Genetic variation between two subspecies of reedfrogs in the genus *Hyperolius* (Anura: Hyperoliidae)

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ABSTRACT

Two subspecies of reedfrogs *Hyperolius marmoratus broadleyi* (eastern Zimbabwe) and *Hyperolius marmoratus verrucosus* (Tsitsikama area - Knysna) were analyzed for variation in nuclear ribosomal DNA by mapping restriction endonuclease sites. Ribosomal DNA analysis showed that *H. m. broadleyi* and *H. m. verrucosus* differ substantially at certain restriction sites in their nuclear ribosomal DNA and the genetic data suggests specific status for these taxa.

INTRODUCTION

Hyperolius marmoratus is a reedfrog which occurs in central and south-east Africa (Poynton & Broadley 1991). It lies in a very tightly knit gradient stretching from Kenya down to the Tsitsikama area in South Africa. The frogs that belong to this complex are morphologically very similar, but polymorphism in dorsal colours and patterns occurs between, as well as within the various populations. Over-all colour patterns, however, correlate to some extent with geographic distribution (Passmore & Carruthers 1979). This variation in colour patterns has been used as a taxonomic character in the classification of these frogs. 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 these frogs with different names being given to the same populations. Poynton, (1964) for example divided the group into the following subspecies in southern-eastern Africa: *H. m. taeniatus*, *H. m. broadleyi*, *H. m. swynnertoni*, *H. m. marginatus*, *H. m. marmoratus* and *H. m. verrucosus*. This demarcation was not accepted by Schiøtz (1975), nor by Laurent (1976). Poynton and Broadley (1987) recorded another two subspecies in southern Africa, *H. m. rhodesianus* and *H. m. aposematicus*, but noted that the delimitation of nearly all subspecies of *H. marmoratus* is still largely a matter that remains open to question.

In this pilot study we determined to what extent genetic differences exist between two subspecies. *H. m. broadleyi* and *H. m. verrucosus* were selected, because they are geographically well separated with other subspecies in between. Individuals of *H. angolensis* were included in the study as a reference group. *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* based on the difference in its advertisement call from that of *H. marmoratus*. Based on the latter evidence *H. angolensis* is here regarded as a separate species.

METHODS

Genetic differences were determined using the molecular technique of restriction site mapping of ribosomal DNA (Hillis & Davis 1986). The sizes of the restriction site fragments were determined by using the computer programme FRITENSKY (Schaffer & Sederoff 1981). Estimation was within an error of ±100 base pairs. The relative positions of the restriction sites were determined by single and double digestion, 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 & Pavon 1982). Maps were constructed manually and confirmed using the computer programme Resolve 3.3 (Harley, unpublished).

MATERIALS

Field collection localities of the various taxa are as follows: *H. m. verrucosus* (n = 9) samples were collected from three different localities - (Keiskammahoek 34° 40' S, 27° 10' E; LakePleasant 34°01' S, 22° 15' E; St. FrancisBay 34° 12' S, 24°55' E), *H. m. broadleyi* (n = 5) 18° 40' S, 35° 05' E; *H. angolensis* (n = 2) 18° 32' S, 21° 50' E.

RESULTS

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 1. The fragment sizes produced by the various enzymes are given in Table 2. Of the 37 sites that were mapped 15 were in the conserved regions (ie., 18S, 5.8S and 28S genes). Twenty sites were informative ie., not identical in all taxa and were present in the NTS (nontranscribed spacer region) except for Sac I (site no. 27) and Bam HI (site no. 28) present in the internal spacer region 1. Maps of the relative position of the restriction sites are given in Figure 1. The ribosomal DNA gene is repeated in tandem in the genome of eukaryotes. The repeat length of *H. angolensis* was calculated as 12 600 ±100 base pairs, *H. m. broadleyi* 12 800 ±100 and *H. m. verrucosus* 12 400 ±100 base pairs.

As shown in Figure 1 the variation in restriction site maps was confined to the non coding regions.

TABLE 1: 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. angolensis*	*H. m. broadleyi*	*H. m. verrucosus*
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	+	+	+

Between *H. m. broadleyi* and *H. m. verrucosus* 33 sites were mapped of which 20 were shared including the sites in the coding regions. Five out of 18 were shared in the variable regions only. A deletion was detected between the Pst I and Dra I sites of *H. m. 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 1. 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*.

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 was 7 out of 19. *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.

Samples from three different localities within the distribution range of *H. m. verrucosus* were examined for interpopulation variation. 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.

TABLE 2: Fragment sizes (base pairs) produced by single and double digests with different enzymes. Fragments that hybridized to the 18s and 28s probes are given.

ENZYME	*H. m. broadleyi*		*H. m. verrucosus*		*H. angolensis*	
	18s	28s	18s	28s	18s	28s
Eco RI	5 346	5162	7362	5162	7423	5162
	2 212					
Sac I	7034	7034	10090	10090	3545	3067
	1073	1192	1073	1192	2772	1161
		1073		1073		
Eco RI + Sac I	3166	3545	6348	3545	3238	3095
	771	1232	771	1232	2426	1161
Pvu II	12940	12940	12574	12574	6648	6648
					5994	5994
Eco RI + Pvu II	5024	4112	6835	4112	4372	4112
	2234	809		809	2706	809
Bgl II	6994	11407	11407	11407	7024	7024
	4506				5598	5598
Eco RI + Bgl II	3582	3235	6820	3235	4969	3235
	2110	1838		1838	3296	1838
Nco I	4378	4378	8102	8102	8230	8230
	3883	3883	4378	4378	4378	4378
	1023					
Eco RI + Nco I	3441	3191	5827	3191	6128	3191
	1545	1982	1257	1982	1257	1982
	1257					
Dra I	3875	3875	3875	3875	5252	5252
	3108	3108	2876	2876	3875	3875
	2515		2515		2245	
Eco RI + Dra I	3166	2866	2528	2866	2959	2866
	1101	2196	1101	2196	2309	2196
	952				1101	
Bam HI	3088	3088	4378	4378	4308	4308
	2406	2406	2406	2406	3128	3128
		1995				1995
Eco RI + Bam HI	2174	2494	2174	2494	2174	2494
		2071		2071		2472
Bcl I	7343	7343	13040	13040	13261	13261
	5518	5518				
Eco RI + Bcll I	3979	4066	7194	4066	7430	4066
	2107					
Bst EII	12247	12247	5786	5786	11086	11086
	1226		5153		1226	
			1226			
Eco RI + Bst EII	2269	5183	5207	5183	6294	5183
	1257		1257		1257	
Pst I	8113	8113	8825	8825	8012	2137
	7890	7890	3484		2137	
	2727					
Eco RI + Pst I	2824	5123	3454	5123	2558	5123
	1811		2765		1981	
	903		903		903	

Analysis

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 the uninformative sites were present in the conserved regions. All three taxa exhibited unique sites. *H. angolensis* displayed 4, *H. m. broadleyi* 5 and *H. m. verrucosus* 3. The number of shared informative sites between *H. angolensis* and *H. m. broadleyi* is 5, *H.*

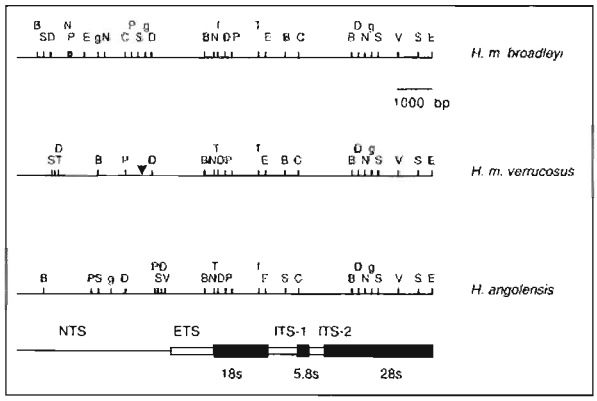

FIGURE 1: Restriction site maps of *H. m. broadleyi*, *H. m. verrucosus* and *H. angolensis* indicating the relative locations of the restriction sites produced by ten different restriction enzymes. B = Bam HI, Bcl I = C, Bgl II = g, Bst-EII = T, Eco RI = E, Dra I = D, Nco I = N, Pst I = P, Sac I = S, Pvu II = V. 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.

angolensis and *H. m. verrucosus* 0 and *H. m. broadleyi* and *H. m. verrucosus* 3.

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 & Davis 1986). Similarities in the maps of *H. m. verrucosus* and *H. m. broadleyi* are mostly confined to the conserved regions of the gene. The 15 sites mapped in the conserved regions were identical to the sites mapped in the conserved regions throughout *Rana* (Hillis & Davis 1986) whereas most differences are located in the NTS regions. The rDNA restriction site maps (Figure 1) indicate substantial differences between *H. m. broadleyi* and *H. m. verrucosus* as well as between them and *H. angolensis*. Out of a total of 18 sites that were mapped in the variable regions between the subspecies, a difference of 72% (13 sites) 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* thus corresponds to differences found between species. The deletion detected in *H. m. verrucosus* contributed to the difference in the repeat length between it and *H. m. broadleyi*. The restriction site map of *H. angolensis* also shows significant differences with both subspecies. The variable region site difference between *H. m. broadleyi* and *H. m. verrucosus* is similar to that recorded between *H. angolensis* + *H. m. broadleyi* (63%) and *H. angolensis* + *H. m. verrucosus* (89%). This suggests that the two subspecies are about as genetically different from each other as either is to *H. angolensis*.

The restriction site maps of the individuals from different localities within the distribution range of *H. m. verrucosus* were identical which is an indication of the concordance of overall colour pattern and genetic composition of *H. m. verrucosus*. Samples of *H. m. broadleyi* and *H. angolensis* also showed no intrapopulation variation.

Sequence divergences

Sequence divergence can be used as measure of genetic differences (Carr *et al.* 1987), using the formula of Nei and Li (1979). Because of the conservative nature of the coding regions, sequence divergence values were calculated only for sites in the variable regions. 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*.

Sequence divergence of mtDNA 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*. Honeycutt *et al.* (1987), recorded 7,5% mtDNA difference between species of the genus *Cryptomys* (African mole rats).

Sequence divergence values found between the subspecies *H. m. broadleyi*, *H. m. verrucosus* and *H. angolensis* are in the same range or higher when compared to separate species in other studies. We suggest that *H. m. broadleyi* and *H. m. verrucosus* represent distinct species.

CONCLUSION

The genetic differences support a specific status for *H. m. broadleyi* and *H. m. verrucosus*, and confirm the species status of *H. angolensis*. However, this study has examined only two taxa that 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. This is essential to determine the boundaries and hence correct taxonomy of the species here recognized. 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 in the group, molecular studies are well suited to unraveling the genetics and past history of the group.

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