et al.* 1980). Two monomorphic loci encoded an anodal, *S-Mdh-1*, and a cathodal, *M-Mdh-1*, form of MDH in southern African *Rhinolophus* and in species from Thailand, Jordan and Japan (Qumsiyeh *et al.* 1988). In southern African *Rhinolophus*, both loci were expressed in all tissues examined. Enzymes encoded by *S-Mdh-1* were most

active in tissues of mesodermal origin and liver, and enzymes encoded by *M-Mdh-1* were predominantly expressed in muscle and liver. Two loci encode MDH in *Cordylus* and are active in tissues similar to those in *Rhinolophus*, except for reduced activity in liver (Brody 1991). In rattlesnakes, however, *S-Mdh* is almost exclusively expressed in heart (Murphy & Crabtree 1985).

Malic enzyme (ME 1.1.1.40) is a tetramer and catalyses the conversion of L-malate to pyruvate. Harris & Hopkinson (1976) report two autosomal loci in humans which encode a slow, anodal mitochondrial enzyme (*M-Me*) and a fast, anodal supernatant enzyme (*S-Me*). In mice, however, the soluble enzyme is less anodal than the mitochondrial enzyme. Two loci, probably homologous to those in bats and humans, encode a mitochondrial and a supernatant form of ME in reptiles (Murphy & Crabtree 1985; Brody 1991). Three loci encode ME in southern African *Rhinolophus* species. The supernatant form of ME was represented by a fast enzyme (*S-Me-1*) and a slower enzyme (*S-Me-2*). These two polymorphic loci are probably homologous to two interconvertible *S-Me* loci in humans (Harris & Hopkinson 1976). The third locus *M-Me-1* encoded a monomorphic, cathodal enzyme of ME. Qumsiyeh *et al.* (1988) report one polymorphic locus, *S-Me*, encoding an anodal enzyme of ME, for *Rhinolophus* from Thailand, Jordan and Japan, but it was impossible to determine which of the two soluble forms of ME it represented. Different groups of animals have different patterns of tissue specificity for enzymes encoded by *M-Me*. In *Rhinolophus* species, *M-Me-1* was strongly expressed in skeletal muscle and was absent from tissues of endodermal and ectodermal origin, except for liver. In humans, the enzyme is expressed in tissues derived from all three germ layers (Harris & Hopkinson 1976). In rattlesnakes, *M-Me* is strongly expressed in heart, kidney and brain and is absent from tissues of endodermal origin. In *Cordylus*, *Me-2* presumably represents the mitochondrial form of ME and is strongly expressed in tissues of endodermal origin (Brody 1991). In *Rhinolophus*, *S-Me-1* was strongly expressed in brain, muscle, heart and kidney. This ME locus is probably homologous to both *S-Me-A* in rattlesnakes where expression of the locus is restricted to heart (Murphy & Crabtree 1985), and *Me-1* in *Cordylus* which is expressed in most tissues (Brody 1991). In *Rhinolophus*, the enzyme encoded by *S-Me-2* was present in all tissues.

Mannose phosphate isomerase (MPI 5.3.1.8) is a monomer and catalyses the conversion of mannose-6-phosphate to fructose-6-phosphate (Harris & Hopkinson 1976). In *Rhinolophus* of southern Africa, one *Mpi-1* locus encoded a polymorphic enzyme which was strongly expressed in all tissues examined, except for heart and lung where the enzyme was moderately active. One autosomal locus encodes a polymorphic enzyme of MPI, expressed in all tissues of phyllostomoid bats (Arnold *et al.* 1982), humans (Harris & Hopkinson 1976), *Cordylus* (Brody 1991) and other reptiles (Murphy & Crab-tree 1985). In rattlesnakes, *Mpi-A* is absent from brain and lung tissue, and a fast electromorph is restricted to expression in skeletal muscle. Murphy & Crabtree (1985) ascribe its presence to some form of post-translational modification.

'Nothing' dehydrogenase (NDH). In *Rhinolophus*, a single cathodal banding zone appeared on gels stained for GPD, MDH and MPI. All of these enzyme stains contained phenozinemethosulphate (PMS), tetrazolium and either NAD or NADP, which could act as a substrate for NDH. Stronger activity was observed on gels with NAD than on those with NADP. Tissue specificity of expression showed strong activity of *Ndh-1* in liver, and slight activity in muscle, kidney, lung and spleen. In *Cordylus*, the enzyme is restricted to expression in liver (Brody 1991).

Nucleoside phosphorylase (NP 2.4.2.1) is a trimer and catalyses the conversion of inosine to hypoxanthine (Harris & Hopkinson 1976). Three autosomal loci encoded NP in *Rhinolophus* of southern Africa (Figure 5). The most anodal enzyme encoded by *Np-1* was monomorphic and was restricted to expression in brain, heart, lung and liver tissues. *Np-2* encoded a slow anodal, polymorphic enzyme which was strongly expressed in skeletal muscle and kidney. The third locus (*Np-3*) encoded a monomorphic enzyme which was active in heart, kidney and lung tissues. No interlocus heteropolymers were formed. In humans, the least anodal banding zone of NP is designated the primary isozyme and secondary

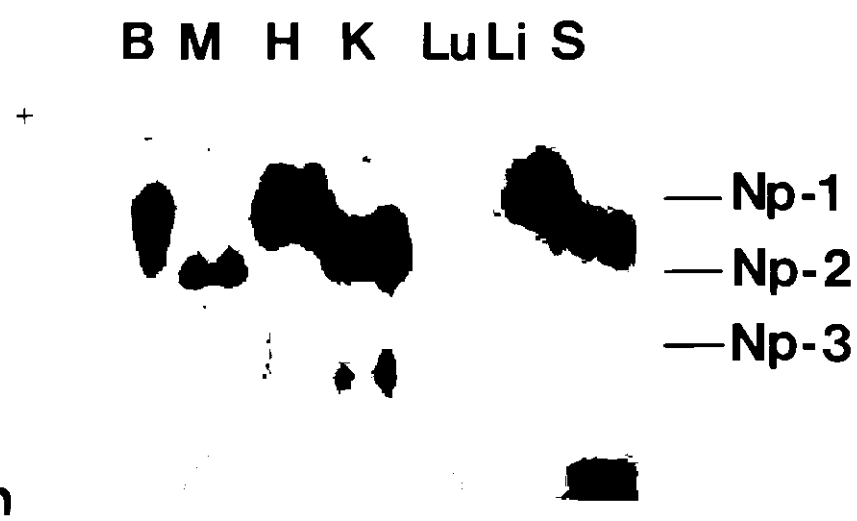

Figure 5 Nucleoside phosphorylase (NP) isozyme patterns resolved from brain (B), skeletal muscle (M), heart (H), kidney (K), lung (Lu), liver (Li) and spleen (S) tissue extracts of *R. clivosus* and *R. landeri* from southern Africa.

isozymes occur anodal to it (Harris & Hopkinson 1976). One locus encodes NP in rattlesnakes and *Cordylus*, and is expressed in tissues derived from ectodermal and endodermal origins in rattlesnakes (Murphy & Crabtree 1985). One locus is slightly active in tissues of mesodermal and endodermal origins in *Cordylus* (Brody 1991).

Peptidase (PEP A,B,C,D 3.4.11 or 13). The staining system for peptidases depends on the release of an L-amino acid using a peptide as a substrate. In humans, peptidase A,B,C,D and E are expressed in virtually all tissues, but there are consistent differences between tissues in overall and relative activities of the different peptidases (Harris & Hopkinson 1976). Peptidase A,B,C and D were successfully stained for in *Rhinolophus* of southern Africa.

Peptidase A is a monomer and was encoded by one autosomal locus (*Pep-A*), using the dipeptide Leucyl-tyrosine (Leu-Tyr) as a substrate. This polymorphic enzyme was most active in brain, muscle and heart, and less active in liver, kidney and spleen. Tissue specificity of expression differs from humans in that the enzyme is very active in kidney tissue of humans. No homologies of this locus could be determined with other bats. Baker *et al.* (1988) report a slow, anodal, polymorphic peptidase (*Pept-1*) in *Lasiurus* using L-leucyl-L-alanine as a substrate, which may be homologous with either *Pep-A* or *Pep-S* of humans. Several dipeptidase isozymes are found in reptiles, for example four loci encoding PEP-A in *Cordylus* with Leu-Tyr as substrate (Brody 1991).

Peptidase B was encoded by one autosomal polymorphic locus (*Pep-B*) in both southern African and Eastern *Rhinolophus* species, using Leucyl-glycyl-glycine (Leu-Gly-Gly) as substrate. Baker *et al.* (1988) report two loci (*Pept-2* and *Pept-3*) encoding anodal enzymes of PEP-B in *Lasiurus*, using the same substrate. It was not possible to determine homologies with the loci encoding PEP-B in humans, but according to substrate specificity it might be two of *Pep-B*, *Pep-E* or *Pep-S*. One *Pep-B* locus is strongly expressed in all tissues of reptiles (Dessauer & Densmore 1983; Brody 1991). In *Rhinolophus Pep-B* was moderately active in tissues of mesodermal origin and the locus was strongly expressed in liver. This may be a derived condition compared to the condition in humans, where *Pep-B* is expressed in all tissues (Harris & Hopkinson 1976).

Peptidase C is a monomer, and was encoded by one autosomal, polymorphic locus (*Pep-C*), most prominent in brain and kidney tissue of southern African *Rhinolophus*. The banding zone was anodal to that of *Pep-A*, and was detected using Leu-Tyr as substrate. In bats of the Brachyphyllinae, one polymorphic locus encodes PEP-C using Glycyl-L-leucine (Gly-Leu) as substrate (Baker *et al.* 1981). In humans, *Pep-C* has several allelic variants which vary in their relative intensities of expression in different tissues (Harris & Hopkinson 1976).

Peptidase D (3.4.13.9) is a dimer and was encoded by one autosomal locus (*Pep-D*) in *Rhinolophus*, using the substrate Phenylalanyl-proline (Phe-Pro). This polymorphic enzyme was active in all tissues examined, but was most active in kidney, skeletal muscle and liver. One *Pep-D* locus is expressed in all tissues in humans (Harris & Hopkinson 1976). One locus encodes PEP-D in rattlesnakes (Murphy & Crabtree 1985) and the whiptail lizard, *Cnemidophorus tigris* (Dessauer & Cole 1984), but two loci (*Pep-D-1* and *Pep-D-2*) are found in alligators (Dessauer & Densmore 1983) and *Cordylus* (Brody 1991).

Phosphoglucomutase (PGM 2.7.5.1) is a dimer and catalyses the conversion of α-D-glucose-1-phosphate to α-D-glucose-6-phosphate. Three autosomal loci encode anodal enzymes of PGM which are expressed in all tissues of humans and presumably most mammals. The contribution of the third most anodal enzymes in humans is very small in all tissues, and sometimes undetectable (Harris & Hopkinson 1976). Two loci encode PGM in southern African rhinolophids. *Pgm-1* encoded a polymorphic, anodal enzyme which was active in all tissue types examined. Each primary band of the enzyme encoded by *Pgm-1* had a satellite band, with the *Pgm-1*(110/110) allele superimposed on the satellite band. The second locus *Pgm-2* encoded a polymorphic enzyme and was strongly expressed in skeletal muscle. Two polymorphic loci (*Pgm-1* and *Pgm-2*) encode PGM in bats of the Phyllostomidae, *Lasiurus* and in *Rhinolophus* species from Thailand, Jordan and Japan (Arnold *et al.* 1982; Baker *et al.* 1988; Qumsiyeh *et al.* 1988). These *Pgm* loci are presumably homologous with *Pgm-1* and *Pgm-2* of southern African rhinolophids. A single locus (*Pgm-2*) with anodal and cathodal products encodes PGM in bats of the Brachyphyllinae (Baker *et al.* 1981). Cathodal migration of isozymes may have been caused by different electrophoretic conditions. Two loci encode PGM in most reptiles such as lizards, snakes, crocodillians and turtles, but the products of only one locus (*Pgm-A*) are resolved in rattlesnakes. This locus may be homologous to the *Pgm-2* locus in *Rhinolophus* judged by the absence of expression of PGM in lung tissue, and may represent a derived condition which resulted from the loss or silencing of a locus (Murphy & Crabtree 1985).

Phosphogluconate dehydrogenase (PGD 1.1.1.44) is a dimer and catalyses the conversion of 6-phosphogluconate to ribulose-5-phosphate in the presence of NADP. One autosomal *Pgd* locus is expressed in all tissues of humans (Harris & Hopkinson 1976). A variable number of loci encoding PGD was reported for various bats. Three loci encoded anodal isozymes of PGD in *Rhinolophus* of southern Africa, and the appearance of allelic variants in all three loci eliminated the possibility of post-translational or epigenetic modification. The first locus (*Pgd-1*) encoded a polymorphic enzyme and was moderately expressed in heart, kidney and liver. The second polymorphic locus (*Pgd-2*) was expressed in all tissues examined, but was most active in brain, kidney, liver and spleen. A third locus (*Pgd-3*) encoded a polymorphic enzyme that was restricted to expression in skeletal muscle and heart, and was detected using a Tris-citrate buffer with phosphate. The absence of this locus in rhinolophids from Thailand, Jordan and Japan may be the result of using a buffer without phosphate. Two polymorphic loci (*Pgd-1* and *Pgd-2*) encode anodal, polymorphic enzymes of PGD in *Rhinolophus* from Thailand, Jordan and Japan (Qumsiyeh *et al.* 1988). These loci are presumably homologous to the two *Pgd* loci of southern African rhinolophids. This may be an ancestral condition to the condition in the Phyllostomidae (Arnold *et al.* 1982), Brachyphyllinae

(Baker *et al.* 1981) and *Lasiurus* (Baker *et al.* 1988) where one polymorphic *Pgd* locus encodes anodal isozymes of PGD. This may be the result of the silencing or loss of one locus. One locus encodes PGD in *Cordylus* (Brody 1991) and alligators (Dessauer & Densmore 1983). Two loci encode PGD in rattlesnakes (Murphy & Crabtree 1985).

Superoxide dismutase (SOD 1.15.1.1) is a dimer and catalyses the conversion of superoxide to hydrogen peroxide and oxygen, or appears co-incidently when staining for other enzymes with PMS or MTT linked reactions (Harris & Hopkinson 1976). A single *Sod-1* locus encoded a monomorphic enzyme of SOD in *Rhinolophus* of southern Africa and was strongly expressed in all tissues examined. One polymorphic locus encodes SOD in *Lasiurus* (Baker *et al.* 1988). Varying numbers of *Sod* loci occur in different chordate groups. SOD is encoded by one locus in diploid fishes, two loci in squamate reptiles and alligators and three loci in turtles, rattlesnakes and most other reptiles (Murphy & Crabtree 1985). A supernatant enzyme (*S-Sod*) and a mitochondrial enzyme (*M-Sod*) of SOD are active in almost all tissues of humans (Harris & Hopkinson 1976). The *Sod-1* locus in *Rhinolophus* may be homologous to *M-Sod* in humans. This may be a derived condition in bats resulting from the silencing of the supernatant *Sod* locus as was the case in some reptiles (Murphy & Crabtree 1985).

Conclusion

Isozyme data other than allele frequencies may facilitate inter-study comparison and may reveal evolutionary relationships between groups. Although no systematic inferences were possible from the isozyme characters of *Rhinolophus* in southern Africa, it may be a basis for comparison with other *Rhinolophus* species and bat genera to determine evolutionary relationships. There were no differences in gene expression between *R. clivosus* and *R. landeri* which represent genetically divergent species. Therefore, it is unlikely that there would be differences between other *Rhinolophus* species. The results of this study showed that most of the examined loci were expressed in heart, liver, kidney and skeletal muscle of *Rhinolophus* bats. These tissues were therefore used to study the phylogenetic relationships and evolution in 10 southern African species of *Rhinolophus* based on allo-zyme variation of 34 loci (Maree & Grant in preparation). The loci were randomly chosen, based on whether or not the banding patterns on the gel could be sufficiently resolved with specific stains, to formulate a genetic model to account for banding variability. However, to obtain a true estimate of the genetic variability within or between taxa, both monomorphic and polymorphic loci must be taken into account, without prior knowledge of their level of polymorphism (Grant 1990). The loci which were polymorphic (Table 1) in this study, may be a useful starting point for further population genetic studies in *Rhinolophus*.

Acknowledgements

Financial support by the Foundation for Research Development (FRD), South Africa is gratefully acknowledged. We thank G. Bronner, I.L. Rautenbach (Transvaal Museum), T.J. Robinson, M. van der Merwe (University of Pretoria) and J.P. Maree (Transnet) for their various contributions towards this study.

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