Phylogenetic relationships and call structure in four African bufonid species

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Four species of toads of the genus *Bufo*, comprising three species endemic to southern Africa and one closely-related species, were examined electrophoretically to infer their phylogenetic relationships. The evolution of advertisement call structure in these species is discussed in relation to this phylogeny. *Bufo rangeri* and *B. gutturalis*, two species with very different call structures, are sister taxa. Two pairs of species with very similar call structures, *B. pardalis* and *B. gutturalis*, and *B. rangeri* and *B. angusticeps*, were only distantly related. Our results suggest that call parameters are poor characters to use in inferring phylogenies among congeneric species, probably because of selection for optimal audibility in different habitats, and because of the role that they play in premating isolation. The phylogeny derived from allozyme data agrees with evidence on albumin immunological distance, karyotype and morphology.

Vier spesies skurwepaddas van die genus *Bufo*, bestaande uit drie wat endemies is in Suid-Afrika en een nou-verwante spesie, is elektroforeties ondersoek om hulle filogenetiese verwantskap af te lei. Die evolusie van die struktuur van die bekendstellingsroep by hierdie spesie word met betrekking tot hierdie filogenie bespreek. *Bufo rangeri* en *B. gutturalis*, twee spesies met baie verskillende roepstrukture, is suster taksa. Twee pare van spesies met baie ooreenstemmende roepstrukture, *B. pardalis* en *B. gutturalis*, en *B. rangeri* en *B. angusticeps*, was slegs ver langs verwant. Ons resultate dui daarop dat roep-parameters swak kenmerke is om te gebruik by die aflei van filogenieë by verwante spesies, waarskynlik as gevolg van seleksie wat plaasvind vir optimale hoorbaarheid in verskillende habitatte, sowel as die rol wat hulle speel by die pre-paringsisolasie. Die filogenie wat van allosiemdata afgelei is, stem ooreen met getuienis oor albumien immunologiese afstand, kariotipe en morfologie.

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Recent studies have explored the phylogenetic origins of different behavioural systems by relating behavioural characters to genetic ones (Högland 1989; Losos 1990). But comparisons of phylogenies derived independently from molecular and behavioural data are rare. Tandy & Keith (1972) found that the topology of a phylogeny based on one call character, passive pulse rate, was congruent with that based on haemoglobin type, karyotype and occurences of hybridization among five African bufonids. Arntzen & Sparreboom (1987) used both behavioural and biochemical data to reconstruct a phylogeny of Old World newts in the genus *Triturus*. They inferred phylogenies with 15 courtship characters and with allozyme frequencies and found that the two phylogenies were similar. Molecular data, however, appear to provide the best reconstructions of phylogenies of amphibians because generally there are few shared derived morphological or behavioural characters to estimate phylogenetic relationships among taxa (Maxson 1984).

The anuran genus *Bufo* has approximately 50 species in Africa. Tandy & Keith (1972) divided these species into 22 groups or complexes on the basis of morphological and karyotypical variation and variation in the acoustic characteristics of calls. There appear to be at least two major lineages in Africa (Bogart 1972): one with a diploid chromosome number of 22 (as in non-African *Bufo*) and another group of endemic species with a 2n number of 20. Species with a 2n number of 20 chromosomes appear to have originated from African *Bufo* with 22 chromosomes (Bogart 1972; Cei, Erspamer & Roseghini 1972). Maxson's (1981) phylogenetic reconstruction of species in 10 of these groups confirms this hypothesis.

In this study, we construct a phylogenetic hypothesis on the basis of allozyme variation for four southern African species of *Bufo*, and then use this phylogeny to infer the evolution of mating calls among these taxa. (Since anuran courtship is simple, there are too few behavioural characters to attempt to infer phylogenetic relationships from them.) Phylogenies derived from allele-frequency data are compared with those derived from data on albumin evolution, call structure, and karyotyping.

Materials and Methods

We collected samples from the following sites in the Cape Province: Rondebosch Common (33°57'S/18°29'E) (n = 5 *B. angusticeps*), Sun Valley (34°07'S/18°24'E) (n = 14 *B. pardalis*), Stellenbosch (33°51'S/18°52'E) (n = 17 *B. rangeri*). *Bufo gutturalis* (n = 12) were collected from Weza State Forest (30°36'S/29°40'E) in the province of Natal. Animals were sacrificed by freezing, and carcasses were kept at –70°C until dissection. Samples of liver, kidney and hindleg muscle were homogenized separately with an equal volume of 0,01 mol dm^{-3} Tris/HCl buffer (pH = 8,0). Tissue homogenates were frozen and centrifuged at 2500g for 5 min prior to use. Horizontal starch gel electrophoresis followed May, Wright & Stoneking (1979), and histochemical staining protocols followed Shaw & Prasad (1970) and

Harris & Hopkinson (1976). Buffer systems and tissues giving the best resolution for each of the 31 loci examined are listed in Table 1. Loci were numbered beginning at the cathodal end of the gel, and cathodally migrating allozymes were indicated by a minus sign. The most common allele in *B. rangeri* was designated 100, and other allozymes were designated by their mobilities relative to this allele.

Relative allelic frequencies for each locus were estimated from the gel banding patterns, which were interpreted to reflect Mendelian variation according to the criteria of Allendorf & Utter (1979). Nei's standard genetic distance (D), corrected for sample size (Nei 1978) and Rogers' (1972) genetic distance were computed from allelic frequencies. We constructed a phenogram from genetic distances with the UPGMA method of cluster analysis (Sneath & Sokal 1973), and inferred a cladistic tree from electrophoretic characters with the program MIX in the package PHYLIP ver. 3.0 of J. Felsenstein. For the cladistic analysis we followed recommendations made by Mickevich & Mitter (1981), Buth (1984), and Green (1986): in constructing allozyme character-state series, the locus was treated as the character. Alleles were scored for presence or absence, and combinations of alleles at each locus were considered to be its character-states. For loci which were polymorphic among species, we assumed that character-state changes proceeded from the allele or alleles in the outgroup taxon to an allele of next most similar electrophoretic mobility. (We assumed that allozymes of the most similar electrophoretic mobilities differed by the fewest number of amino-acid substitutions.) Branched pathways were resolved into single-path components, yielding a data-matrix of unidimensional, multistate vectors which were further resolved into binary (0,1) vectors. A tree based on these data was computed using Camin-Sokal parsimony criteria (Camin & Sokal 1965) which allow a root to be specified because ancestral states are presumed to be known. *Bufo angusticeps* was designated the outgroup on morphological grounds (Poynton 1964).

Calls were recorded in the field with either a SONY Professional TCD5 cassette recorder or a UHER 4200 Report tape recorder, and a SENNHEISER MK2 directional microphone. Calls were analysed using a KAY 6061B sonagraph with a wide band (300 Hz) filter.

Table 1 Buffer systems and tissues used for starch-gel electrophoresis on 31 polymorphic loci in four species of southern African *Bufo*. R = Ridgway *et al.* (1970), TC = Tris-citric acid, after Whitt (1970), and MF = Markert & Faulhaber (1965)

Enzyme	Locus	E.C. number	Tissue	Buffer
adenosine deaminase	ADA	3.5.4.4	kidney	MF
adenylate kinase	AK	2.7.4.3	muscle	TC
alcohol dehydrogenase	ADH	1.1.1.1	liver	R
aldolase	ALD	4.1.2.13	kidney	R
aspartate aminotransferase	AAT-1	2.6.1.1	muscle	R
aspartate aminotransferase	AAT-2		muscle	TC
creatine kinase	CK-1	2.7.3.2	kidney	R
creatine kinase	CK-2		kidney	R
esterase	EST-2	3.1.1.1	kidney	R
esterase	EST-3		kidney	R
glucose-phosphate isomerase	GPI	5.3.1.9	muscle	R
glyceraldehyde-phosphate dehydrogenase	GAP	1.2.1.12	muscle	TC
glucose-6-phosphate dehydrogenase	GPD-1	1.1.1.49	kidney	TC
glucose-6-phosphate dehydrogenase	GPD-2		kidney	TC
haemoglobin	HAEM		kidney	TC
isocitrate dehydrogenase	IDH-1	1.1.1.42	muscle	TC
isocitrate dehydrogenase	IDH-2		liver	TC
lactate dehydrogenase	LDH-1	1.1.1.27	muscle	R
lactate dehydrogenase	LDH-2		muscle	R
lactate dehydrogenase	LDH-3		liver	R
malic enzyme	ME	1.1.1.40	kidney	TC
mannose-phosphate isomerase	MPI	5.3.1.8	kidney	MF
peptidase (substrate: gly-leu)	PEP-GL	3.4.11.1	liver	MF
peptidase (substrate:leu-gly-gly)	PEP-LGG		liver	MF
peptidase (substrate:phe-pro)	PEP-PHP	3.4.13.9	kidney	MF
phosphoglucomutase	PGM-1	2.7.5.1	muscle	R
phosphoglucomutase	PGM-2		muscle	R
6-phosphogluconate dehydrogenase	PGD	1.1.1.44	kidney	TC
sorbitol dehydrogenase	SDH-1	1.1.1.14	liver	R
superoxide dismutase	SOD	1.15.1.1	muscle	R
xanthine oxidase	XO	1.2.3.2	liver	MF

Results

Electrophoretic variation

We identified the gene products of 31 loci in the four toad species. All of the isozyme loci examined were polymorphic within or among species (Table 2). *Bufo gutturalis* was the species in which the greatest percentage of loci (35,5%)

Table 2 Allozyme frequencies detected at 31 polymorphic loci in four southern African *Bufo* species

		Sample			
Locus	Allele	*B. angusticeps*	*B. pardalis*	*B. rangeri*	*B. gutturalis*
ADA	90	–	–	–	–
	95	–	–	–	1
	98	–	1	–	–
	100	–	–	1	–
AK	100	–	0,964	1	1
	200	–	0,036	–	–
	250	1	–	–	–
ADH	40	–	–	0,029	–
	80	–	0,143	–	–
	90	1	–	–	–
	100	–	–	0,971	–
	150	–	0,857	–	1
ALD	85	–	1	–	–
	87	1	–	–	–
	95	–	–	0,059	1
	100	–	–	0,941	–
AAT-1	-100	–	–	1	1
	-95	1	–	–	–
	-90	–	1	–	–
AAT-2	-900	–	–	–	–
	50	0,25	0,464	–	–
	100	0,75	0,536	1	0,958
	180	–	–	–	0,042
CK-1	80	1	–	–	–
	100	–	1	1	1

Table 2 Continued

Locus	Allele	Sample			
		B. angusticeps	*B. pardalis*	*B. rangeri*	*B. gutturalis*
CK-2	0	1	–	–	1
	100	–	1	0,971	–
	103	–	–	0,029	–
EST-2	90	–	1	–	–
	95	–	–	–	0,042
	98	–	–	0,118	–
	100	–	–	0,794	0,042
	102	–	–	0,088	0,042
	105	–	–	–	0,874
	115	1	–	–	–
EST-3	95	–	0,036	–	–
	97	0,1	–	–	–
	98	–	–	0,029	1
	100	0,9	0,964	0,971	–
GPI	-120	0,3	–	–	–
	-100	0,7	–	1	–
	-95	–	1	–	–
	-50	–	–	–	1
GAP	-500	1	–	–	–
	100	–	–	1	–
	115	–	–	–	–
	500	–	1	–	–
	600	–	–	–	0,042
	700	–	–	–	0,042
	700	–	–	–	0,958
GPD-1	100	1	0,964	1	1
	180	–	0,036	–	–
GPD-2	90	–	1	–	–
	92	1	–	–	–
	95	–	–	–	1
	100	–	–	1	–
HAEM	70	–	–	–	1
	100	–	1	1	–
	150	1	–	–	–
IDH-1	50	–	–	–	1
	100	–	1	1	–
	200	0,9	–	–	–
	300	0,1	–	–	–
IDH-2	100	–	–	1	1
	103	1	–	–	–
	105	–	1	–	–
LDH-1	-300	–	–	0,029	–
	-100	1	1	0,971	1
LDH-2	85	–	1	–	–
	90	1	–	–	–
	100	–	–	1	1
LDH-3	100	–	–	1	1
	110	1	1	–	–
ME	90	–	–	0,662	–
	100	0,6	0,857	0,938	1
	105	–	0,036	–	–
	107	–	0,107	–	–
	110	0,4	–	–	–
MPI	80	0,1	–	–	–
	82	0,2	–	–	–
	85	0,6	–	–	–
	92	–	–	–	1
	100	–	–	0,735	–
	102	–	1	–	–
	107	0,1	–	–	–
	110	–	–	0,265	–

Table 2 Continued

Locus	Allele	Sample			
		B. angusticeps	*B. pardalis*	*B. rangeri*	*B. gutturalis*
PEP-GL	90	–	–	–	0,083
	100	1	0,964	1	0,917
	115	–	0,036	–	–
PEP-LGG	100	–	1	1	0,636
	120	–	–	–	0,364
	125	–	–	–	–
	130	1	–	–	–
PEP-PHP	65	1	–	–	–
	70	–	–	–	1
	80	–	1	–	–
	100	–	–	1	–
PGM-1	60	–	–	0,088	–
	90	–	–	0,03	0,042
	95	–	0,25	–	–
	100	1	–	0,882	0,958
	110	–	0,536	–	–
	120	–	0,214	–	–
PGM-2	0	–	–	–	–
	97	0,625	–	–	–
	100	–	–	0,625	0,792
	103	0,375	–	–	–
	105	–	–	0,375	0,208
	110	–	0,679	–	–
	112	–	0,071	–	–
	115	–	0,25	–	–
PGD	-20	–	1	–	–
	80	–	–	–	0,042
	100	1	–	1	0,916
	120	–	–	–	0,042
SDH	87	–	–	–	0,045
	92	1	1	–	–
	95	–	–	–	0,955
	100	–	–	1	–
SOD	90	–	0,571	–	–
	95	1	–	–	–
	97	–	0,429	–	–
	100	–	–	1	–
	105	–	–	–	0,75
	115	–	–	–	0,25
XO	100	1	–	1	0,833
	102	–	1	–	0,167
	104	–	–	–	–

were polymorphic. In both *B. rangeri* and *B. pardalis* 32,3% of loci were polymorphic, whereas in *B. angusticeps* only 22,6% of loci were polymorphic. One enzyme (XO) appeared to have octomeric subunit construction. Eight enzymes (Adh, Ald, Gap, Ldh-1, Ldh-2, Ldh-3, Me and Sdh) appeared to have quaternary tertiary structure, and a further seven enzymes (Ak, Est-2, Est-3, Lgg, Mpi, Pgm-1 and Pgm-2) had banding patterns that were consistent with a monomeric subunit construction. The remaining 15 enzymes had banding patterns typical of dimeric enzymes.

Expected average heterozygosities are listed in Table 3 along with both Nei's (1978) unbiased genetic distances and Rogers' (1972) genetic distances. Standard errors of Nei's distances (Nei & Roychoudhury 1974) were of the order of

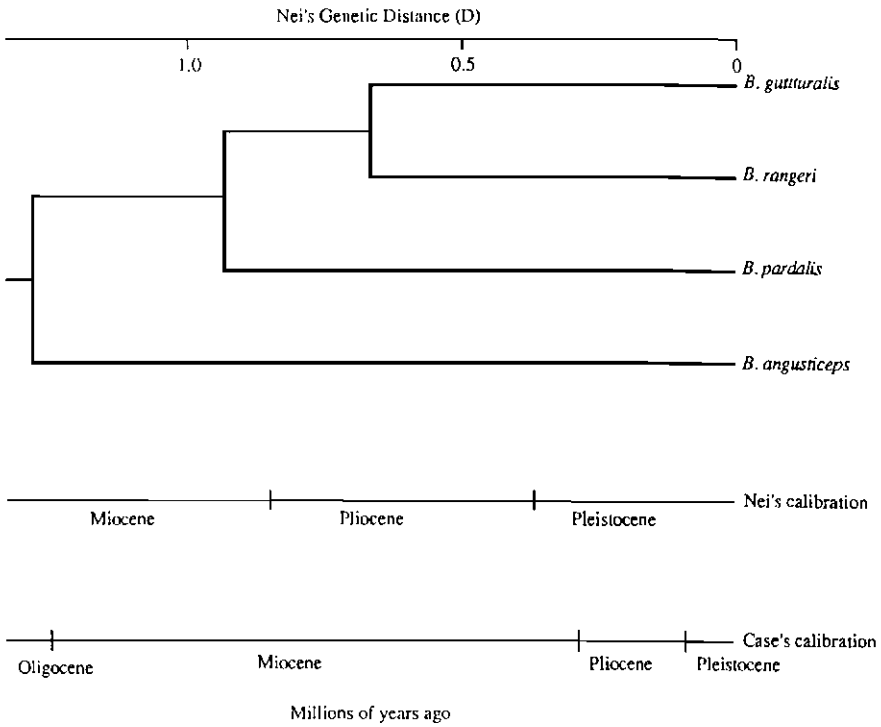

Figure 1a UPGMA phenogram of Nei's genetic distance for four species of southern African *Bufo* based on data in Table 3.

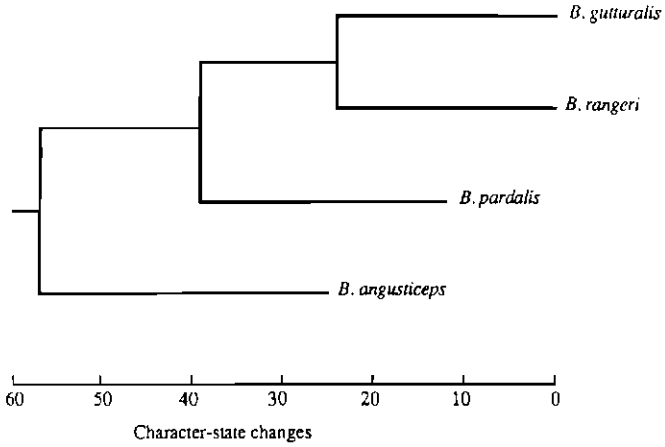

Figure 1b Cladogram of relationships among four species of southern African *Bufo* based on allozyme character-state data as listed in Table 4 and calculated with assumption of known ancestral states. Branch lengths drawn proportional to numbers of character-state changes required. Tree has a total of 94 steps.

Table 3 Nei's genetic distance (upper right), Rogers' genetic distance (lower left), and average heterozygosity estimates (diagonal) for samples of four southern African *Bufo* species (based on allozyme data in Table 2)

Sample		1	2	3	4
1	*B. angusticeps*	**0,087**	1,402	1,174	1,261
2	*B. pardalis*	0,734	**0,092**	0,941	1,28
3	*B. rangeri*	0,69	0,608	**0,06**	0,67
4	*B. gutturalis*	0,712	0,717	0,501	**0,074**

1,8 and 3,2% of the distance values. Of the four species, average heterozygosity was lowest in *B. rangeri* (H = 0,06) and highest in *B. pardalis* (H = 0,092), but all values fell within the range previously recorded in toads (Feder 1979; Green 1984).

Figure 1 shows the phenetic and phylogenetic trees computed with the methods outlined previously: the UPGMA phenogram in Figure 1a was computed from genetic distances, and Figure 1b is based on the character-state series constructed for the 31 loci and listed in Table 4. The topology of both trees is identical: *Bufo rangeri* and *B. gutturalis* appear to have diverged most recently from a common ancestor, with *B. pardalis* diverging from that line at an earlier stage. A time calibration of the tree in Figure 1a using a time scale based on the average rate of codon substitution detectable by electrophoresis of 1D = 5 Myr (Nei 1987), suggests that the *B. angusticeps* line diverged from ancestral stock during the late Miocene, followed shortly afterwards by the lineage leading to *B. pardalis*. The separation of the lines leading to *B. gutturalis* and *B. rangeri* took place as recently as the mid-Pliocene. If a calibration of 1D = 19,7 Myr, based on empirical evidence for hylid evolution (Case, Haneline & Smith 1975) is used, the calculated divergence time of the line leading to *B. angusticeps* is 25 Myr ago, that to *B. pardalis* 22 Myr ago, and the separation of the lines leading to *B. rangeri* and *B. gutturalis* as long ago as 13 Myr. The first of these dates is just prior to the start of the Miocene 24,6 Myr ago, whereas the last two fall within this epoch (Harland, Cox, Llewellyn, Pickton, Smith & Walters 1982).

Acoustic behaviour

Figure 2 presents sonograms of the advertisement calls of the four species, all of which display only passive

Table 4 Data matrix of allozyme character states for four species of southern African *Bufo*, derived from isozyme loci (characters) as indicated in Table 2

Locus	Character-state series	*B. angusticeps*	*B. pardalis*	*B. rangeri*	*B. gutturalis*
ADA	1	0	2	3	1
AK	2	0	1	2	2
ADH	3	0	1	2	0
	4	0	2	1	2
ALD	5	0	1	0	0
	6	0	0	2	1
AAT-1	7	0	0	1	1
	8	0	1	0	0
AAT-2	9	0	0	0	1
	10	0	0	1	1
CK-1	11	0	1	1	1
CK-2	12	0	1	2	0
EST-2	13	0	3	2	1
	14	0	3	1	2
EST-3	15	0	2	1	2
	16	0	1	0	0
GPI	17	0	2	1	3
GAP	18	0	2	1	3
GPD-1	19	0	1	0	0
GPD-2	20	0	0	2	1
	21	0	1	0	0
HAEM	22	0	1	2	2
IDH-1	23	0	1	1	2
IDH-2	24	0	1	0	0
	25	0	0	1	1
LDH-1	26	0	0	1	0
LDH-2	27	0	1	0	0
LDH-3	28	0	0	1	1
	29	0	0	1	1
ME	30	0	1	3	2
MPI	31	0	2	3	1
	32	0	1	2	3
PEP-GL	33	0	1	0	0
	34	0	0	0	1
P-LGG	35	0	2	2	1
PEP-PHP	36	0	2	3	1
PGM-1	37	0	1	0	0
	38	0	0	1	1
PGM-2	39	0	1	2	1
	40	0	0	1	1
PGD	41	0	2	0	1
	42	0	0	0	1
SDH	43	0	0	1	1
	44	0	0	1	1
SOD	45	0	1	0	0
	46	0	1	2	3
XO	47	0	1	0	2

modulation (Martin 1972). Broughton (1963) defined the physical elements of three basic grades of complexity to analyse temporal structures of calls: pulses, pulse trains and complex pulse trains. The call of *B. angusticeps* (Figure 2a) consisted of a simple pulse train (i.e. only one call per performance), whereas that of *B. rangeri* consisted of a complex pulse train of between 6 and 273 pulse trains (n = 76 bouts from 31 individuals), one of which is illustrated in Figure 2b. In both cases the pulse rates were so fast (138 pulses per second for *B. angusticeps*, and 189 pulses per second for *B. rangeri*: Figure 2a and b) that the calls sounded like rasping squawks. The duration of the simple pulse train varied considerably; in the former species it was just over 0,5 s (Figure 2a), whereas in *B. rangeri* it had an average duration of 0,118 s (Cherry 1993). The dominant frequency range was between 1300 and 1850 Hz for *B. rangeri* (Cherry 1993), and was slightly higher for *B. angusticeps* (Figure 2a).

In *B. pardalis* and *B. gutturalis* the pulse rates were 28 and 16 pulses per second, respectively, and were slow enough to sound 'pulsatile'. Figures 2c and 2d show only a section of one pulse train in each species. The total duration of a simple pulse train was 1,3 s for the *B. gutturalis* call in Figure 2c, and an average of 1,2 s for *B. pardalis* (Cherry 1989). The call of *B. pardalis* has a dominant frequency of between 500 and 800 Hz (Cherry 1989), whereas that of *B. gutturalis* is slightly higher (Figure 2c).

When two parameters, the duration of a complex pulse train and the passive pulse rate, are examined, the result is a progression in the first case from longest to shortest from *B. gutturalis* to *B. pardalis* to *B. angusticeps* to *B. rangeri*, and the same sequence from slowest to fastest in the second. With *B. angusticeps* as an outgroup, neither of these characters produces phylogenies that are congruent with the allozyme data, as it is the calls of *B. pardalis* and *B. gutturalis* that are most similar, even though they are only distantly related.

Discussion

Phylogenetic affinities of species

The family Bufonidae is represented by 16 species in southern Africa. A grouping of these species after Tandy & Tandy (1976), but incorporating the work of Channing (1978) and Grandison (1980), is presented in Table 5. Phylogenetic relationships based on the allozyme data agree with evidence on chromosome number (Bogart 1972; Griffin, Scott & Papworth 1970): *B. rangeri* and *B. gutturalis* have a chromosome number of 2N = 20, whereas *B. pardalis* has one of 2N = 22. Although the chromosome number of *B. angusticeps* has not been determined, it is likely to be 2N = 22, as it is for *B. gariepensis*, which is in the same species group (Tandy & Keith 1972). If this is the case, both electrophoretic and karyotype data support Bogart's (1972) suggestion that species of *Bufo* with 20 chromosomes were derived from an ancestral African bufonid with 22 chromosomes.

There is some dispute as to the taxonomic placement of *B. pardalis*. Using morphological criteria, Poynton (1964) placed this species in the *regularis* group (see Table 5), whereas Tandy & Keith (1972) placed it in the *latifrons* complex, because it shares a distinctive pattern of dorsal markings with other members of the latter group. Subsequently, Tandy (1972) placed it in a monospecific group on the grounds that it had a diploid chromosomal number of 22, whereas the rest of the *latifrons* complex had a complement of 20 chromosomes. The present study supports this conclusion as it refutes Poynton's placing of *B. pardalis* in the *regularis* group. As neither *B. angusticeps* nor *B. pardalis* were included in Maxson's (1981) study of albumins in

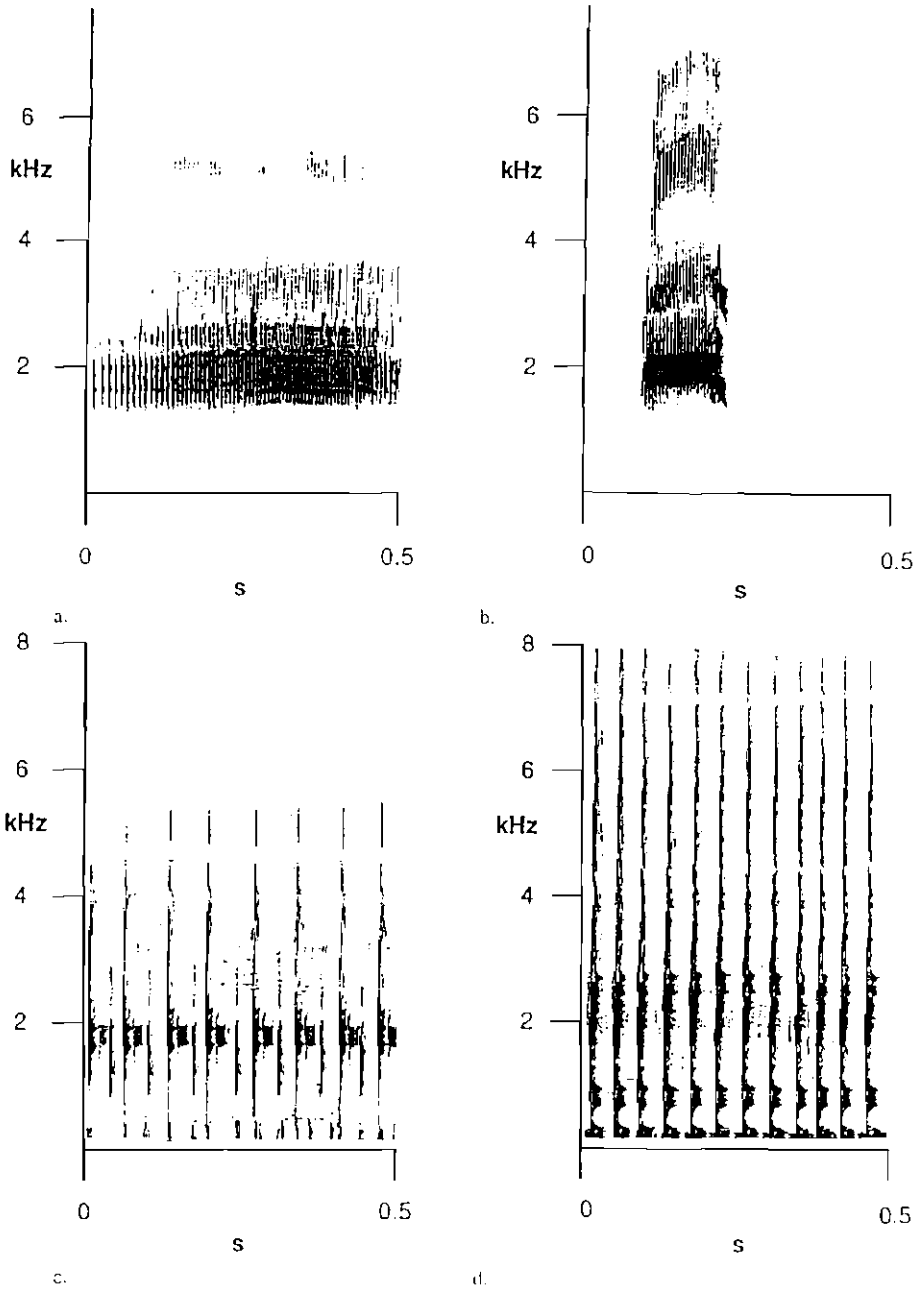

Figure 2 Sonograms of advertisement calls of (a) *B. angusticeps* near Stellenbosch, July 1986, (b) *B. rangeri* near Stellenbosch, October, 1985, (c) *B. gutturalis* at Weza State Forest, September 1986, and (d) *B. pardalis* at Rondevlei, near Cape Town, August 1985.

African bufonids, the present study serves to complement that of Maxson. Insofar as they overlap, the topologies of Maxson's and the phylogenetic trees in Figure 1 agree.

Time-scale calibration of phylogeny

The calibration of the 'molecular clock' remains a subject of some controversy (Thorpe 1982): various workers have calibrated the clock empirically for different groups of animals. Different calibrations for albumin immunological distance (AID) have been made for two anuran families: in the Hylidae, Case, Hanceline & Smith (1975) estimated that a Nei D of 1 equalled 36,5 AID units, whereas Case (1978) suggested it equalled 46,5 AID units for the Ranidae. In the absence of an empirical calibration for the Bufonidae, the calibration of Case *et al.* (1975) has been used, as the Bufonidae are more closely related to the Hylidae than to the Ranidae (Duellman 1988). With this value and the calibration of Wilson, Carlson & White (1977), which equates 1 AID unit to 0,54 Myr, we derived a calibration of 1D = 19,7 Myr.

This calibration can be applied to the phylogeny in Figure 1a, and this phylogeny compared to that obtained by Maxson (1981). Unfortunately her phylogenetic tree does not include *B. gutturalis*, but does include *B. regularis*, which is closely related to it (only 10 AID units distant). Using the calibration of Wilson *et al.* (1977) she estimated that the lines leading to *B. rangeri* and *B. regularis* diverged from a common ancestor 12 Myr (21,5 AID units) ago. This is close to the estimate of 13 Myr for the divergence time between the *B. gutturalis* and *B. rangeri* lineages displayed in Figure 1a. *Bufo gutturalis* and *B. regularis* are very

Table 5 The family Bufonidae in southern Africa

Group/complex	Species	Diploid chromosome number
Schismaderma	*S. carens*	22
Capensibufo	*C. rosei*	?
	C. tradouwi	?
Stephopaedes angusticeps	*S. anotis*	?
	B. angusticeps	?
	B. gariepensis	22
	B. amatolicus	?
	B. inyangae	?
vertebralis	*B. vertebralis*	?
taitanus	*B. taitanus*	?
pardalis	*B. pardalis*	22
regularis	*B. gutturalis*	20
	B. rangeri	20
	B. garmani	20
	B. maculatus	20
Group uncertain	*B. lemairi*	?

closely related: *Bufo gutturalis* was formerly regarded as an eastern subspecies of *B. regularis*, but has recently been given specific status (Tandy & Keith 1972; Blair 1972), so one would expect their respective times of divergence from *B. rangeri* to be similar, which they are if empirical calibration is used. By contrast, if we use Nei's (1987) calibration for the genetic distance for our data, then the topologies in the present study and those of Maxson concur, but not the time scales.

Bufonid radiation in southern Africa

We suggest that two major episodes of bufonid radiation took place in southern Africa, one in the Miocene and the second during the climatic fluctuations in the Pliocene and Pleistocene. The Miocene (5,1 until 24,6 Myr before present) was characterized by aridity and replacement of forest with savannah and other drier ecosystems (Axelrod & Raven 1978). We suggest that during this period (Figure 1a), the ancestral form of the *angusticeps* group colonized drier areas. All four species in this group are presently allopatric (Poynton 1964), and, whereas little is known about *B. amatolicus* and *B. inyangae*, *B. angusticeps* and *B. gariepensis* are both explosive breeders, breeding in temporary rainpools (Passmore & Carruthers 1979; Visser 1979). This adaptation allows for rapid utilization of these ephemeral breeding sites.

By the late Miocene, the coastal belt from Cape Town to Natal was covered by laural forest cut off from forest further north by savannah (Greenway 1970). *Bufo pardalis* appears to have effectively exploited this wetter environment, as the current distribution of this species (Figure 3) coincides roughly with this belt, although it is absent from all areas in which rainfall is less than 400 mm per annum (rainfall data from Jackson 1960). In contrast to the *angusticeps* group, *B. pardalis* breeds in large permanent waterbodies, but remains an explosive breeder, breeding over a period of 4–5 days

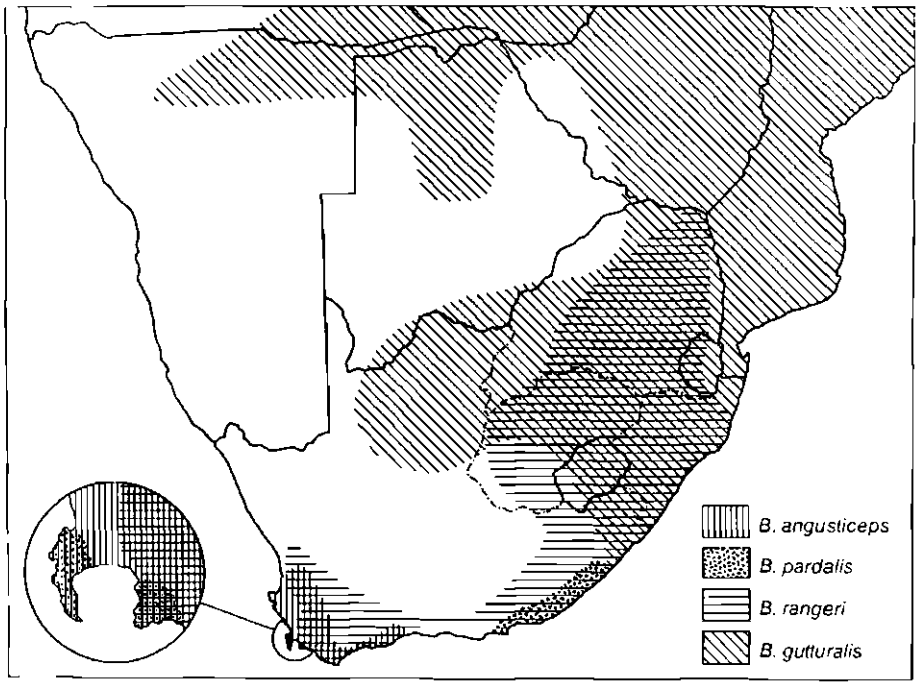

Figure 3 Distribution of (a) *B. angusticeps*, (b) *B. pardalis*, (c) *B. rangeri*, and (d) *B. gutturalis* in southern Africa. This figure is based on Poynton (1964) and Auerbach (1987), but has been updated using the collections of the SA Museum and the Cape Department of Nature and Environmental Conservation. *Bufo angusticeps* is restricted to the winter rainfall area of the south-western Cape, where it breeds in temporary rain pools between June and August. *Bufo rangeri* is a temperate form which is endemic to southern Africa, and is present in the eastern highlands as well as along the coastal belt from Natal to the south-western Cape. There are two isolated populations of *B. pardalis* in the western and eastern Cape Provinces. *Bufo gutturalis* in contrast, is a tropical form which reaches the southern limit of its distribution in South Africa.

(Cherry 1992). It appears likely that the present distribution represents relic populations of a formerly more widespread species.

The present study supports the view of Tandy & Keith (1972) that the radiation of the *regularis* group is the most recent, although it questions their contention that it took place as recently as the Pliocene and Pleistocene. Of the members of this group, *B. regularis*, *B. maculatus* and *B. rangeri*) have long breeding seasons (Tandy & Keith 1972; Cherry 1993), whereas *B. gutturalis* displays behavioural elasticity in its breeding behaviour, being an explosive breeder in Zimbabwe (Telford & van Sickle 1989), but a prolonged breeder in the Transvaal, South Africa (Balinsky 1985). Tandy & Keith (1972) postulate that the ancestral species of this group was an eastern forest form, which gave rise to a moist savannah form and a moist forest form. The former line gave rise to *B. regularis* and *B. gutturalis*, and the latter to *B. rangeri* (and *B. garmani*, which is closely related to *B. rangeri* but inhabits warm, dry areas). The present study corroborates the view of Maxson (1981) that the separation of these lineages took place between 12 and 13 Myr ago.

Inferring phylogeny from call characters

Bufo gutturalis and *B. rangeri* are closely related, and hybridize over part of their zone of sympatry (Guttman 1967; Passmore 1972). But despite their close relatedness, the calls of these two species are completely different (Figures 2b and 2c). This may reflect different optimal audibility in their respective ancestral habitats: *B. gutturalis* has a lower, drawn-out call typical of many savannah species, whereas *B. rangeri* has a higher-pitched call of shorter duration and is typical of many forest forms (Morton 1975; Passmore 1985), although Zimmerman (1983) suggested that microhabitat may be a more important factor in the evolution of anuran calls (see below). Selection for premating isolation would have been likely to maintain or increase the divergence in call structure between these two species when their ranges began to overlap (Figure 3), as conspecific recognition is one of the main functions of calling in anurans (Littlejohn 1977).

Tandy & Keith (1972) found that the topology of a phylogeny based on passive pulse rate was congruent with one based on haemoglobin type, karyotype and occurrences of hybridization among *B. rangeri*, *B. garmani*, *B. brauni*, *B. regularis* and *B. gutturalis* (with *B. viridis* as an outgroup). The present study shows that this apparent congruence may reflect a fortuitous choice of species: the calls of *B. gutturalis* and *B. pardalis*, and of *B. angusticeps* and *B. rangeri* are similar, despite the distant relatedness of these pairs of species. These relationships are best interpreted in terms of Zimmerman's (1983) argument that microhabitat is the most important factor influencing the evolution of anuran calls: the first mentioned two species call from dense reed beds, whereas the last two call from open sites. Thus an analysis of call parameters can be misleading in trying to estimate phylogenetic relationships among species, because of convergent and divergent evolution.

One call parameter which can be used in phylogenetic studies is the mode of amplitude modulation, because this is related to specific anatomical structures. Passive amplitude modulation is produced by vibration of the arytenoid cartilages, whereas active modulation is produced by quasi-periodic contraction of the thoracic musculature, which permits the reversal of air-flow between the lungs and the vocal sac. Martin (1972) defined three patterns of amplitude modulation in the family Bufonidae and the South American leptodactylid *Odontophrynus occidentalis*; Type II modulation, which he considers to be primitive, contains both passive and active components, from which have evolved both Type I (lacking active pulsation within the call), and Type III, lacking passive modulation. All four species under consideration in the present study have Type I calls. Only four species of African *Bufo* (all in different species groups) have Type II calls, whereas Type III calls are found only in American bufonids (Tandy & Tandy 1976). Thus, although the mode of modulation may be an instructive character for inferring phylogenies on a global basis, it is of limited value in estimating the evolution of *Bufo* in Africa.

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