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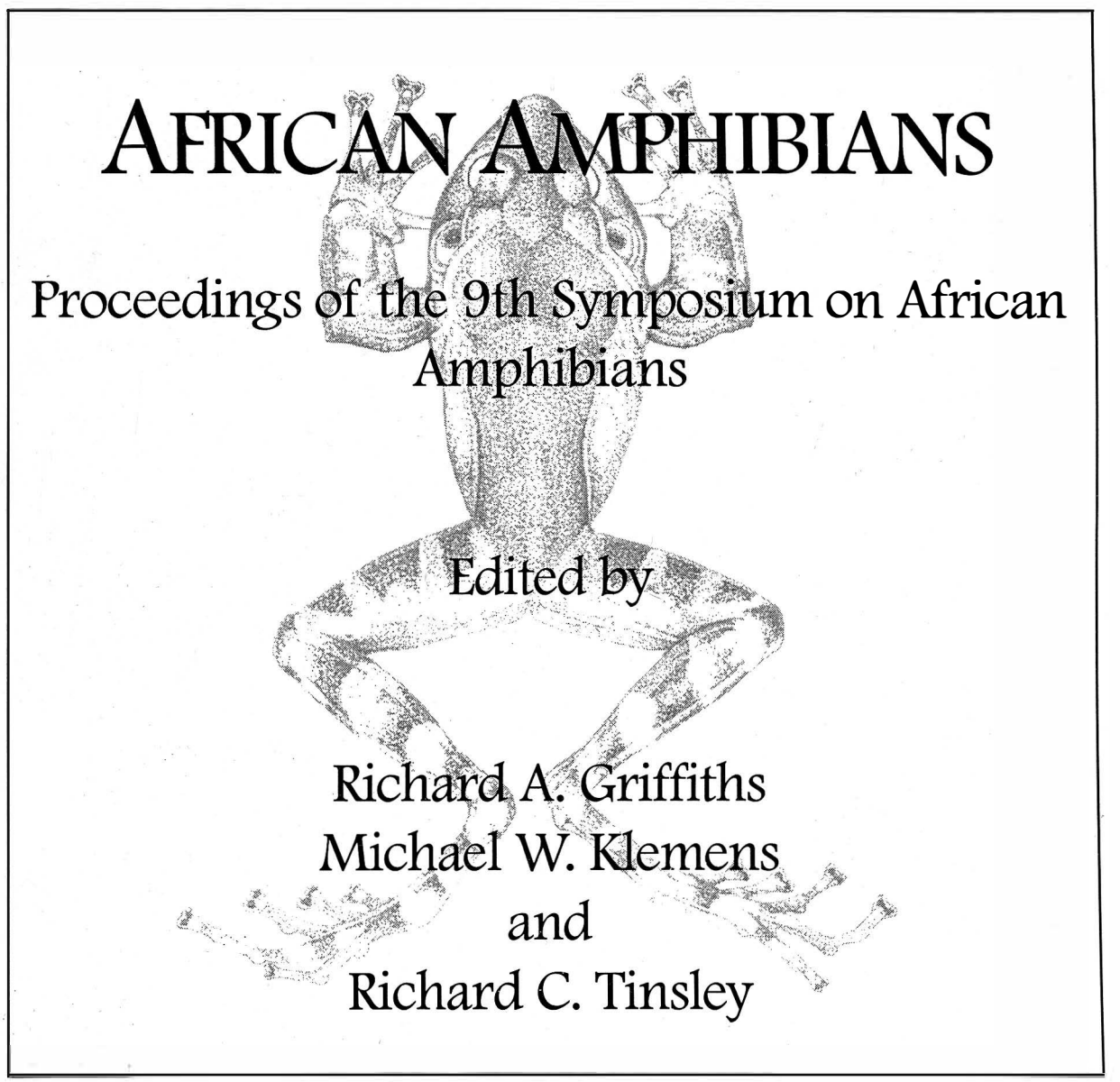

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BRITISH HERPETOLOGICAL SOCIETY

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PROCEEDINGS OF THE 9TH SYMPOSIUM ON AFRICAN AMPHIBIANS, UNIVERSITY OF BRISTOL, 9-12 SEPTEMBER 1996

PREFACE

The African Amphibians Working Group was established in 1969 when its inaugural meeting was held in Paris. Its aims, expressed at the 1969 meeting, are to promote personal relationships between scientists working with African amphibians; to facilitate exchange of material, ideas and publications; and to prepare studies and revisions, on both a systematic and a regional basis, towards a Fauna of African Amphibia.

Successive meetings have been held in France (twice), Denmark, Italy, South Africa, USA, Zimbabwe, Namibia and, most recently, the UK. The 9th Symposium was held at the University of Bristol, on the 9th-12th September 1996. Thirty-one delegates attended the meeting, and the scientific programme occupied two full days with 27 oral presentations and 1 poster, and an evening discussion session. The programme provided a snap-shot of current research based on African amphibians comprising *Ecology and Behaviour* (7 papers); *Biogeography, Diversity and Conservation* (6 papers); *Evolutionary Biology* (including systematics and phylogeny) (9 papers); *Reproductive Biology* (4 papers); and *Ultrastructure* (1 poster). These presentations reflect the increasing focus of biological research on molecular techniques, seven papers dealing with the molecular systematics of various anuran groups. Other phylogenetic studies concerned the use of parasites as indicators of host evolutionary relationships and the evolution of the immune system. Biodiversity and conservation represented a major theme of the meeting, and a series of current projects to produce atlases of the geographical distribution of amphibian species were described. The programme had a significant bias, atypical of representation at most amphibian meetings, nine papers dealing with the biology of *Xenopus* (including population ecology, behaviour, reproductive biology, parasitology, immunology and molecular phylogeny).

The papers presented in this volume provide a selection of the subjects discussed at the Symposium. Some of the presentations gave résumés of recently published studies and are not included here. One session, chaired by Dr Arne Schiøtz, considered conservation activities in Africa and led to a consensus that a dedicated group should formulate an action plan, preferably within the IUCN/SSC system. It was also considered important to contribute to the IUCN's Red Data Books, where some of the species presently included (apart from those from South Africa) do not seem to be adequately justified.

It was observed that some geographical gaps in our knowledge could be filled by opportunistic involvement in biodiversity assessments which are becoming part of larger development projects: amphibians are excellently suited for rapid assessment. Dr Schiøtz (who is the co-ordinator for African Amphibia for a project currently in progress at the Centre for Tropical Biodiversity in Denmark to map species distributions in a WorldMap format) emphasized the importance of amphibians as indicators of species diversity and habitat change. This derives from a series of characteristics: that most amphibian species are strictly habitat dependent; their taxonomy is reasonably well understood; a fast assessment of the fauna can be obtained by recording mating calls in the breeding season; and the number of species is large enough to allow numerical treatment, but not so large that identification problems interfere with assessment. Indeed, the meeting acted as something of a catalyst for the official reconstitution of the IUCN Species Survival Commission Specialist Group on African Reptiles and Amphibians, at the Third World Congress of Herpetology in Prague, some 11 months later. Under the chairmanship of Bill Branch, and deputy chairmanship of Michael Klemens, this group is already moving forwards towards a more consolidated strategy for herpetofauna conservation in Africa.

The meeting was held in the Burwall's Centre of Bristol University, overlooking the spectacular Avon Gorge, and included a day spent at the nearby city of Bath - a World Heritage Site - and an informal evening at the Tinsley home.

The Symposium concluded with discussion concerning the timing and venue of the next Symposium: this is to be held in the Cape, South Africa, in late 1999, organized by Dr Alan Channing and Dr Louis Du Preez.

We are grateful to Heather Tinsley for assistance with the organization of the meeting. On behalf of the African Amphibians Working Group, we are indebted to the British Herpetological Society for supporting the publication of a permanent record of this 9th Symposium in the *Herpetological Journal*. We wish to thank all those who contributed to the programme, including session chairs, and also Arne Schiøtz who, as a founder member of the working group, provided information on its development. The Group has grown during the 27 years between the 1st and the 9th Meetings, but not to the extent of most comparable scientific organisations. However, publicity for the meeting was mailed to over 200 people known to have involvement with the study of African amphibians and to have attended recent meetings. The Proceedings could never have seen the light of day had it not been for the many referees who patiently undertook reviews of the submitted papers to a tight schedule.

Richard A. Griffiths
Michael W. Klemens
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TANZANIAN BUFONID DIVERSITY: PRELIMINARY FINDINGS

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The toad family Bufonidae is being studied as part of a review of Tanzanian amphibians. The current list includes 6 genera (one undescribed), and 28 described and undescribed species. This represents 40% of the bufonid genera and 31% of the bufonid species known in sub-Saharan Africa. Two genera and fifteen species are considered to be endemic. Tanzania appears to have the richest bufonid fauna of any sub-Saharan country. Bufonid distribution conforms with the tendency in Tanzania towards marked differences between upland and lowland biotic communities. Separation of the Afromontane *Nectophrynoides* and the lowland *Mertensophryne* and *Stephopaedes* is particularly striking. Fifty-seven per cent of the bufonid species recorded in Tanzania appear to be associated with forest. Precise data concerning the rate and extent of forest disturbance and deforestation are lacking, and the potential for forest-associated bufonids to survive these perturbations is unknown. Effective conservation action requires more information and improved understanding of the many factors involved.

INTRODUCTION

The author is part of an informal network of scientists currently working on the amphibian fauna of Tanzania. This paper reports on the first phase of a study of the toad family Bufonidae, which brings out the extraordinary richness of the Tanzanian amphibian fauna. Work is based on material in the Natural History Museum, London (NHM), augmented by loans particularly from the American Museum of Natural History, New York (AMNH); Museum of Comparative Zoology, Cambridge (MCZ); and Muséum National d'Histoire Naturelle, Paris (MNHN).

Bufonid taxonomy throughout sub-Saharan Africa lacks proper analysis, and inadequate sampling gives a very incomplete picture of species' ranges. I estimate that some 89 bufonid species can presently be discerned in the subcontinent. Twenty-eight of these (31% of the total) occur in Tanzania, a number which surpasses even Cameroon's reported total of some 24 species to reveal Tanzania as having the richest bufonid fauna in Africa according to current taxonomic assessment.

Fifteen endemic bufonid species can so far be recognized in Tanzania (54% of the total), although some of these may yet be found in northern Mozambique or north-eastern Zambia, areas that are still poorly collected. The following species list indicates Tanzanian endemics by an asterisk, and outlines total range and habitat type. In Tanzania, diversity within the genus *Bufo* is considerable not only at species level. To indicate the degree of high-ranking diversity, species are grouped according to the 'divisions' of Poynton (1996). Classification of land cover follows Thompson (1996).

BUFO LAURENTI

(1) *FUNEREUS* DIVISION

Bufo fuliginatus Witte. Upland areas of south-western Tanzania, northern Zambia and southern Zaire. Woodland to forest.

**Bufo reesi* Poynton. Kihanzi-Kilombero floodplain, southern Tanzania. Floodplain grassland.

Bufo steindachneri Pfeffer. North-eastern Tanzania, Kenya, eastern Uganda, Somalia across to northern Nigeria. Wooded grassland and bushland.

(2) *KERINYAGAE* DIVISION

Bufo kerinyagae Keith. Highland areas of Tanzania, Uganda, Kenya, Ethiopia. Grassland and cleared forest.

(3) *MACULATUS* DIVISION

Bufo maculatus Hallowell. Sub-Saharan Africa excluding most of Botswana, Namibia and South Africa. Wooded grassland to forest edge.

(4) *REGULARIS* DIVISION

Bufo gutturalis Power. South-eastern Uganda and Kenya to the Eastern Cape, westwards to Angola-Namibia. Wooded grassland to forest edge.

Bufo kisoloensis Loveridge. Highland areas of Kenya, Uganda, Rwanda, eastern Zaire, western Tanzania, northern Malawi. Forest.

Bufo xeros Tandy, Tandy, Keith & Duff-MacKay. Tanzania to Senegal. Sparsely wooded grassland and thicket.

(5) *SUPERCILIARIS* DIVISION

**Bufo brauni* Nieden. Eastern Arc Mountains of Tanzania, but not the southern highlands. Forest to forest edge.

(6) *TAITANUS* DIVISION

Bufo lindneri Mertens. Eastern Tanzania, northern Mozambique, south-eastern Malawi. Wooded grassland.

Bufo taitanus Peters. South-western Kenya, mainly western Tanzania, Malawi, northern Zambia. Wooded grassland.

**Bufo uzunguensis* Loveridge. South-western Tanzania. Swampy grassland.

**Bufo* sp. (*lonnbergi* complex). South-western Tanzania. Probably grassland.

(7) *URUNGUENSIS* DIVISION

Bufo urunguensis Loveridge. Mountains in Tanzania and Zambia bordering southern end of Lake Tanganyika. Woodland to forest.

(8) *VERTEBRALIS* DIVISION

Bufo parkeri Loveridge. Lake Natron Basin of Tanzania and Kenya. Sparsely wooded grassland.

SCHISMADERMA SMITH

Schismaderma carens (Smith). Tanzania, south-eastern Zaire, to Eastern Cape border. Wooded grassland.

NECTOPHRYNOIDES ROUX

**Nectophrynoides cryptus* Perret. Uluguru Mountains above 2000 m. Forest.

**Nectophrynoides minutus* Perret. Uluguru Mountains at about 1500 m. Forest.

**Nectophrynoides tornieri* (Roux). East Usambaras through to the Udzungwas between about 1500 m and 500 m; not lowland forests. Forest or forest margins, including plantations of exotics.

**Nectophrynoides viviparus* (Tornier). Southern Tanzanian highlands and (possibly a different form) the Uluguru Mountains. Forest.

**Nectophrynoides wendyae* Clarke. Udzungwa escarpment. Forest.

**Nectophrynoides* sp. West Usambaras. Forest.

**Nectophrynoides* sp. Uluguru North Forest Reserve. Forest.

**Nectophrynoides* sp. Kihansi Falls, Udzungwa Range. Forest.

UNDESCRIBED GENUS

**Nectophrynoides*-like species, undescribed. High Ukaguru mountains. Forest.

STEPHOPAEDES CHANNING

**Stephopaedes loveridgei* Poynton. South-eastern Tanzania. Forest, forest edge and woodland.

**Stephopaedes* sp. North-eastern Tanzania (including Mafia Island). Forest.

MERTENSOPHRYNE TIHEN

Mertensophryne micranotis (Loveridge). Eastern Tanzania (including Zanzibar and Songo Songo islands) and south-eastern Kenya. Forest or woodland.

BUFO AND *SCHISMADERMA*

Species of the genus *Bufo* occupy a wide spectrum of habitats in Africa ranging from semi-desert to forest, although the highest species diversity is in moist and relatively cool areas (Poynton, 1996). Tanzania provides a complete range of these habitats, apart from semi-desert, as reflected by the absence of members of the *arabicus* and *blanfordii* divisions of the arid Horn of Africa. During drier phases of the Quaternary, Tanzania was evidently part of an 'arid corridor' which connected the flora and fauna of north-eastern and south-western Africa (Poynton, 1995). Among bufonids, residual fragments of this arid corridor appear to be represented by the very limited, disjunct distribution in Tanzania of *B. xeros,* an arid savanna member of the ecologically diverse *regularis* division. *B. garmani,* another member of the *regularis* division inhabiting dry savannas, shows a patchy distribution with no confirmed records in Tanzania; its distribution pattern may also represent fragments of the arid corridor (Poynton, 1995). Tanzania is also included in a break in the known distribution of the mainly dry-savanna *vertebralis* division of small-sized toads (Poynton, 1995). *B. parkeri* is the only member of this division known to occur in Tanzania, recorded in the Lake Natron Basin.

The *taitanus* division of small-sized toads, whose members show a preference for open but moister situations, is represented by *B. lindneri, B. taitanus, B. uzunguensis,* and a hitherto unrecognized species of the *lonnbergi* complex from Mbeya Mountain in the south (Poynton, in press). This complex is currently recorded from grassy highlands of Malawi and Kenya (Poynton, in press); a single NHM specimen which cannot be assigned to the Malawian or Kenyan taxa indicates that this complex is also represented in high open situations in Tanzania.

Grassy highland is an important element of the Afromontane region (Meadows & Linder, 1993); *B. kerinyagae,* apparently most closely related to *B. asmarae* of Ethiopia, is limited to it. The southern record of this species is the Ngorongoro Crater rim in Tanzania.

The widespread savanna member of the *regularis* division, *B. gutturalis,* is well represented in Tanzania, as is the similar *B. maculatus. B. reesi* of the ecologically diverse *funereus* division is known only from a small area of open floodplain. Its limited known range is probably a reflection of limited collecting in Tanzania. *B. steindachneri,* another member of the *funereus* division, reflects the same situation; this species is based on a single specimen collected near the eastern edge of the Nguru Mountains in 1888, deposited in the Hamburg Museum but destroyed in World War II. It is still the only Tanzanian record of the species. Specimens that appear to belong to this species have been collected from the Kenyan coastal lowlands and beyond, but their identification needs to be confirmed by fresh topotypic material. Another member of the *funereus* division, *B. fuliginatus,* appears to be a vicariant of *B. funereus* centred in Zaire; hitherto unrecorded in Tanzania, the NHM has a specimen collected in the last century apparently in highlands west of Lake Rukwa.

B. fuliginatus may be included among the four mainly forest-associated *Bufo* species in Tanzania, the remainder being *B. kisoloensis, B. brauni* and *B. urunguensis. B. kisoloensis* is a montane member of the *regularis* division, hitherto unrecorded in Tanzania but represented by two NHM juveniles from the Mahale Mountains bordering Lake Tanganyika; the species could well occur in several patches of montane forest in under-collected western Tanzania. *B. brauni,* common in forests above 500 m, is closely related to *B. superciliaris* of West African forests. The affinities of *B. urunguensis* have yet to be determined.

This rich mix of *Bufo* species has exploited all terrestrial and freshwater habitat types in Tanzania with the exception of lowland forest; no known species of *Bufo* is restricted to it and none enter deeply into it. The known distributions of all *Bufo* species are patchy and show the need for systematic sampling; nevertheless, the available data suggest a separation of much of the toad fauna into lowland and upland or Afromontane elements, whether in wooded or open habitats. This separation has been recognized for many years (Loveridge, 1937; Poynton, 1962), but locality data are still insufficient to allow the degree of analysis and quantification now possible in more southern areas (Poynton & Boycott, 1996).

Schismaderma carens, another large-sized toad, reaches its known northernmost limit in Tanzania. At present no ecological explanation is available for its limitation in the region, although its preference for deep pools or impoundments for breeding may limit its distribution in the drier north.

NECTOPHRYNOIDES, MERTENSOPHRYNE AND *STEPHOPAEDES*

Of special interest is the occurrence of the montane *Nectophrynoides* and an undescribed related Ukaguru Mountain form, and the more lowland *Mertensophryne* and *Stephopaedes.* All these small-sized bufonids are associated with forest and do not breed in open water. *Nectophrynoides* (*sensu* Dubois, 1987) is recorded only from Tanzania. Eggs are retained in the oviduct in species whose larval stage is known, and fully formed individuals emerge from the vent. The selective advantage of this retention still has to be investigated in detail. The ranges of most species are limited to particular forest blocks, no doubt the result of vicariation in ecologically isolated areas of montane forest. The number of known species is rapidly increasing as more forests become explored.

In contrast to the ovoviviparous *Nectophrynoides, Mertensophryne* and *Stephopaedes* lay eggs in water-filled treeholes and other small containers that are normally available only in forests (references in Poynton, 1991). The tadpoles of both genera are very similar, although the adults differ sufficiently to suggest generic separation (Poynton, 1991). The distribution of these toads is in lowland or transitional forest in south-eastern Kenya, Tanzania, and eastern Zimbabwe (Grandison & Ashe, 1983; Poynton & Broadley, 1988; Poynton, 1991). The ranges of the East African species are more extensive than those of most *Nectophrynoides* species, which is evidently linked to the more continuous distribution of lowland forest throughout the Quaternary (Livingstone, 1993). In the case of the two Tanzanian *Stephopaedes* species, vicariation may have resulted from range separation caused by the Great Ruaha-Rufiji Basin. The undescribed species that occurs north of this gap shows a very fragmented range which suggests it is verging on extinction.

The altitudinal separation between the *Nectophrynoides* group and *Mertensophryne*/*Stephopaedes* is part of a general pattern shown by eastern Tanzanian forest amphibians. *Nectophrynoides* appears to belong to a mainly Afromontane group whose members are distributed from Table Mountain in the Cape through East Africa and Ethiopia to West African mountains (Grandison, 1981; Gauld & Underwood, 1986). The taxonomic affinities of *Mertensophryne* and *Stephopaedes* have yet to be clarified; a knowledge of their history should contribute to understanding the broader issues of biogeography and evolution in East Africa.

SURVIVAL PROSPECTS

Some 57% of the Tanzanian bufonid species are associated with forest. While destruction of forest in Tanzania is widespread, there is little precise information about where it is occurring and how rapidly it is taking place (Anon. 1995). Also lacking is precise information about the extent to which forest-associated amphibians can survive deforestation. Howell (1993) recorded *B. brauni* remaining in deforested areas 'as long as adequate cover of some dense vegetation persists,' and the same appears true of many other 'forest' amphibians (Poynton, 1996). It is, however, not known how long such species may continue breeding in disturbed conditions. Informed and effective conservation action requires an improved knowledge of the rate, extent, and the short and long-term effects of forest disturbance and deforestation. It may be hoped that this will be achieved before the opportunity to study the wide range of natural communities is lost.

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This study was centred in the Natural History Museum, London; the staff of the Amphibian and Reptile Section, particularly B. T. Clarke, are thanked for ready assistance in many ways. New collections and relevant information were provided by those involved in the Frontier-Tanzania project, particularly N. Burgess, K. Howell and P. Clarke. M. Klemens and M. Cherry have made available their Tanzanian collections and have provided valuable information and discussion.

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A PRELIMINARY REVIEW OF THE AMPHIBIANS OF ETHIOPIA

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Provisionally, 60 named species of amphibian are recognized in Ethiopia, of which 14 have been described since 1970. Six genera and 23 species are currently listed as endemic, the great majority of these being clearly associated with montane forest, grassland or moorland at altitudes above 1800 m. The Ethiopian Plateau has evidently provided a refuge for some taxa which have a relict distribution in the mountains of Africa and a major centre for occupation and adaptive radiation by others. Huge areas of Ethiopia remain to be explored biologically and it seems certain that future fieldwork will substantially increase the number of amphibian species recorded. Meanwhile, as destruction of natural habitats by an ever-growing human population becomes increasingly widespread, it is inevitably the threat to montane and forest endemics which gives greatest cause for concern.

THE FAUNA

At the present time, I recognize 60 named amphibian species in Ethiopia, 14 of which have been described since 1970 (Table 1). In fact, the total number of taxa has remained remarkably constant throughout this period; the addition of new species being more or less balanced by the elimination of old names now synonymized or shown to be based upon misidentifications. Hopefully, this equilibrium will soon begin to change, since few suspect names seem to remain on the current list but there is still ample scope for new discoveries. Descriptions of two further species of *Ptychadena* are expected to appear shortly (Largen, in press), included in a paper which also explains the author's reasons for doubting the validity of *P. largeni*. On the other hand, the genus *Phrynobatrachus* is desperately in need of revision, both in Ethiopia and further afield, and the status of *P. zavattarii* and *P. sciangallarum*, each known only from its type material, remains particularly obscure.

In May 1993, as a result of political rather than taxonomic decisions, Ethiopia's former northern province became the independent nation of Eritrea and *Bufo pentoni* Anderson, 1893 disappeared overnight from the Ethiopian faunal list. Two other species, *B. asmarae* and *Conraua beccarii*, ceased to be Ethiopian endemics, although both have distributions clearly centred on the Ethiopian Plateau; as indeed does *Xenopus clivii*, which is reputed to extend also into northwestern Kenya.

An eco-geographic analysis of the Ethiopian species (Table 1) indicates that 52% of the amphibian fauna is primarily associated with savanna habitats - being either widespread in sub-Saharan Africa (17%), distributed more particularly in the Sahelian region (15%), derived from the East African savannas (10%), or centred on those semi-desert areas of Somalia and eastern Ethiopia known as the Somali-arid zone (10%).

In marked contrast, the remaining 29 species in Ethiopia are predominantly montane and/or forest forms, 23 of which (38% of the total) are currently considered to be endemic. Seven of the eight families include endemic representatives, amongst which are six endemic genera: *Sylvacaecilia, Altiphrynoides, Spinophrynoides, Ericabatrachus, Balebreviceps* and *Paracassina*.

At the species level, *Bufo langanoensis* is at present known only from the Ethiopian Rift Valley, but it seems unlikely to be a true endemic and may eventually prove to be a member of the Somali-arid fauna. Three species, *Sylvacaecilia grandisonae, Leptopelis vannutellii* and *Afrixalus clarkei*, appear to be confined to the tropical deciduous forests of south-western Ethiopia at altitudes below 2200 m, but the great majority of endemics are species most clearly associated with montane forest, grassland or moorland at elevations of 1800 m and above.

Why does Ethiopia boast such a significant array of high altitude endemics? Quite simply, because the country affords an impressive area of montane habitat (Fig. 1). South of the Tropic of Cancer, almost 50% of all land above 2000 m and nearly 80% of all land above 3000 m lies within the borders of Ethiopia (Yalden, 1983). This vast plateau (Fig. 2), divided by the Rift Valley into north-western and south-eastern massifs, with an average altitude of about 2200 m but rising to more than 4600 m in the Simien Mountains, has had a profound effect upon the evolution, composition and distribution of the flora and fauna in north-east Africa. It not only provides a habitat for a unique assemblage of montane species, including numerous endemics, but also delimits the ranges of many other taxa which extend into the marginal lowlands from neighbouring territories. Species characteristic of the East African savannas, of the sub-Saharan savanna belt, of the North African deserts and of the Somali-arid zone meet and, in varying degrees, mingle around the edges of the Ethiopian Plateau.

For several years, I somewhat arbitrarily defined this plateau as being land above 2000 m, but it has gradually become apparent that, for many of the montane endemics (including mammals and reptiles as well as

TABLE 1. Provisional checklist of Ethiopian amphibians. *, status uncertain; E, Endemic (38%); EAM, E. African montane (5%); EAS, E. African savanna (10%); EP, Ethiopian Plateau (5%); PAS, Pan-African savanna (17%); SA, Somali-arid (10%); WAS, W. African savanna (15%). Species now recognized: 60

	SPECIES	ZOOGEOGRAPHIC CODE	ALTITUDINAL RANGE (m)
Caecilidae			
	Sylvacaecilia grandisonae (Taylor, 1970)	E	1500-2180
Pipidae			
	Xenopus clivii Peracca, 1898	EP	
	Xenopus largeni Tinsley, 1995	E	2500-2650
Bufonidae			
	Bufo asmarae Tandy *et al.*, 1982	EP	
	Bufo blanfordii Boulenger, 1882	SA	
	Bufo dodsoni Boulenger, 1895	SA	
	Bufo garmani Meek, 1897	EAS	
	Bufo kerinyagae Keith, 1968	EAM	
	Bufo langanoensis Largen *et al.*, 1978	E?	800-1585
	Bufo lughensis Loveridge, 1932	SA	
	Bufo maculatus Hallowell, 1854	PAS	
	Bufo regularis Reuss, 1834	WAS	
	Bufo steindachneri Pfeffer, 1893	WAS	
	Bufo xeros Tandy *et al.*, 1976	WAS	
	Altiphrynoides malcolmi (Grandison, 1978)	E	3200-4000
	Spinophrynoides osgoodi (Loveridge, 1932)	E	1950-3520
Ranidae			
	Cacosternum boettgeri (Boulenger, 1882)	EAS	
	Conraua beccarii (Boulenger, 1911)	EP	
	Ericabatrachus baleensis Largen, 1991	E	2400-3200
	Euphlyctis occipitalis (Günther, 1858)	PAS	
	Hildebrandtia macrotympanum (Boulenger, 1912)	SA	
	Hylarana galamensis (Duméril & Bibron, 1841)	PAS	
	Phrynobatrachus bottegi (Boulenger, 1895)	E?	
	Phrynobatrachus minutus (Boulenger, 1895)	E?	
	Phrynobatrachus natalensis (Smith, 1849)	PAS	
	Phrynobatrachus sciangallarum (Scortecci, 1943)*		
	Phrynobatrachus zavattarii (Scortecci, 1943)*		
	Ptychadena anchietae (Bocage, 1867)	EAS	
	Ptychadena cooperi (Parker, 1930)	E	2500-3100
	Ptychadena erlangeri (Ahl, 1924)	E	1300-2500
	Ptychadena largeni Perret, 1994*		
	Ptychadena mascareniensis (Duméril & Bibron, 1841)	PAS	
	Ptychadena nana Perret, 1980	E	2000-3000
	Ptychadena neumanni (Ahl, 1924)	E	820-3800
	Ptychadena porosissima (Steindachner, 1867)	EAS	
	Ptychadena pumilio (Boulenger, 1920)	WAS	
	Ptychadena schillukorum (Werner, 1907)	PAS	
	Ptychadena schubotzi (Sternfeld, 1917)	WAS	
	Rana angolensis Bocage, 1866	EAM	
	Rana wittei Angel, 1924	EAM	
	Tomopterna cryptotis (Boulenger, 1907)	PAS	
Hemisotidae			
	Hemisus marmoratus (Peters, 1854)	PAS	
	Hemisus microscaphus Laurent, 1972	E	1500-2700

TABLE 1. (continued...)

Microhylidae		
Balebreviceps hillmani Largen & Drewes, 1989	E	3200
Phrynomantis somalicus (Scortecci, 1941)	SA	
Rhacophoridae		
Chiromantis petersii Boulenger, 1882	SA	
Hyperoliidae		
Afrixalus clarkei Largen, 1974	E	820-1800
Afrixalus enseticola Largen, 1974	E	1800-2750
Afrixalus fulvovittatus (Cope, 1861)	WAS	
Afrixalus vittiger (Peters, 1876)	WAS	
Hyperolius balfouri (Werner, 1907)	WAS	
Hyperolius kivuensis Ahl, 1931	EAS	
Hyperolius nasutus Günther, 1864	PAS	
Hyperolius viridiflavus (Duméril & Bibron, 1841)	WAS	
Kassina senegalensis (Duméril & Bibron, 1841)	PAS	
Leptopelis bocagii (Günther, 1864)	EAS	
Leptopelis gramineus (Boulenger, 1898)	E	1900-3900
Leptopelis ragazzii (Boulenger, 1896)	E	1930-3100
Leptopelis susanae Largen, 1977	E	2600-2700
Leptopelis vannutellii (Boulenger, 1898)	E	1500-2200
Leptopelis yaldeni Largen, 1977	E	2000-2700
Paracassina kounhiensis (Mocquard, 1905)	E	2400-3000
Paracassina obscura (Boulenger, 1894)	E	820-3000

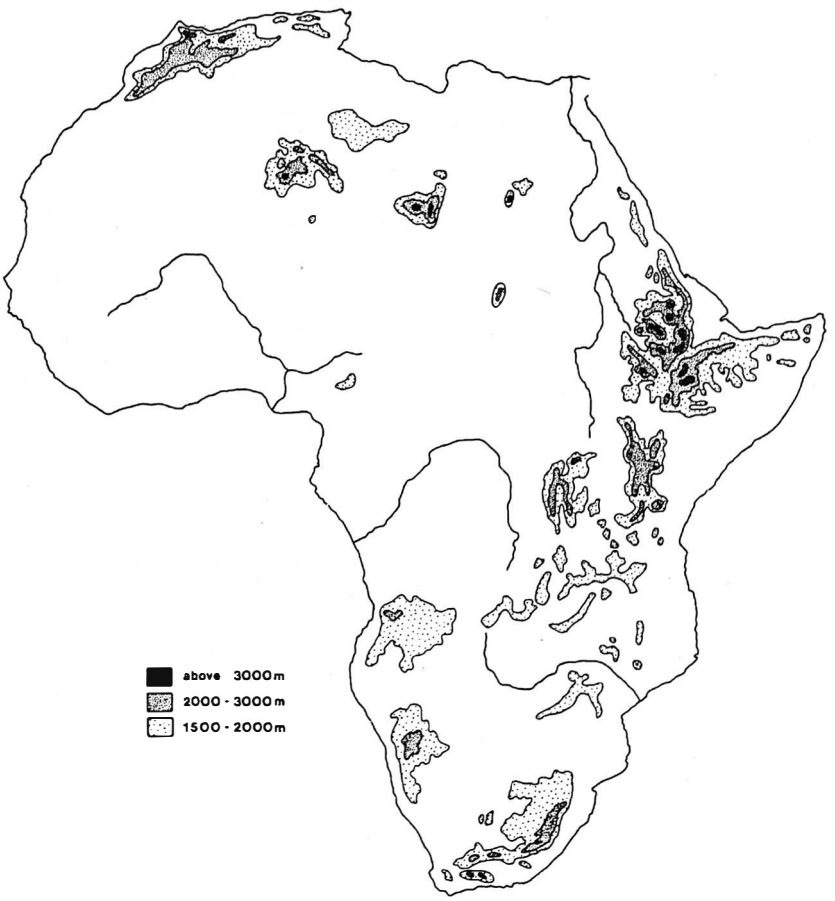

FIG. 1. Distribution of high ground on the continent of Africa.

amphibians), an elevation of about 1800 m is of much greater significance. In fact, only four of the 17 amphibian species categorized as montane endemics are known to occur below this critical limit.

Hemisus microscaphus extends down to 1500 m in the forests of south-western Ethiopia, and it seems that the type locality of *Ptychadena erlangeri* may have been as low as 1300 m. *P. neumanni* appears to have the greatest altitudinal range of any Ethiopian amphibian, assuming that populations found in the Godare Forest at 820 m are conspecific with those from Afro-alpine moorland at 3800 m (and I can find no convincing morphological evidence to suggest that they are different). *Paracassina obscura* is also reported to be present at 820 m in the Godare Forest (Drewes & Roth, 1981).

Even these exceptions conform to a rule that montane species in Ethiopia tend to reach unusually low elevations only at forested sites in the south-western sector of the country, while being conspicuously absent from similar altitudes in the arid grasslands of the south and east. Perhaps species particularly associated with cool, moist environments in the Ethiopian highlands have their wider distributions limited more by humidity than by temperature?

The Ethiopian Plateau has clearly provided a refuge for some taxa which have a relict distribution in the mountains of Africa, and simultaneously an important centre for occupation and adaptive radiation by others, including such large and currently successful genera as *Ptychadena* and *Leptopelis*.

Throughout most of its considerable range in sub-Saharan Africa, *Leptopelis* occurs at low to moderate elevations and rarely extends much above 2000 m, yet in Ethiopia the genus is represented by no fewer than four endemic species that are regularly found higher than 2600 m, including one, *L. gramineus*, which reaches an altitude of 3900 m (Largen, 1977).

Such adaptability appears all the more impressive when it is shown to be far from universal. *Hyperolius*, for example, is known to be represented in Ethiopia by just four species, none of which are endemic and even *H. viridiflavus* seems to be restricted mostly to the foothills of the plateau and not often found much above 2000 m. *Ptychadena, Leptopelis* and even *Afrixalus* demonstrate a capacity to occupy and diversify in montane environments, not excluding Afro-alpine moorland, that *Hyperolius* conspicuously lacks.

Amongst relict populations in the Ethiopian highlands is the brevicipitine species *Balebreviceps hillmani*, found just below the treeline at 3200 m in the Bale Mountains (Largen & Drewes, 1989). Here it is widely separated, both geographically and ecologi-

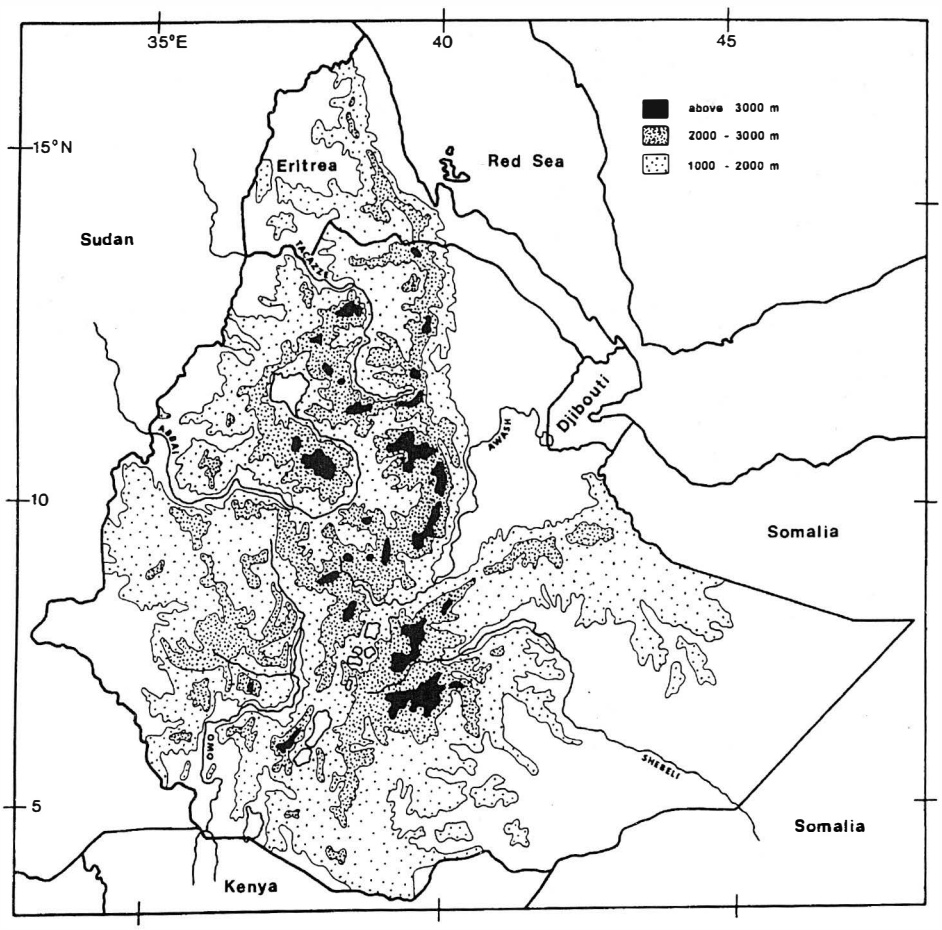

FIG. 2. Distribution of high ground in Ethiopia and Eritrea.

cally, from its nearest relatives - *Probreviceps* in the mountains of Tanzania and Zimbabwe, and *Breviceps* which ranges from Tanzania southwards to the Cape. The discovery of *Balebreviceps* in 1986 raised the still unanswered question of why this subfamily is unknown in the central highlands of Kenya, where there appears to be an abundance of suitable habitat.

It seems significant that a similar discontinuity is encountered when one considers the distribution of the specialized bufonids represented by the monotypic genera *Spinophrynoides* and *Altiphrynoides* on the Ethiopian Plateau, by at least five species of *Nectophrynoides* in the mountains of Tanzania, and by two species of *Nimbaphrynoides* in the Mt Nimba region of West Africa. Are montane populations in southern Ethiopia really so isolated from those in north-eastern Tanzania? Thirty years ago, it might have been claimed that the Kenya highlands were among the more thoroughly-explored regions of Africa, but perhaps our knowledge of this area is more deficient than we sometimes realize.

A final example is provided by the small group of petropedetine frogs with bifid toe tips, that is represented by seven species of *Petropedetes* in the forests of West Africa and by three montane isolates in eastern Africa - including, in this case, a Kenyan population which does provide a geographical link between those in Ethiopia and Tanzania. There are sufficient similarities between *Petropedetes* and *Arthroleptides martiensseni* Nieden, 1910 from the Tanzanian mountains to support a belief that they are congeneric, but *Ericabatrachus* from the Bale Mountains in southern Ethiopia is clearly distinct (Largen, 1991). *Arthroleptides dutoiti* Loveridge, 1935 is known only from the two type specimens obtained on Mt Elgon in Kenya (where it may now be extinct). I have not seen either of these examples, so it is only a reading of the rather brief description of the species which leads me to suspect that *dutoiti* may be more closely allied to *Ericabatrachus* than to *Arthroleptides*.

CONSERVATION OF THE FAUNA

I do not believe that protection of amphibian species is an issue which can yet be meaningfully addressed in most African countries, except within the framework of initiatives aimed at achieving environmental conservation for the benefit of the flora and fauna as a whole. Unfortunately, the historical record sometimes makes it difficult to feel much optimism about the future of any wildlife conservation programme in Ethiopia (Yalden, Largen, Kock & Hillman, 1996).

During the past 50 years, Ethiopia's human population is estimated to have increased eight-fold, from 7 million people in 1940 to 55 million in 1992 (O.P.H.C.C., 1984), and massive destruction of natural habitats has resulted from an ever-growing demand for land and its produce. Forests and woodlands are perhaps most seriously in decline, being felled at an alarming rate to provide timber, fuel-wood and cleared ground for farming. In both the arid lowlands and on the high plateau, more and more marginal land is being forced into temporary cultivation by subsistence farmers, even at altitudes in excess of 3000 m and on slopes so steep that ploughing without adequate terracing makes severe erosion inevitable.

It is now over 30 years since Brown (1965) lamented that "in all of Africa no country has been so brutally ravaged by man", and meanwhile the pace of destruction has scarcely diminished. Huge tracts of once-fertile land have now become so degraded that a relatively slight climate fluctuation is sufficient to render them incapable of supporting the human population, and the result has been the appalling famines and mass migrations of recent decades. All the best efforts of governmental and international organizations with responsibility for wildlife conservation have been thwarted by a seemingly endless succession of social, political and military disasters that have conspired to thrust environmental problems ever further down the list of national priorities. Inevitably, it is the welfare of the most precious elements of the flora and fauna - the montane and forest endemics - which gives the most immediate cause for concern.

It now remains to be seen whether the new civilian government, which has only recently supplanted a long-discredited military dictatorship, will be successful in fostering the social, economic and environmental renewal which the country so desperately needs.

FUTURE PROSPECTS

The preliminary nature of the present review, though due in part to the fact that current research on Ethiopian amphibians has yet to be completed, is far more a reflection of the reality that large areas of the country still remain to be explored by any biologist, let alone visited by a herpetologist.

During three weeks in August 1986, the Harenna Forest Expedition conducted a transect survey on the southern slopes of the Bale Mountains (Largen & Drewes, 1989), obtaining in the process two new genera and three new species of anuran (in addition to a new chameleon, two new shrews and numerous extensions to the previously known ranges of other taxa). If such a brief visit to a comparatively limited area can be so rewarding, it is not difficult to imagine the great wealth of novelties which must await discovery elsewhere on the vast Ethiopian Plateau. A particularly obvious target is offered by mountains in the provinces of Wello and northern Shoa, where extensive tracts of land above 3000 m still require investigation.

I mentioned earlier three amphibian species which appear to be endemic in the dense tropical forests of south-western Ethiopia. This seems a very small number, considering the geographical extent, isolation and ecological diversity of the available habitat, and may well be attributable as much to the inaccessibility of the region in question as to any genuine impoverishment of its fauna.

Neither is it only the mountain and forest areas of Ethiopia which are inadequately known. Arid lowlands

in the extreme east of the country, where the Ogaden region threatens to bisect the Somali Republic, remain a virtual *terra incognita* as far as herpetology is concerned, and no fewer than six amphibian species recorded from central and northern Somalia (Lanza, 1990) are still unknown in Ethiopia. Most, if not all, of these taxa [*Lanzarana largeni* (Lanza, 1978); *Ptychadena mossambica* (Peters, 1854); *Pyxicephalus adspersus* Tschudi, 1838; *P. obbianus* Calabresi, 1927; *Kassina parkeri* (Scortecci, 1932); *K. somalica* Scortecci, 1932] will surely be found in the Ogaden salient, once this region of the country comes to be adequately explored.

I expect the number of amphibians recorded from Ethiopia to be substantially increased in future years - but only after considerable effort has been invested in further fieldwork. Though it may be a long time before the herpetofauna is thoroughly documented and understood, there is no reason to suppose, given the necessary commitment and resources, that this will not eventually be achieved. But what proportion of the Ethiopian fauna, and particularly of the many unique endemics (both known and yet to be discovered), will survive the next century? That seems much more difficult to predict with any degree of confidence, given this country's miserable record of failing to conserve its natural assets.

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MITOCHONDRIAL RDNA PHYLOGENY IN *XENOPUS*

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Based on morphological, biochemical and karyological characters, the genus *Xenopus* can be divided into two main groups (subgenera), *Silurana* and *Xenopus*, and the latter into five subgroups. The relationships among these five subgroups are less clear. Since all except one species are allopolyploid (tetra-, octo- and dodecaploid), they are, by definition, not monophyletic. In principle, sequence data would permit unravelling of these complex relationships, provided that all duplicated genes were conserved. However, that is not the case: redundant genetic information tends to become lost, interrupting phylogenetic lines of descent of the genes. Since the mitochondrial genome is inherited in a purely matrilinear manner, problems linked to polyploidy are seemingly avoided. However, this character is not monophyletic either. At least at the start of an allopolyploid speciation, mitochondria of both parental species can be present though one or the other type eventually becomes extinct. Which one is conserved is probably random. Nevertheless, it may be interesting to compare the phylogeny of mitochondria to species trees based on nuclear characters. We sequenced about 600 bp of mitochondrial 12s and 16s rRNA genes of the diploid *X. tropicalis*, of most tetraploid species, and of the octoploid *X. wittei*. Trees obtained with Neighbor Joining, Maximum Likelihood and Maximum Parsimony methods essentially confirm the *tropicalis, laevis* and *muelleri* groups and subgroups, whereas the *fraseri* subgroup is less well defined. Mitochondria of *X. clivii* and *X. largeni*, members of the *muelleri* and the *laevis* subgroup respectively, show only a low bootstrap score when connected to any subgroup, thus forming a polytomy of several species. Divergence of the same sequence between *Rana catesbeiana* and *R. temporaria*, for which immunological and zoogeographic considerations suggest a possible age of roughly 30-40 Ma, was used for tentative calibration of the *Xenopus* mitochondrial tree. This calibration is necessary for comparison with other phylogenetic data on this genus.

INTRODUCTION

Polyploid *Xenopus* are thought to have arisen through interspecific hybridization. The main evidence for this is that natural and laboratory-made hybrid females spontaneously produce endoreduplicated eggs in variable proportions. Backcrosses with both parental species may result in tetraploid animals (tetraploid with respect to the ploidy of the parental species). Both females and males occur in this F 3 generation, because the dominant female determining genetic mechanism of sex determination is abolished in favor of an environmental mode of sex determination in such experimental allopolyploids (Kobel, 1996).

Although *Xenopus* species can be assigned to several groups and subgroups (Fig. 1) as defined by morphological, karyological, biochemical and parasitological characteristics (reviewed by Kobel *et al.*, 1996; Tymowska, 1991; Graf, 1996; Tinsley, 1996), relationships between these groups remain obscure. In a few cases, hypothetical parental species or parental subgroups can be ascribed to higher polyploids (Fig. 1). However, for the three subgroups at the tetraploid level (4X=36), no diploid species survived that could be studied as potential parental species, though a number of such species certainly did occur at one time.

Theoretically, sequences of duplicated genes that are present in polyploids permit one to look back at dichotomies of extinct diploid parental species. Thus, sequence divergence between 17 duplicated genes of *X. l. laevis* suggests an age of 27-35 Ma of its parental species as measured by the 80 Ma old divergence of the homologous genes between man and rodents (Hughes & Hughes, 1993). In an earlier comparison of globin cDNA sequences (Knöchel *et al.*, 1986) within and between the diploid *X. tropicalis* and the tetraploid *X. borealis* and *X. l. laevis*, diploid and tetraploid species showed a very distant relationship. Whereas the diploid ancestors of the tetraploids diverged possibly 50 Ma ago, dichotomy of the two tetraploid species is thought to have occurred more recently (15-20 Ma) at the tetraploid level. However, the data probably better fit a model comprising three diploid ancestral species and two separate allopolyploidization events as origin of the two tetraploids. Hence, sequence data are not apt to distinguish speciation events between the different ploidy levels nor do they contain information on the allopolyploidization events themselves. Nevertheless, sequence data are valuable in providing insight into the speciation of the ancestral species of allopolyploids. Polyploidy also means redundancy of genetic information which is prone to loss of duplicated genes, thereby interrupting phylogenetic lines in a gene tree. It has been estimated that 25-50% of duplicated genes have been silenced in the tetraploid *X. l. laevis* (Graf & Kobel, 1991; Hughes & Hughes, 1993). The situation is worse in higher polyploids because their parental species might have already lost some duplicated genes

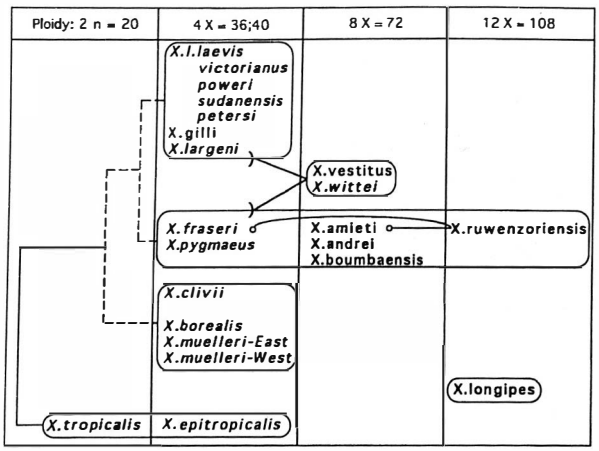

FIG. 1. Composition of the genus *Xenopus*. The species are arranged in groups of related taxa (Kobel *et al.*, 1996), and with respect to ploidy (Tymowska, 1991). The two main branches can be regarded as subgenera *(Xenopus*, *n*=9; *Silurana,* comprising *X. tropicalis and X. epitropicalis*, *n*=10). The tentative connections at the diploid level between tetraploids indicate that these groups descend from extinct diploid species of the subgenus *Xenopus*. The three subgroups might have originated from different hybrid combinations between the postulated diploid species. Species in italics were included in this study.

before the next round of allopolyploidization occurred (Kobel & Du Pasquier, 1986). Also, tetraploids of the various subgroups seem to have silenced genes in an independent manner, each species conserving its own collection of duplicated genes.

Sequences from the mitochondrial genome, which is inherited in a purely matrilinear manner, would seem to avoid problems linked to polyploidy. However, this character also is not monophyletic. At least at the start of an allopolyploidization, mitochondria of both parental species can be present, though one or the other type eventually will be lost. Which one is conserved is probably random. Consequently, different mitochondrial trees may fit equally well to a particular "true" species tree (Fig. 2).

A first attempt to unravel relationships between polyploid *Xenopus* species using mitochondrial genomes (Carr *et al.*, 1987), resulted in a number of contrasting trees of similar parsimony. The study revealed rather pronounced differences between restriction maps of the species, which prompted us to sequence parts of the mitochondrial 12s and 16s rRNA genes from 15 *Xenopus* taxa. Although mitochondrial gene trees, as stated above, do not necessarily parallel allopolyploid species trees, additional information of this kind contributes to an understanding of the complex reticulate evolution of this genus.

MATERIALS AND METHODS

ANIMALS

For the 15 species and subspecies of *Xenopus* analysed, see Fig. 1, names in italics. All animals were laboratory bred at the University of Geneva. Eggs of a wild caught *Rana temporaria* were also included in order to have, together with the published sequence of *R. catesbeiana* (Nagae *et al.*, 1988) a pair of *Rana* species for comparison and calibration.

CLONING

The mitochondrial genome of most *Xenopus* species contains only two Sac II restriction sites, delimiting a fragment of about 1700 bp that comprises parts of the 12s and 16s rRNA genes (Carr *et al.*, 1987). Mitochondria were isolated from eggs by differential centrifugation, and their DNA purified by proteinase K treatment and ethanol precipitation. After digestion with Sac II or Ksp I nucleases, fragments were inserted into Bluescript II KS vector at its Sac II site. Transformed DH 5a *E. coli* were selected and their vectors used to transform JM 105 strains in order to induce ssDNA for sequencing by the dideoxy method. All ma-

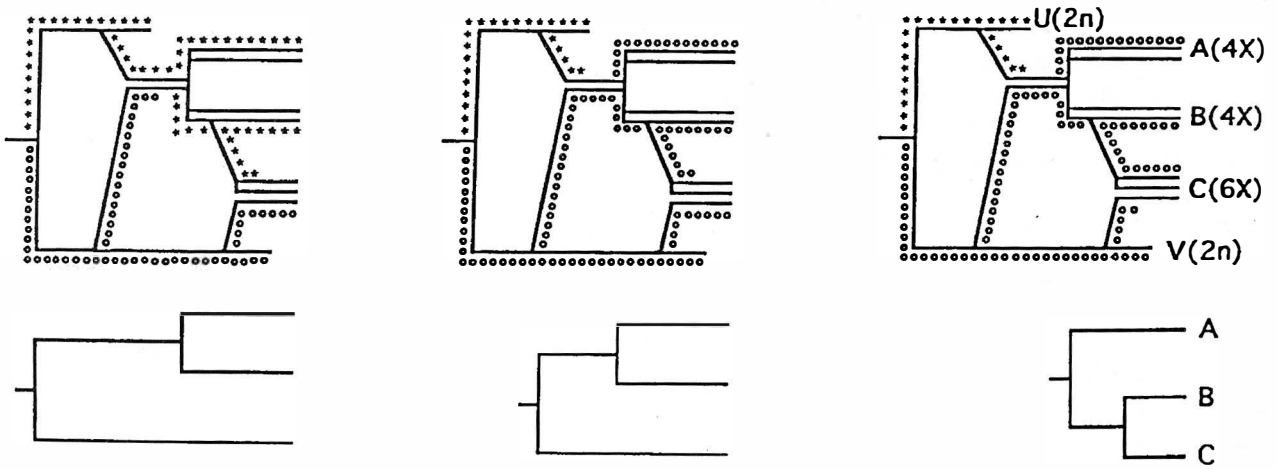

FIG. 2. Model trees demonstrating the limited parallelism between mitochondrial trees and the "true" species trees in reticulate evolution. Several contrasting mitochondrial trees may fit to the same species tree, because of the random loss of parental mitochondrial types. Upper row: — identical nuclear trees; ***and ooo mitochondrial lines. Second row : corresponding mitochondrial trees as inferred from sequence data; diploid species omitted.

nipulations were done following Sambrook *et al.* (1989).

SEQUENCE ANALYSIS

Sequences were aligned by hand, consulting secondary structure models for rRNAs (Dams *et al.*, 1988; Gutell & Fox, 1988; Springer & Douzery, 1996) and different alignments proposed by the Clustal V program (Higgins *et al.*, 1992). Trees were constructed by Neighbor Joining (NJ) (Saitou & Nei, 1987), Maximum Likelihood (ML) (Felsenstein, 1993) and Maximum Parsimony (MP) (Swofford, 1993), using the GDE 2. 2 software (Larson *et al.*, 1993). Robustness of the NJ and MP trees was estimated with 1000 bootstrap replications (Felsenstein, 1985).

RESULTS AND DISCUSSION

ALIGNMENT, VARIABLE SITES

About 600 bp of rDNA were sequenced; 12s : 2319-2618, 16s : 3742-4041 (bp numbering of Roe *et al.* 1985). Five bp (3907, 3976, 3997, 4044, 4028) could not be found in any species; on the other hand, two bp (2551+, 4035+) not listed there, were present in all species.

For most parts of the sequences, alignment is straightforward by introducing gaps where necessary. The consensus length, including *Rana* species, is 598 bp + 16 indels. Some species have shorter sequences, e.g. *X. wittei* 592 bp, *R. temporaria* 572 bp. However, for a few short stretches, especially the loops connecting stem 17 with 18 (Springer & Douzery, 1996), alignment remains ambiguous. These loops (18 bp and gaps) were therefore deleted from the analysis. Among the remaining 596 bp and gaps, 235 are variable sites (12s : 106, incl. 54 positions to accommodate for *Rana*; 16s : 129, incl. 48). Distribution of variable sites appears not to be random, e. g. more than 70% of the variable sites of the 12s fragment are found in the 5' half. Transitions are up to 3. 5x more frequent than transversions.

The secondary structure model for mammalian 12s (Springer & Douzery, 1996) is not applicable exactly to *Xenopus;* some helices contain more, others fewer potential stem nucleotides than the mammalian model shows. A number of compensatory replacements have occurred between *Xenopus* species as well as with respect to mammalian helices.

TOPOLOGY OF TREES

NJ (Fig. 3) and ML gave essentially the same mitochondrial rDNA trees. The subgenera *Silurana* and *Xenopus* are well separated. Within the latter, the *muelleri* subgroup represents the sister group to the remainding species, of which the *laevis* subspecies form a defined entity. However, neither *X. clivii* (*muelleri* subgroup) nor *X. largeni (laevis* subgroup) are placed with their respective subgroup (Fig. 1). Instead, these two species, together with the three species of the *fraseri* subgroup form an ill-defined cluster with very short common branches. Omitting the two species

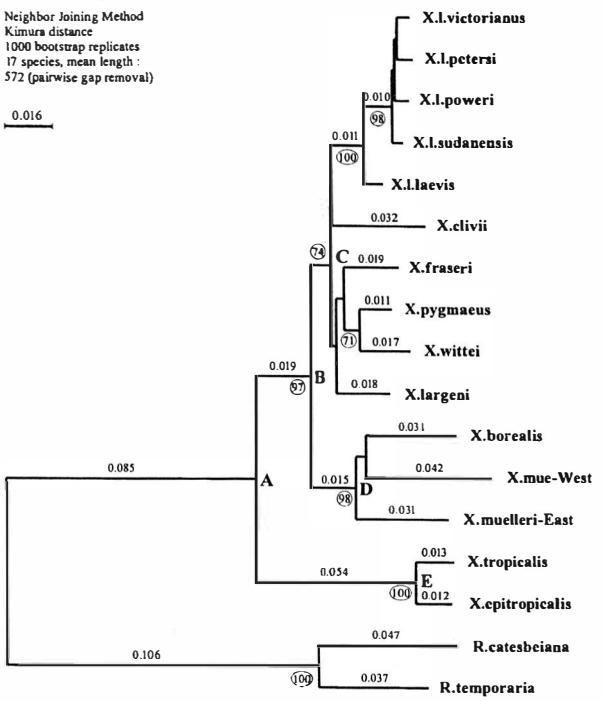

FIG. 3. Neighbor Joining tree with branch lengths in nucleotide replacements per site and bootstrap scores (circled, values above 70 only).

gives better support for the relationship [*muelleri* subgroup *((fraseri* subgroup) (*laevis* subgroup))]. MP, applying a bootstrap 50% majority-rule, places the five species together with the *laevis* branch as a polytomy, with the *muelleri* subgroup as sister group.

Within the *muelleri* subgroup, *X. muelleri*-East and *X. muelleri*-West show an amount of sequence divergence such that both have to be regarded as separate species, confirming evidence from other traits such as biochemical characters and parasite fauna (Tinsley, pers. comm.). *X. borealis* appears more closely related to *X. muelleri*-West, but their common branch is short, pointing to a trichotomy, i. e. a more or less simultaneous speciation. That *X. clivii* is not attached to this branch of the tree, is not a contradiction to the species grouping of Fig. 1. The four species could still have emerged from the same allopolyploidization event, *X. clivii* having conserved its mitochondrial type from the one parental species while the remaining species of this subgroup conserved the mitochondrial type of the other parent. The same argument holds also for *X. largeni*. This is a matter of interpretation since, as stated above, a mitochondrial gene tree shows only half of the parental relationships of allopolyploids.

The clade of the *laevis* subgroup comprises five taxa that are regarded as subspecies with *X. l. laevis* as the basal subspecies having diverged earlier than the other four. Therefore, it seems appropriate to upgrade the rank of this taxon to the species level. The remainder are so similar in their rDNA sequences that subspecies status seems adequate in spite of the distinctness in mating calls (Vigny, 1979) and genetic make-up (Graf, 1989). This contradictory situation demands further investigation.

How can the topology of these mitochondrial rDNA trees be interpreted? Since the sequence data contain no information about the ploidy level on which the various dichotomies occurred, one can only speculate. While there is no doubt that nodes A and E (Fig. 3) occurred at a diploid level, dichotomy B could have occurred either at a diploid or tetraploid level. In the former case, the two post-B branches would thus represent two parental species among an unknown number of diploid species, such as must be postulated to account for the duplicated nuclear genes of tetraploids. Sequences of duplicated genes of *X. l. laevis* (see below) possibly coalesce at a point which coincides with node B of the mitochondrial tree, enhancing the view that the two branches represent a diploid level indeed. Nodes C and D, on the other hand, are polytomies rather than dichotomies, a natural outcome if allopolyploidization triggered a burst of speciation. This is easily conceivable since allopolyploidy assembles adaptations of two parental species and creates genetic redundancy with which evolution may experiment. The emergence of polyploid *Xenopus* then likely took place somewhere between nodes B and C, D. How many polyploidization events occurred cannot be known. Also, more diploid species might have been implicated in allopolyploidization than appear in the tree, but for which mitochondria were lost.

Branch Lengths, Calibration

Branch lengths vary between species and between subgroups; *tropicalis* and *muelleri* groups together with *Rana* have 15% longer branches than *fraseri* and *laevis* subgroups.

Measured by a mammalian standard, sequence divergences in *Xenopus* are rather low, as is the case in some other non-mammalian vertebrates (Avise *et al.*, 1992; Martin *et al.*, 1992). In order to propose a more appropriate calibration, we collected similar data also for *Rana temporaria*. Comparison with the published sequence of *R. catesbeiana* (Nagae *et al.*, 1988) gives a dichotomy for which external evidence exists to determine age. First, it has been proposed (Duellman & Trueb, 1986) that *Rana* was transported by the drifting Indian subcontinent from Africa to Asia and arrived there about 34 Ma ago, from which the ancestors of the two species migrated in opposite directions to Europe and to North America. Second, immunological distances (Post & Uzzell, 1981; Uzzell, 1982) suggest an age of 33-43 Ma for the separation between these two branches. This gives an estimate of the substitution rate for these rDNA fragments of roughly 0.12% per Ma. Considering that this figure is based on only two species, this calibration cannot be more than tentative and has to be taken cautiously.

Using this value for *Rana*, separation between the two anuran families goes back about 130 Ma, which is an estimate that agrees with other evidence. Fossils of Pipidae are known from 120 Ma (Baz, 1996). Since these already show adaptations to an aquatic life style, the age of the family must be older. Mitochondrial rDNA sequences of extant *Xenopus* species coalesce at 48 Ma, contrasting with values of immunological distance (33 Ma; Bisbee *et al.*, 1977) and globins (110 Ma; Knöchel *et al.*, 1986). However, as demonstrated in Fig. 2, mitochondrial gene trees do not necessarily show the deepest nodes of species trees. In this figure, diploid species were omitted from the mitochondrial trees for the sake of simplicity. By including species V, for example in the third tree, it can be seen that the resulting tree does not show a coalescence point as deeply rooted as that of the duplicated nuclear genes of the polyploid species. Nevertheless, it seems unlikely that the *Silurana* line is directly implicated in the polyploidy of the subgenus *Xenopus*.

Node B (Fig. 3) has an age of 30 Ma, which is very close to the age (27-35 Ma) of 17 duplicated nuclear genes of the tetraploid *X. l. laevis* (Hughes & Hughes, 1993). The divergence between duplicated genes in an allotetraploid specifies the dichotomy that led to the two diploid parental species of that allotetraploid. Hence, the coincidence in the ages of mitochondria and nuclear genes strongly suggest that mitochondrial node B represents a dichotomy at the diploid level also. The first polyploid *Xenopus* species then would have originated posterior to node B. Nodes C and D represent polytomies rather than dichotomies. As discussed above, such bursts of speciation are not unexpected consequences of allopolyploidizations.

The youngest diploid node (10 Ma; node E) lies in the subgenus *Silurana*. If *X. epitropicalis* is also an allotetraploid, as differences within its chromosome quartets suggest (Tymowska, 1991), the dichotomy at node E then indicates the existence of a second diploid species. A relatively recent origin of *X. epitropicalis* can also be inferred from the presence of duplicates of almost all genes analysed so far in this species (unpublished; L. Du Pasquier, pers. comm.). This second species has not been found though not many localities have been sampled in the Congo rainforest. However, since only a single diploid species is known in the entire genus, one has to envisage the possible capability of allopolyploids to outcompete their diploid ancestors, and that the second diploid *tropicalis*-like species may be extinct.

CONCLUSION

The relatively short rDNA fragments yielded a surprising amount of information. Although mitochondrial gene trees do not necessarily parallel species trees in the case of allopolyploid (reticulate) speciation, the trees (NJ, ML, MP) confirm postulated subdivisions in the subgenus *Xenopus*. However, the similarity of the mitochondria of the *fraseri* and *laevis* subgroups indicates they may be more closely related to each other than was previously thought. Two other features in the trees are of special interest. Node B, by using a *Rana* mitochondrial calibration, appears to coincide with the coalescence point of duplicated genes of the tetraploid *X. l. laevis*. One may infer therefore that this deeper part

of the tree still represents a diploid level. On the contrary, the polytomies that follow could reflect bursts of speciation caused by allopolyploidization events.

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POLYPLOIDY AND PARASITIC INFECTION IN *XENOPUS* SPECIES FROM WESTERN UGANDA

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Three *Xenopus* taxa occur with sympatric or parapatric distributions in western Uganda. Two of these, *X. wittei* and *X. vestitus*, are polyploid relative to the third, *X. laevis*, and are considered to have arisen by allopolyploidization. This is a rare mechanism of speciation by which doubling of the genome in interspecific hybrids leads to the production of a self-maintaining hybrid lineage. As one of the parental lineages of both higher polyploid species may have been related to *X. laevis*, the co-occurrence of the three taxa might show the effects of hybrid host genomes on parasite specificity. Data are reported on the natural distribution of a monogenean fluke, *Protopolystoma*, occurring in these hosts at five sites in western Uganda. It is established that *P. xenopodis*, a common parasite of *X. laevis*, is absent from wild populations of *X. vestitus* and *X. wittei* in this area. Another *Protopolystoma* species infects all three hosts. The results of other recent host-specificity studies of parasites occurring in central African *Xenopus* species are also reviewed. Observed host-parasite relationships can be related to the genealogical origin of *X. wittei* and *X. vestitus*. Hybrid organisms might inherit susceptibility to parasites from both parents and thus be exposed to infection by a greater number of species. However, the available information suggests that allopolyploid clawed toads do not show such an effect and are resistant to some potential parasites from their presumed parental lineages. The possible use of parasites as "tags" for investigating the distribution and relationships of cryptic host species is also considered.

INTRODUCTION

The pipid genus *Xenopus* includes a series of polyploid representatives, at least some of which are allopolyploid, having originated from the hybridization of separate lineages (Tymowska, 1991; Kobel, 1996). Highland lakes in western Uganda are inhabited by three taxa (Tinsley, Kobel & Fischberg, 1979). These include two octoploid species, *X. wittei* Tinsley, Kobel & Fischberg and *X. vestitus* Laurent that may have arisen from interbreeding between tetraploid ancestors related to *X. fraseri* Boulenger and *X. laevis* (Daudin) (see Tymowska, 1991). *X. wittei* and *X. vestitus* occur sympatrically with *X. l. victorianus* Ahl in western Uganda (Tinsley *et al.*, 1979): both higher polyploids thus co-occur or show adjacent distributions with each other and with a species related to one of their likely parental lineages. *X. fraseri*-like toads do not occur sympatrically with *X. wittei* or *X. vestitus* and are restricted to lowland tropical forest, a habitat which exists in the Zaire basin immediately adjacent to highland regions of the western Rift Valley occupied by *vestitus* and *wittei*. As the *Xenopus* taxa mentioned above are found within a restricted geographical area, sometimes in the same water bodies (Tinsley *et al.*, 1979), they might frequently be exposed to infective stages of the same parasite species. This is particularly likely given the great ecological similarity of clawed toads, which are all fully aquatic and feed underwater (Tinsley, Loumont & Kobel, 1996). The distribution of *Xenopus* species in western Uganda and surrounding areas therefore presents a "natural experiment" on the effects of large scale host genetic changes on parasite specificity (Tinsley, 1981).

Protopolystoma is a polystomatid monogenean which occurs as an adult in the urinary bladder of *Xenopus* species. The life histories of monogeneans make them particularly suitable for a study of interspecific variation in host resistance to parasitism. They typically show restricted host ranges (e.g., Du Preez, Kok & Seaman, 1997; Tinsley & Jackson, 1998) and a direct life-cycle with transmission by actively swimming infective stages. The parasites' distribution is therefore unaffected by a reliance on intermediate hosts which might show varying ecological interactions with different final host species (Tinsley, 1981).

The present paper reports the distribution of *Protopolystoma* species in relatively large samples of *X. wittei*, *X. vestitus* and *X. l. victorianus* from five sites in western Uganda. Existing information on the host-specificity of these polystomatids from central African *Xenopus* (based on laboratory experiments and records of natural distributions) is summarized and data for other parasite groups are also reviewed. The significance of host ploidy level and hybrid origin on parasite distributions, and the possible uses of parasites in studies of the host group are considered.

MATERIALS AND METHODS

GENERAL

Xenopus species were collected from five localities in western Uganda (Kigezi District) in September, 1996, and immediately killed and preserved (in forma-

lin) for return to the laboratory in Bristol. Samples were taken at L. Bunyonyi (*X. wittei*, *n*=63); L. Chahafi (*X. l. victorianus*, *n*=40); L. Echuya (*X. wittei*, *n*=37); L. Mulehe (*X. vestitus*, *n*=95; *X. wittei*, *n*=6) and L. Mutanda (*X. vestitus*, *n*=74). The urinary bladders of all toad specimens were removed and examined for the presence of *Protopolystoma* spp. Terminology for infection statistics follows Margolis, Esch, Holmes, Kuris & Schad (1982).

PARASITES

The two *Protopolystoma* species found in this study will be described or redefined elsewhere (Tinsley & Jackson, 1998). One species corresponds to the existing taxon *P. xenopodis* (Price, 1943), while the other form is referred to here as species A.

HOST IDENTITY

Present localities for *X. wittei* and *X. vestitus* are amongst those listed in the studies describing *X. wittei* and *X. kigesiensis* Tinsley (a synonym of *X. vestitus*) (see Tinsley, 1973a, Tinsley *et al.*, 1979). Where *X. wittei*-like toads originate from other sites they are termed *X. wittei* aff., as distinct morphological variants occur in some areas (R.C.T. unpublished observations). Unidentified *X. fraseri* subgroup species (see Kobel, Loumont & Tinsley, 1996) are termed *X. fraseri* aff.

RESULTS

Infection statistics for polystomatids recovered at five western Ugandan sites are presented in Table 1. Two *Protopolystoma* species were found: *Protopolystoma* species A occurred at all sites and infected all three host taxa, while *P. xenopodis* was present in *X. l. victorianus* at L. Chahafi but absent from *X. wittei* and *X. vestitus*. Population levels of species A were lower in *X. wittei* from L. Bunyonyi and Echuya (prevalence <10%, abundance 0.1) than in *X. vestitus* from L. Mulehe and L. Mutanda (prevalence >30%, abundance 0.7-0.9). The parasite was absent from *X. wittei* in the only *vestitus/wittei* mixed sample (from L. Mulehe): however, only a small number of toads (*n*=6) was examined, so that no significance can be attached to this observation. Tinsley (1973*b*) reported *Protopolystoma* species A (as *P. xenopi*) in *X. vestitus* (=*X. kigesiensis*) from L. Mutanda, based on a collection made in November, 1969. The presence of a particular *Protopolystoma* species may therefore be stable over relatively long periods of time. However, species A occurred at a lower prevalence (10%, *n*=241) and abundance (0.2) in 1969 than in 1996, indicating that its populations might show significant seasonal or longer term fluctuations. As such variations between sites could account for the higher infection levels found in *X. vestitus* from the present study, it is not possible to draw further conclusions from this feature of the data. At L. Chahafi, species A occurred at a moderate level in *X. l. victorianus* (prevalence 18%, abundance 0.2), infecting more hosts but showing fewer worms in the overall sample than *P. xenopodis* (prevalence 10%, abundance 0.4; see Table 1).

DISCUSSION

Present results establish that *Protopolystoma xenopodis* is absent from natural populations of *X. wittei* and *X. vestitus* in western Ugandan. Both *P. xenopodis* and an undescribed species (species A) co-occurred in *X. laevis* at L. Bunyonyi, while *wittei* and *vestitus* were only infected by species A, which was present at all five sites investigated. The pattern of host-specificity revealed in this survey is consistent with data from a wider study of *Protopolystoma* biogeography (Tinsley & Jackson, 1998). This showed that species A is endemic to central Africa, having been recorded from *X. laevis* subspecies at localities in western Kenya, western Uganda, south and eastern Zaire and Rwanda (Tinsley & Jackson, 1998). It was also found in *X. wittei* and *X. vestitus* at some of the present sites (Tinsley, 1973*b*; Tinsley *et al.*, 1979; Tinsley & Jackson, 1998) and in *X. wittei*-like hosts from Rwanda and Burundi. *P. xenopodis*, which appears to be narrowly specific to *X. laevis*, is common in the Rift Valley region of central Africa and frequently co-occurs with Species A.

X. wittei and *X. vestitus* are octoploid forms which may have speciated as the result of allopolyploidization (Tymowska, 1991). This a mechanism by which interspecies hybrids show doubling of their genome, leading to the production of a new, self-maintaining hybrid lineage. Both species are believed to descend from tetraploids related to *X. laevis* and *X. fraseri*-like

TABLE 1. *Protopolystoma* spp. from *Xenopus* spp. in Ugandan lakes: infection statistics. (Standard deviations in parentheses.)

Locality	Host	Parasite	Prevalence	*n*	Mean intensity	Abundance
L. Chahafi	*laevis*	*P. xenopodis*	10.0%	40	4.0 (1.6)	0.4 (1.3)
		sp. A	17.5%	40	1 (0)	0.2 (0.4)
L. Bunyonyi	*wittei*	sp. A	9.5%	63	1 (0)	0.1 (0.3)
L. Echuya	*wittei*	sp. A	8.1%	37	1.3 (0.6)	0.1 (0.4)
L. Mutanda	*vestitus*	sp. A	37.8%	74	1.8 (0.9)	0.7 (1.0)
L. Mulehe	*vestitus*	sp. A	31.6%	95	2.7 (1.9)	0.9 (1.6)
	wittei	sp. A	0	6	0	0

toads (Tymowska, 1991). Susceptibility to *Protopolystoma* species A may therefore have been inherited by *X. vestitus* and *X. wittei*-like forms from their *X. laevis*-like parental lineage. At the same time, the absence of *P. xenopodis* from octoploid species, even though it does infect the sympatric *X. l. victorianus*, suggests that monogenean specificity may be controlled by subtle host genetic differences. Experimental studies (Tinsley & Jackson, 1998) indicate that there is a physiological barrier to the maturation of *P. xenopodis* infections in *X. wittei* from western Uganda. Apart from the two species occurring in western Ugandan clawed toads, a third representative of *Protopolystoma* was found in *X. wittei* aff. from southern Rwanda by Tinsley & Jackson (1998). This form is usually associated with *X. fraseri* aff. from lowland forest in the Zaire basin and west Africa: its occurrence in *wittei* aff. may therefore result from the genealogical relationship of this toad to a *fraseri*-like lineage.

In addition to work on *Protopolystoma* reviewed above, host specificity studies on a number of other parasite groups have recently been carried out (Tinsley 1981; Tinsley, 1996), or are in progress. Three species of the gyrodactylid monogenean *Gyrdicotylus* are found in *X. wittei*-like hosts, showing a mixture of morphological characteristics (Tinsley, 1996). One is closely related to *G. gallieni* from *X. laevis*, while the others show affinities to a species occurring in *X. fraseri* aff. This pattern may again result from the inheritance of parasite lines by the hybrid host species.

The occurrence of monogenean species in *X. wittei*-like hosts also shows geographical variation (Tinsley, 1996). Although *Protopolystoma* species A is widespread, the other species from this host was only found in southern Rwanda. Of the three *Gyrdicotylus* species mentioned above, each form occurs separately in geographically distant *X. wittei*-like populations (from western Uganda, eastern Zaire and southern Rwanda). It therefore seems possible that octoploid populations show variable susceptibilities to monogenean parasites. In the absence of experimental data confirming the resistance/susceptibility pattern of these populations it is impossible to be sure that the distributions of monogeneans are not influenced by host-independent ecological factors. In one case, however, experimental evidence suggests that the species of *Gyrdicotylus* from *X. wittei* aff. in southern Rwanda is not able to sustain population growth on type-locality *X. wittei* from western Uganda (Tinsley, 1996).

Studies of interspecific hybrid individuals have shown that levels of parasitism may be increased (Sage, Heyneman, Lim & Wilson, 1986; Dupont & Crivelli, 1988). Also, parasites from either parental host might potentially infect the hybrid offspring, so that these are exposed to larger numbers of parasite species. In the case of monogeneans, hybrid fish hosts have been found to be susceptible to parasites primarily specific to either parent species (Dupont & Crivelli, 1988; Cloutman, 1988). However, for the octoploid toads it appears that, at least at single localities, numbers of parasite species are not increased, and some parasites from either or both related tetraploid lineages (*X. fraseri* and *X. laevis*-like) may be excluded. Apart from the example of *P. xenopodis*, this is illustrated by the distribution of the pseudophyllidean cestode *Cephalochlamys,* which occurs in all African pipids which have been examined except for *X. wittei* and *X. vestitus* (see Tinsley, 1981; also unpublished records from *X. tropicalis* and hymenochirine hosts). Such novel resistance in allopolyploid species may be the result of their hybrid genetic constitution. Immune system genes (which might be of particular importance in determining parasite specificity) can be expressed from both parental genotypes (Du Pasquier, Wilson & Robert, 1996). Genetic reorganizations subsequent to allopolyploidization have been shown to include silencing of some major histocompatibility complex genes (Du Pasquier, Miggiano, Kobel & Fischberg, 1977) and the loss of immunoglobulin genes (Du Pasquier *et al.* 1996). Hybridization might carry a penalty of increased parasitism in the short term (as seems possible from studies of other systems). However, in octoploid clawed toads, present observations suggest that either immediately, or over periods of evolutionary time, the hybrid lineages develop resistance to parasites which infected their parental species. Regional variations in *X. wittei* susceptibility to infection by monogeneans may suggest that isolated populations have undergone a differing history of the genetic rearrangements mentioned above or even originate from independent allopolyploidization events. Further experimental host specificity studies of monogenean parasites are in progress which might further clarify regional variations in the susceptibility of *X. wittei*-like toads.

Another parasite group showing clear discontinuities of distribution involving octoploid hosts is the seuratoid nematode genus, *Chitwoodchabaudia.* As the sole representative within its family (Chabaud, 1978) it does not show close relationships with any known form (Puylaert, 1970; Chabaud, 1978) and its lineage is of ancient, perhaps Mesozoic, origin (Baker, 1984). The distribution of *Chitwoodchabaudia*, which is only found in *X. wittei* and *X. vestitus*, is therefore remarkable given the relatively recent evolution of its hosts (Tymowska, 1991). This age disparity between the octoploid *Xenopus* and parasite lineages indicates that *Chitwoodchabaudia* cannot have evolved *in situ.* It must, therefore, have been inherited from an unknown parental toad species or have "jumped" from a non-*Xenopus* host.

The present results show how the study of parasite taxonomy and specificity might provide information useful in host identification. The host range of high-specificity parasites with direct life-cycles may reflect genetic differences in closely related, ecologically similar, sympatric hosts. Shared parasites may also illuminate the evolutionary relationships of their host species. For instance, the discovery of a *Chitwoodchabaudia* species amongst *X. fraseri*-like

taxa might well indicate the ancestry of *X. wittei* and *X. vestitus*. Preliminary studies of some *Protopolystoma* species have also revealed small morphological differences between samples from different hosts and locations. The variation, primarily in sclerotized structures of the attachment organ and terminal genitalia, may be genetically determined or affected by the host environment. In either case, polystomatid morphotypes might be of use in the identification of cryptic host species established by molecular methods or mating call variation (e.g. amongst the complex of *X. fraseri*-like species). Identification of parasites, which can be examined by light microscopy with minimal preparation, may be more practical than the determination of host samples by mating call or cytogenetic and molecular techniques. It is also possible to recover specimens from existing preserved museum collections of amphibians whose specific identity cannot be determined from morphological characters.

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FERAL *XENOPUS LAEVIS* IN SOUTH WALES

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Despite its prominence as the "standard laboratory amphibian", the ecology of the African clawed frog, *Xenopus laevis*, has been neglected. Feral populations have been documented in several countries with Mediterranean climates, but established populations are also known from the UK. Long term studies of individually-marked *X. laevis* in South Wales reveal large demographic fluctuations and the ability to migrate overland. Maximum longevity recorded from recapture of marked individuals was 14 years. Diet analysis demonstrates a major reliance on benthic invertebrates and zooplankton components of the pond fauna. Skeletochronological studies of growth rings in bone showed that lines of arrested growth are formed annually and allow calculation of age. Data on population age structure indicate successful recruitment is infrequent, with dominant cohorts originating in perhaps only four summers during the past 20 years.

INTRODUCTION

The African clawed frog, *Xenopus laevis*, has been intensively studied in the areas of developmental, cell and molecular biology (Gurdon, 1996). However, ecological studies of this principally aquatic amphibian remain scarce. Accidental and deliberate introductions of *X. laevis laevis* to alien environments are associated with its use in human pregnancy diagnosis, as a laboratory animal, and in the pet trade in the 1950s and 1960s. A recent review of feral populations (Tinsley & McCoid, 1996) noted that this species has colonized a variety of lentic habitats world-wide, but principally in Mediterranean climates similar to that of its native southern Africa.

The most intensively studied feral populations are those in California (McCoid & Fritts, 1980*a,b*; 1989; 1993; 1995; McCoid, Pregill & Sullivan, 1993; McCoid, 1985) where *X. laevis* has colonized several river drainages (Tinsley & McCoid, 1996). California has a Mediterranean climate (Walter, Harnickell & Muller-Dumbois, 1975), and McCoid & Fritts (1995) found that *X. laevis* grows year-round in optimal conditions, maturing in eight months, and has an extended breeding season.

The climate of the UK appears to be ill-suited to this southern African species. However, feral populations of *X. laevis* in the UK include a number of isolated reports as well as two established populations, on the Isle of Wight (now possibly extinct) and in South Wales (Tinsley & McCoid, 1996; Lever, 1977). This report summarizes a long-term study of the Welsh population of *X. laevis* from 1982 to 1996 and a shorter more intensive study of one site within the area (1994 to 1996). Detailed accounts of these studies are described elsewhere (Measey & Tinsley, in prep.).

MATERIALS AND METHODS

Long-term studies of populations of *X. laevis* were conducted in two adjacent watercourses in South Wales, from 1982 to 1996. The catchment areas of these watercourses are below 100 m a.s.l. and lie within 8 km of the coast, exposed to westerly winds, which markedly increase the salinity of the water. The catchments are well drained; much of the area is either cultivated or pasture. A 2 km length of the river and associated ponds, confined to a steep sided U-shaped valley (hereafter referred to as the "Valley"), was sampled. A small calcareous stream runs through open pasture and then through a number of man-made terraces (hereafter referred to as the "Stream"). A man-made pond, studied intensively from November 1994 to August 1996, lies 0.5 km south-west and 15 m above the river on the top of a hill (hereafter referred to as the "Pond").

X. laevis were trapped using modified fyke nets baited with liver. Traps were set at dusk and collected at dawn. *X. laevis* caught were sexed and weighed, the snout-vent length (SVL) was recorded, and then they were marked using a dye injector ("Panjet", Wright Health Group Ltd, Dundee) with unique combinations of spots on the ventrum (Wisniewski, Paull, Merry & Slater, 1979). Beginning in 1994, *X. laevis* caught were freeze-branded with three digit numbers (Daugherty, 1976).

Population estimates were calculated using the triple catch method (Begon, 1979) and a zero-truncated geometric model based on numbers of marked animals recaptured from the population (Seber, 1973) at the Pond.

The Pond was visited at two-weekly intervals. Traps were set for two consecutive nights and physical parameters (water temperature, pH, conductivity and oxygen concentration) were measured. Water samples were taken for chlorophyll *a* analysis and algal composition. Replicate samples of benthic invertebrates were collected with 0.00125 m^2 cores and zooplankton with a 250 µm mesh net. Samples were preserved in 10% formalin and were subsequently identified, weighed, and sorted. Stomach contents were removed from 20 *X. laevis* (or as many as numbers captured permitted) per visit by stomach-flushing (Legler & Sullivan, 1979)

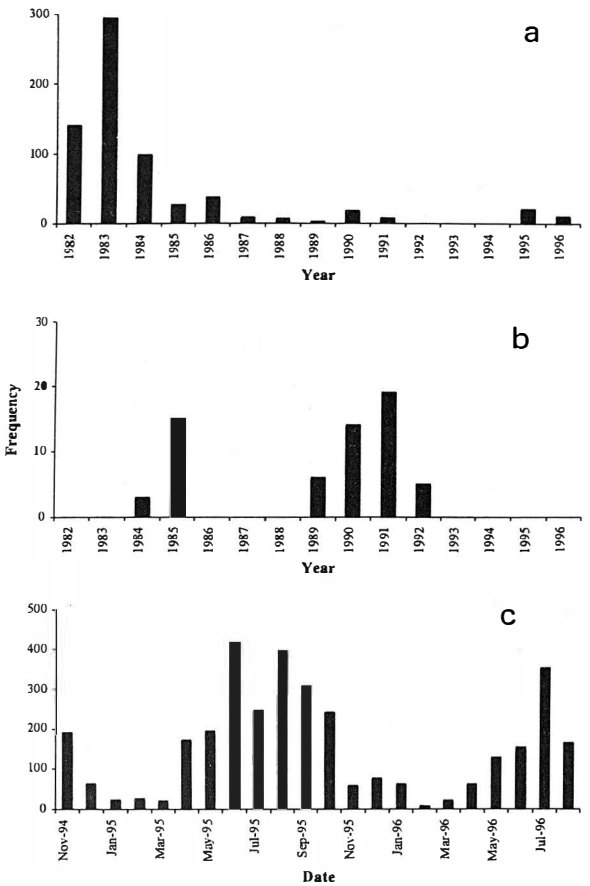

FIG. 1. Frequency distributions of numbers of *X. laevis* captured in (a) the Valley, (b) the Stream and (c) the Pond.

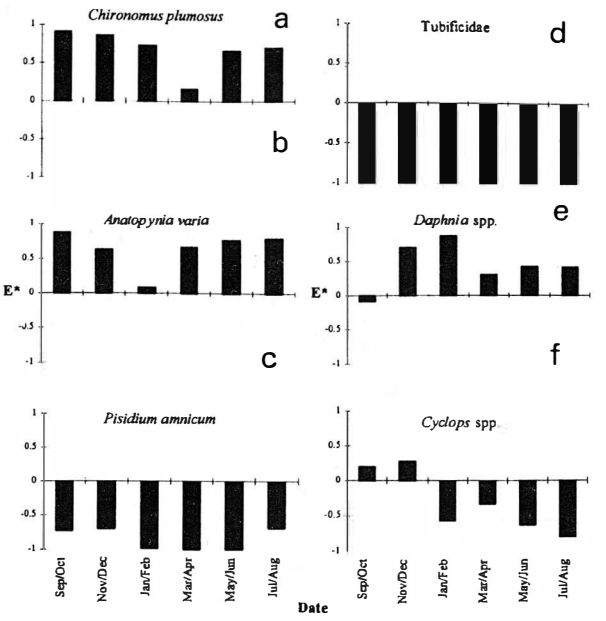

FIG. 2. Vanderploeg & Scavia's (1979) electivity index (E*) for numbers of zoobenthic and zooplanktonic invertebrates recorded in the stomach contents of *X. laevis* from the Pond. Each bar represents the average of two months of two-weekly samples.

within four hours of capture, and preserved immediately in 10% formalin. A measure of relative abundance of prey in the diet compared to relative abundance in the pond was made using Vanderploeg & Scavia's (1979) relativized electivity index (E*) for zooplankton and benthic invertebrates.

For age analysis, standard skeletochronological techniques (see Castanet & Smirina, 1990) were employed, based on the second phalange of the fifth toe of 60 *X. laevis* from the Pond in November 1994 and a further 60 in June 1996. Histological preparations were also made from 16 femurs of *X. laevis* captured in the Valley from 1982 to 1990 and seven femurs from the Stream from 1988 to 1993. Toes were processed from 10 *X. laevis* caught in the Valley in 1995.

RESULTS

XENOPUS LAEVIS CAPTURES

A total of 264 *X. laevis* was caught in 677 captures in the Valley from February 1982 to August 1996 (Fig. 1a). In the Stream, there were 62 captures of 38 individually-marked animals from October 1983 to December 1994 (Fig. 1b). Movements between capture sites were recorded for 21% of animals within the Valley, with distances of 0.2-2 km, and 36% of animals from the Stream, with distances less than 100 m. No *X. laevis* were found to move between catchments, and for this reason the sites were treated as separate populations. In 46 visits to the Pond, 3358 captures were made of 913 individually-marked *X. laevis* (Fig. 1c). Two animals marked in the Pond were subsequently found in other localities within the Valley 0.75 and 1.5 km away (direct distances). Time elapsed between recaptures at different locations was generally greater than one month, but one female was recorded as travelling 0.2 km (direct distance) in less than 48 hrs.

Throughout the entire period, 47% of *X. laevis* trapped in the Valley were caught only once, whilst others were caught up to 12 times including two individuals whose captures spanned all 14 years of the study. Individual panjet marks made on these animals in 1982 were readily identifiable in 1996. All numbers freeze-branded onto animals in 1994 were still easily distinguished at the end of the study in August 1996.

POPULATION ESTIMATES

Population estimates of *X. laevis* in the Valley, by the triple catch method on contiguous trapping data, revealed a rapid decline from 408 in 1982 (upper and lower confidence limits not calculable) to 64 (+152, -45) in 1984, and a very small population in 1995 (16±2). The population in the Stream was estimated by the triple catch method as 15 (±1) in 1990. From November 1993 to December 1994, despite intensive trapping, no *X. laevis* were caught in the Stream. The population at the Pond was found to be a good fit to a zero-truncated geometric model of numbers recaptured ($\chi^2 < 0.001$, df = 22) to predict numbers left uncaught, and together with those caught gave a total population of 1276 *X. laevis*.

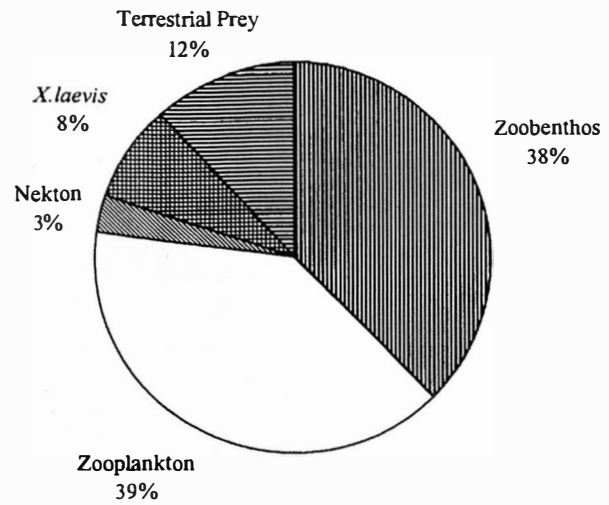

FIG. 3. Proportions of dietary components in the stomach contents of *X. laevis* from the Pond averaged over 12 months.

DIET

Densities of common benthic invertebrates in the Pond were seen to fluctuate from August 1995 to August 1996. The major components were tubificid worms, chironomid larvae (*Chironomus vr. plumosus* and *Anatopynia varia*), and pea mussels (*Pisidium amnicum*). Electivities for numbers of chironomids (Fig. 2) are generally positive, while those of *P. amnicum* are negative. Tubificids showed total negative electivities as they were completely absent from the stomach contents of *X. laevis*.

The densities and composition of zooplankton were seen to fluctuate from August 1995 to August 1996. The major components were cladocerans (*Daphnia* spp.), whose density peaked in summer, and copepods (*Cyclops* spp.), whose density peaked in autumn. Zooplankton sizes varied during the year, with sizes of both *Daphnia* spp. and *Cyclops* spp. greater in the winter than the summer. Electivities for numbers of zooplankton taxa (Fig. 2e) showed positive selection of *Daphnia* spp. for most of the year, while *Cyclops* spp. showed positive selection mostly in the autumn when density peaked.

Data on stomach contents of *X. laevis* from the Pond were divided into groups by prey habitat and consequently by predation mechanism: zoobenthos, zooplankton, nekton, and terrestrial (Measey & Tinsley, in prep.). The prey category *X. laevis* has been placed in a separate group which comprised larvae and eggs of *X. laevis*. Zoobenthos and zooplankton consistently made up the largest components of stomach contents (Fig. 3), with terrestrial prey items (e.g. beetles, flies and woodlice) peaking in spring and summer. *X. laevis* made an important contribution to stomach contents only during summer 1996 when breeding occurred in the Pond. The contribution of nektonic prey items was consistently low throughout the year. Each prey group showed a large taxonomic range of invertebrate (and vertebrate) prey items, but the majority of prey was made up of very few taxonomic groups.

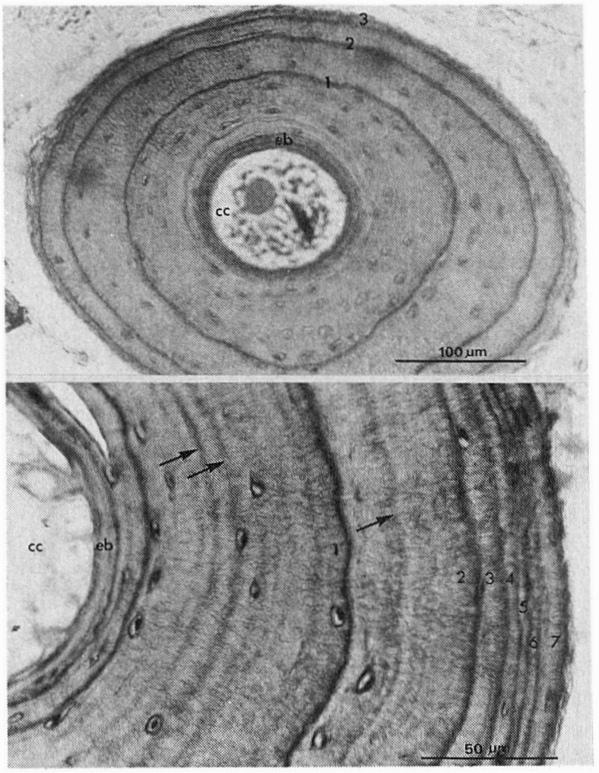

FIG. 4. Transverse sections of phalanges of *X. laevis* from South Wales. (a) a female with SVL 73 mm and 3 LAG from the Pond, and (b) a female with SVL 101 mm and 7 LAG from the Valley. *cc* central cavity; *eb* endosteal bone; numbers indicate LAG and arrows false LAG.

MORPHOMETRICS

From first collections at the Pond in November 1994, sex could be determined externally for only 12% of individuals. No internal examinations were made. Mean snout-vent length for juveniles was 51 mm (SE = 0.38, n = 113), and for those identified as females 61 mm (SE = 0.30, n = 13) and males 62 mm (SE = 0.88, n = 3). By July 1996, sex could be determined externally for all individuals, and females were significantly greater than males in both length (mean difference 3.90 mm) and weight (mean difference 2.34 g) (length $F_{1,347}$ = 79.72, $P < 0.001$; weight $F_{1,347}$ = 29.96, $P < 0.001$).

SKELETOCHRONOLOGY

Lines of arrested growth (LAG) were easily discernible in all bones from the Pond population with no false (intraseasonal) LAG at this site (Fig. 4a). The age structure of the 60 individuals toe clipped in October 1994 is shown in Fig. 5: alongside 28 % of individuals estimated to be ages 4-8 years, there is a dominant cohort of animals reflecting metamorphosis in 1993. Of the 60 *X. laevis* toe-clipped in June 1996, 98% had 3 LAG. One individual which was toe clipped on both occasions was found to have increased numbers of LAG from 1 to 3. All bones collected in the Valley contained clear LAG within the periosteal as well as some with false LAG (Fig. 4b). Fig. 6 shows the frequency of the year of metamorphosis (i.e. years preceding LAG_1) to be grouped in the mid and late 1970's, mid 1980's and

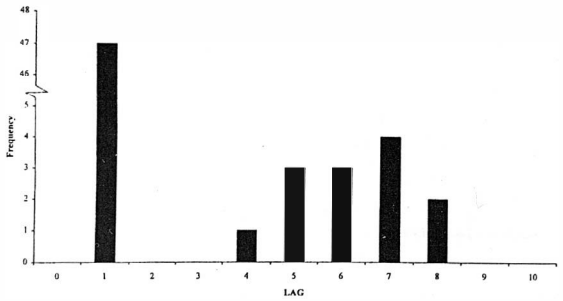

FIG. 5. Frequency distribution of LAG in phalanges of *X. laevis* from the Pond, toe clipped in October 1994.

late 1980's and early 1990's for toes and femurs from the Valley (Fig. 6a). Years of metamorphosis of *X. laevis* from the Stream are shown to be grouped in the late 1980's and early 1990s (Fig. 6b).

DISCUSSION

Population estimates of *X. laevis* in both the Valley and the Stream suggest that numbers have declined sharply from those at the start of the study period. It is thought that contributory factors include predation, particularly the lack of recruitment into the population due to cannibalism (Tinsley, Loumont & Kobel, 1996), and loss of appropriate lentic habitats. Skeletochronological results show conclusively that LAG formation is annual, enabling accurate demographic analysis of the Pond population. This confirms the reproductive potential of *X. laevis*: more than 97 % of the population could be traced to metamorphosis during a single summer (1993) (Measey & Tinsley, in prep.).

Documentation of movements of individually-marked animals demonstrates that, although *Xenopus* is principally adapted to aquatic life, travel between habitats involved overland movement. Direct distance between capture sites may not accurately reflect the actual distance and obstacles encountered during these journeys. For the individual found to move 0.2 km in 48 hrs, the route would have included an initial distance overland (150 m), crossing the river and then overland through woodland with dense undergrowth, over a metalled road and into a quarry, where the pond was situated. For the animals marked in the Pond, the most likely route to the habitats in which they were found would be using the river as a corridor. These ponds were both within 20 m of the river, but were not visible from the water surface, and in both cases travel would have involved movement up steep banks and walls. Movement may possibly be stimulated by olfactory cues emanating from the pond, and Savage (1961) has suggested that for *Rana temporaria* the cues may come from phytoplankton. True navigation, homing without landmarks, is known in amphibians (Phillips, Adler & Borland, 1995) and may involve magnetoreception.

The composition of gut contents was very similar to that found in previous studies of *X. laevis* in an impoundment in Transkei (Schoonbee, Prinsloo & Nxiweni, 1992) and from a stream in California (McCoid & Fritts, 1980*a*). In common with the present

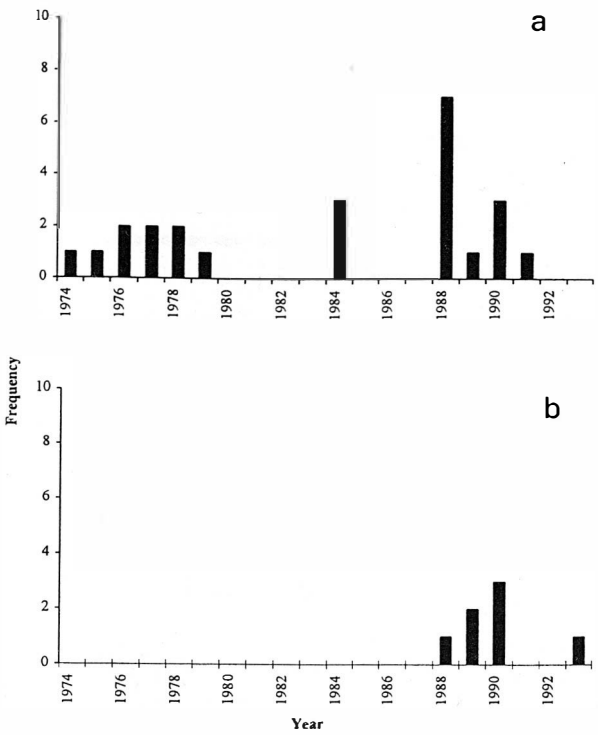

FIG. 6. Frequency distributions of years of metamorphosis (i.e. year preceding LAG_1) from skeletochronological analysis of femurs and phalanges of *X. laevis* from (a) the Valley and (b) the Stream.

study, tubificids found to be abundant in the environment in Transkei were completely absent from stomach contents. This phenomenon has been observed in fish diets, and is attributed to the unavailability of tubificids, which are found to move deeper into the sediment in the presence of predators (Kornijow, Moss & Measey, in prep).

In a recent review of diet in *Xenopus*, it was considered unlikely that the large amount of terrestrial prey previously reported in stomach contents originates solely from invertebrates which have fallen or been swept from overhanging vegetation into the water (Tinsley *et al.*, 1996). In the present study, items of terrestrial origin make up a quarter of the weight of all ingested prey items. Many may have been ingested after falling inadvertently into the pond, where they may have become trapped in the surface tension or fallen to the bottom. However, some may have been caught whilst on land: current laboratory studies have confirmed that *X. laevis* can prey terrestrially on invertebrates (Measey & Tinsley, in press).

The record of two animals recaptured 14 years after first being marked with a panjet represents the longest period reported for *X. laevis*, or any anuran, recaptured from the wild, as well as a record period of panjet mark retention. This also represents a record for the longevity of *X. laevis*, if it is assumed that each animal must have been at least two years old on first capture (Tinsley *et al.*, 1996; Tinsley & McCoid, 1996).

Mark-recapture data for the age of a male which was first caught as an adult in 1982 and toe-clipped in 1995 does not correspond with the skeletochronological re-

sults (11 LAG). The underestimated LAG count was probably due to the difficulty of differentiating LAG at the periphery of periosteal bone in older individuals (Smirina, 1994; Castanet & Smirina, 1990).

Ages of *X. laevis* in both the Valley and the Stream suggest these populations resulted from three groups of years; the mid to late 1970's, mid 1980's and early 1990's (see Fig. 6). With the errors associated with counting outer LAG, it may be that each of these groups of spawning years represents a single successful spawning occasion. This would indicate that there has been only sporadic success in recruitment during the past 20 years, despite more frequent observation of spawning. Reasons for this intermittent recruitment may include cannibalism and dwindling habitats. Cannibalism, of eggs and larvae of *X. laevis*, is well known (Tinsley *et al.*, 1996; Measey & Tinsley, in prep.). Three of the original habitats available to *X. laevis* in the Valley in 1982 are no longer available, forcing adults into other ponds where reproduction is not successful. If there is no recruitment to a population, and predation pressure is persistent, a rapid decline in population numbers would be expected. However, the existence of refugia make the last individuals difficult to eliminate, and as *X. laevis* are explosive breeders, like most other amphibians, there is always a potential for another large expansion in numbers.

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A REVIEW OF THE TAXONOMY OF THE *HYPEROLIUS VIRIDIFLAVUS* COMPLEX

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This paper reviews the problematic taxonomy of the superspecies *Hyperolius viridiflavus* (the senior synonym of *H. marmoratus*) (Anura: Hyperoliidae). The classifications proposed by various authors for the complex are summarized and compared. Significant differences in classification have resulted in the complex being divided into as few as three species (with numerous subspecies) or as many as 18 species (with a number of subspecies).The review shows that the taxonomy of this complex has not been resolved, as a consequence of dependence on the use of dorsal colour patterns as diagnostic characters. Colour pattern is polymorphic within taxa, and shows both geographical as well as non-geographical variation. Recent work has shown molecular techniques to be a valuable means of distinguishing taxa previously considered to be subspecies. It is proposed that the use of molecular techniques and phylogenetic analysis of these data is the most suitable means of resolving the taxonomy of the members of this complex.

INTRODUCTION

The Old World tree frogs of the family Hyperoliidae are a diverse and colourful group, found throughout much of Africa and the islands of Madagascar and the Seychelles. Although many hundreds of species have been named, currently about 200 are recognized. Adults vary in size and most are arboreal. Although the geatest diversity occurs in the tropics, they are found throughout sub-Saharan Africa.

One of the widespread taxa in this family is known as *Hyperolius viridiflavus*. In the southern part of its range the name *Hyperolius marmoratus* is applied. There is little morphological variation discernible, and dorsal colour patterns have been used as a basis for subspecific taxonomy. Various populations appear to have dominant colour patterns, although pattern polymorphism is the rule. The group has been the subject of extensive debate (Broadley, 1965). The taxonomic confusion arises out of the apparently stable pattern polymorphisms within each population, and the difficulty of reconciling different taxonomies based upon populations from west, east, central and southern Africa.

The present review was prompted because this complex of taxa is of evolutionary interest, comprises a considerable proportion of the African amphibian fauna, and has conservation value. We review the major literature, highlight existing confusion, and show that molecular techniques have started to solve some long-standing problems.

THE OLD WORLD TREE FROGS

The first major taxonomic revision of the Arican tree frogs placed them into a single family with 527 species in 12 genera (Ahl, 1931). Laurent (1951) examined the osteology of the African and Asiatic tree frogs, and proposed a split into two families, the Hyperoliidae and the Ranidae. Liem (1970) recognized two families: the Hyperoliidae and the Rhacophoridae, believing them to be diphyletically derived from primitive ranoids. His proposal was based on an analysis of internal and external morphological characters. Drewes (1984) extended the study by incorporating material from more taxa, and including more characters. Current taxonomy (Frost, 1985) retains the Hyperoliidae and Rhacophoridae. The systematics of this group, however, are uncertain, particularly in genera like *Hyperolius*, and the question of whether subfamilies such as Hyperoliinae are monophyletic remains unanswered.

THE FAMILY HYPEROLIIDAE

Liem (1970) proposed that the Hyperoliidae evolved from ranoid stock in Africa. Detailed investigations by Drewes (1984), based on a cladistic analysis of skeletal, myological and cartilaginous characters, placed *Leptopelis* as the most primitive genus in the family. *Hyperolius* and *Afrixalus* constituted a monophyletic clade, with the Malagasy *Heterixalus* as sister group. A re-analysis of the available data by Channing (1989) supported the monophyly of the Hyperoliidae, but suggested minor rearrangements, such as placing *Hyperolius* as the sister group to *Cryptophylax* and *Chrysobatrachus*. Richards & Moore (1996) were able to use molecular methods to contribute to this debate.

The status of genera within the family is not stable. Perret (1988) erected the genera *Nesionixalus* for *Hyperolius thomensis*, *Alexteroon* for *H. ostetricans*, *Arlequinus* for *H. krebsi* and *Chlorolius* for *H. kohleri*.

The family also has a number of taxonomic problems at species level, for example, within the genus *Hyperolius* (Poynton & Broadley, 1987). This interest-

ing genus has been considered to be 'one basic morphological type' with many dorsal colour patterns (Poynton, 1964).

THE *HYPEROLIUS VIRIDIFLAVUS* COMPLEX

The taxa included in this species have been placed in *Hyperolius marmoratus*, *H. parallelus*, *H. tuberculatus* or *H. viridiflavus* by various authors. The taxa included may indeed belong to only one or to a number of different species. No generic-level phylogeny is available to adequately resolve all these issues. For the purposes of this review, we will regard all the taxa within this group (sometimes referred to as a superspecies) to be nominally part of *Hyperolius viridiflavus*.

Schiøtz (pers comm.) is no longer convinced of the need to maintain specific status for *H. parallelus*. The recognition of this species was based on five reported cases of supposed sympatry between two forms in the complex. Poynton & Laurent (in press) doubt four of these cases, leaving only the sympatry at Sango Bay, Uganda. The Sango Bay case can be explained by a recent immigration to the west of Lake Victoria fom the south and north.

BIOGEOGRAPHY

Hyperolius viridiflavus occurs throughout sub-Saharan Africa. It is absent from arid areas, and the highlands of Ethiopia and southern Africa. Various fac-

TABLE 1. Comparison of various taxonomies for the *Hyperolius viridiflavus* complex.

Laurent (1951)	Poynton (1964)	Schiøtz (1971)	Laurent (1976)	Poynton & Broadley (1987)
Hyperolius marmoratus angolensis	*Hyperolius angolensis*	*Hyperolius parallelus angolensis*	*Hyperolius marginatus angolensis*	*Hyperolius marmoratus angolensis*
Hyperolius marmoratus aposematicus	*Hyperolius aposematicus*	*Hyperolius parallelus aposematicus*	*Hyperolius marginatus aposematicus*	*Hyperolius marmoratus aposematicus*
Hyperolius marmoratus argentovittis		*Hyperolius parallelus argentovittis*	*Hyperolius marginatus argentovittis*	*Hyperolius marmoratus argentovittis*
	Hyperolius marmoratus broadleyi	*Hyperolius parallelus broadleyi*	*Hyperolius marginatus broadleyi*	*Hyperolius marmoratus broadleyi*
	Hyperolius marmoratus marginatus	*Hyperolius marginatus*	*Hyperolius marginatus marginatus*	*Hyperolius marmoratus marginatus*
Hyperolius marmoratus nyassae		*Hyperolius viridiflavus nyassae*	*Hyperolius marginatus nyassae*	*Hyperolius marmoratus nyassae*
Hyperolius marmoratus rhodoscelis		*Hyperolius viridiflavus rhodoscelis*	*Hyperolius marginatus rhodoscelis*	*Hyperolius marmoratus rhodoscelis*
Hyperolis marmoratus rhodesianus	*Hyperolius rhodesianus*	*Hyperolis parallelus rhodesianus*	*Hyperolius marginatus rhodesianus*	*Hyperolius marmoratus rhodesianus*
Hyperolius marmoratus swynnertoni		*Hyperolius parallelus swynnertoni*	*Hyperolius marginatus swynnertoni*	*Hyperolius marmoratus swynnertoni*
Hyperolius marmoratus taeniatus	*Hyperolius marmoratus taeniatus*	*Hyperolius viridiflavus taeniatus*		*Hyperolius marmoratus taeniatus*

TABLE 2. Subspecies of *Hyperolius* listed by Schiøtz (1975) (excluding *Hyperolius sheldricki* from southern and eastern Kenya for which no subspecies were recognized, and *Hyperolius marginatus*, which is of doubtful status).

Hyperolius tuberculatus from forest in central and west Africa	*Hyperolius viridiflavus* from savanna in west, east and southern Africa	*Hyperolius parallelus* from savanna in western central Africa
H. t. tuberculatus	*H. v. spatzi*	*H. p. parallelus*
H. t. hutsebauti	*H. v. nitidulus*	*H. p. angolensis*
H. t. nimbae	*H. v. pallidus*	*H. p. argentovittis*
	H. v. pachydermis	*H. p. melanoleucus*
	H. v. viridiflavus	*H. p. epheboides*
	H. v. variabilis	*H. p. pyrrhodictyon*
	H. v. coerulescens	*H. p. aposematicus*
	H. v. karissimbiensis	*H. p. rhodesianus*
	H. v. xanthogrammus	*H. p. broadleyi*
	H. v. ferniquei	*H. p. albofasciatus*
	H. v. grandicolor	*H. p. swynnertoni*
	H. v. ommatostictus	
	H. v. goetzei	
	H. v. rhodoscelis	
	H. v. mariae	
	H. v. nyassae	
	H. v. taeniatus	
	H. v. marmoratus	
	H. v. verrucosus	

tors have been suggested to account for frog distribution in southern Africa (Van Dijk, 1971; Poynton & Bass, 1970). Detailed distributional data for *H. viridiflavus* is poor for some areas, notably central Angola and southern Zaire.

SPECIAL CHARACTERISTICS

A number of unique characteristics serve to define and delimit the complex. The members of the complex are distributed (1) in tropical savannas in Africa at low and medium altitude; (2) populations are usually large and a conspicuous component of frog breeding aggregations; (3) members of the group share a number of morphological and behavioural characters, although they are extremely variable in dorsal colour pattern, both within and between populations (Schiøtz, 1971).

Various colour phases are recognized: a juvenile (J) phase, which is typically cryptic, and an adult female (F) phase, which may be cryptic or aposematic (Schiøtz, 1971). Mature females are always phase F, but mature males may be either phase J or F. In *H. parallelus* (*sensu* Schiøtz, 1971), the F phase is polymorphic, with three morphs showing independent geographic variation. The variation in the F phase has been suggested to be the result of recent hybridization (Schiøtz, 1971). Zimmerman (1979) showed that more than 45 hybrid colour patterns could be obtained within a few generations from four breeding adult *Hyperolius*. The role of hybridization as the source of the pattern polymorphism has not been experimentally demonstrated.

Typical external features of *H. viridiflavus* are the short, almost truncated snout, giving a 'pug-face' appearance, and extensive webbing reaching the distal subarticular tubercle of the fourth toe on both sides (Poynton, 1964). The vocal sac and gular gland are large relative to the snout-vent length, and females have a transverse gular fold (Schiøtz, 1971). Taxonomy based on morphological characters is difficult in this group, owing to the fact that there are many similarities shared by the members of the complex. Some characters, such as the dorsal colour pattern, are covenient to describe but are highly variable, which makes them of questionable use in taxonomy.

TAXONOMY

Broadley (1965), in a review of *H. viridiflavus* in central and southern Africa, described the group as a 'taxonomic nightmare'. In Table 1 we compare the nomenclature applied to certain taxa by different authors. Schiøtz (1971) suggested that the complex may be in the process of rapid evolution with many morphologi-

cally similar species exhibiting great variation with a propensity for geographical splitting. He lists five species (Schiøtz, 1975) with many subspecies (Table 2). A molecular-level genetic study is required to determine if the group consists of old or recent species.

Laurent's (1951) concept of the group regarded *H. tuberculatus* as being an isolated, possibly primitive, species occuring in clearings within the forest belt. *Hyperolius marmoratus* was considered to be a polytypic species occuring in savanna south of a line through Kivu, Burundi, northern Tanzania and the Tana river valley of Kenya. The form occurring north of the line was named *H. viridiflavus*. Table 1 illustrates how various authors have subsequently regarded these taxa, with many being shifted between the species *marmoratus*, *parallelus* and *marginatus*, or being regarded as full species in their own right.

Schiøtz (1971) questioned the validity of the northern and southern species, as the only character used to separate them was a supposed difference in J phase. The value of the J phase as a taxonomic character is doubtful (Schiøtz, 1971; Richards, 1981). Although *H. marmoratus* was considered a junior synonym of *H. viridiflavus* (Schiøtz, 1971), he states that more than one species is involved in the material he examined.

Not all the taxa are allopatric, as classical subspecies concepts suggest. *H. v. argentovittis* occurs together with various other 'subspecies': for example in Uganda with *H. v. bayoni*, and in Rwanda with *H. v. schubotzi* (Schiøtz, 1971). Further complicating the issue, Schiøtz (1971) emphasizes a great similarity in pattern between a number of forms covering a huge area in the Republic of Congo, Angola, Zambia, Zimbabwe and Malawi. Although the geographical pattern polymorphism in *H. viridiflavus* is great, a character such as the brown dorsum is found in all taxa from west and north central Africa (Schiøtz, 1971). Schiøtz (1971) demonstrated that individual characters (1) have a wider distribution than the conventional subspecies; (2) change gradually over the area; (3) change independently of one another; and (4) do not show steep clinal steps. He recommends the use of the subspecies category only as a covenient short-hand way of describing distinct forms.

The pattern of polymorphism may be maintained as a predator avoidance strategy, or may merely be the result of ongoing hybridization in relatively recent savanna, for example between the Congo forest and Lake Victoria. Laurent (1983) defended his earlier view of a north-south split, based on morphometric analyses of a number of body proportions. This kind of numerical approach has not gained general acceptance.

Poynton (1985) investigated the subspecies *H. m. taeniatus* and *H. m. broadleyi,* and attempted to determine whether they were deserving of subspecific status. He stated that there is general agreement that there are one or more species of marmorate reedfrog in the southern half of Africa, and that their dorsal pattern shows a vicariate distribution. He then goes on to indicate that although there is agreement on which taxa may be erected on the basis of presently collected material, there is widespread disagreement in the literature as to which species these should be assigned to. The problem appears to be largely related to the use of the subspecies as a taxonomic unit (Poynton, 1985). The subspecies concept is largely based on allopatric but relatively similar taxa, and whether they intergrade or not when their distributions overlap. The *H. marmoratus* complex confounds this definition, as various forms may be collected from one locality with distinct differences, while at other localities, complete series from one form to another may be collected. Poynton's (1985) study of subspecies arose from confusion surrounding these taxa. As a result of there being intergrades with each other in the south of their distribution, a series including forms that may be *H. parallelus* or *H. marmoratus* is observed, even showing intergrades with "non-marmorate" *H. marginatus* in central and western Zimbabwe. This contrasts with Schiøtz (1971), however, who separated *taeniatus* and *broadleyi*.

This decision evidently results from confusion of material examined, and Poynton (1985) suggests that the divisions of Schiøtz (1975) and Laurent (1976), placing intergrades under various species are incorrect, and that the name *marmoratus* should be applied.

In a more recent paper, Poynton & Broadley (1987) confirm that, although the members of the complex share morphology that separates them from the rest of the genus *Hyperolius*, the delimitation of all currently recognized subspecies in the complex is open to question. They suggest that the featureless plateau areas inhabited by these frogs in the west, and the degree of variation and merging of forms, may frustrate the nomenclatural process in that part of Africa, as clear-cut ranges can not be defined.

The use of colour patterns to determine relationships and taxonomy in this group may lead to confusion if the Mendelian basis for pattern inheritance is not understood. Richards (1981) showed that three colour variants of *H. viridiflavus*, 'striped', 'hourglass', and 'female', were allelic. She hypothesized that 'striped' was dominant over 'hourglass', and that 'hourglass' and 'female' patterns were recessive, producing both patterns in a 1:1 ratio in crossing experiments. Similar findings have been reported for other anurans (Pyburn, 1961). Adult colour patterns are switched on hormonally at maturity. Richards (1982) was able to force colour pattern changes at metamorphosis by appropriate hormone treatment. A particular individual frog may show either a juvenile or adult pattern depending on its state of sexual maturity.

MOLECULAR TECHNIQUES

Molecular techniques have made important contributions in resolving similar issues in other species of anurans (e.g. Hillis & Davis, 1986) and they may be of

use in providing much-needed data on some of the systematic problems in the *Hyperolius viridiflavus* complex.

Hess *et al.* (1995) used RFLPs (Restriction Fragment Length Polymorphisms) to compare *H. v. broadleyi* from eastern Zimbabwe and *H. v. verrucosus* from South Africa. They used 37 restriction sites on nuclear genes, and concluded that the differences between these two taxa warrented recognition at the species level. They did not attempt to determine the geographical ranges of these two subspecies. Wieczorek & Channing (in press) present DNA sequence data from a mitochondrial gene that confirms the differences found by Hess *et al.* (1995), and also indicates that some presently recognized 'subspecies' are genetically identical. Molecular work on other species within the Hyperoliidae (Richards & Moore, 1996) has demonstrated that DNA sequence data is a valuable approach to producing hypotheses of phylogeny.

Molecular techniques thus seem able to produce sufficient data that can be objectively evaluated to lead to a phylogeny of the group. The resolution of the problems of taxonomy will follow.

CONCLUSIONS

The Hyperoliidae is a monophyletic family, derived from ranoid stock. The Hyperoliinae consists of arboreal species inhabiting trees or other vegetation. The genus *Hyperolius* contains a number of colourful and diverse species. It appears that the complex of forms presently included in the *Hyperolius viridiflavus* complex may be of relatively recent origin. This hypothesis is supported by the presence of unstable hybrid populations (Schiøtz, 1971) and the large number of subspecies recognized. This complex is distributed throughout the tropical African savanna, with a closely related form, *H. tuberculatus*, patchily distributed in forests.

Taxonomy in the *Hyperolius viridiflavus* complex is largely based on dorsal colour pattern. However, there is a large amount of variation both between and within populations which confuses the taxonomy. Many pattern intergrades exist, which adds to the taxonomic uncertainty that plagues this taxon.

A phylogeny that adequately resolves the taxa included in the *Hyperolius viridiflavus* complex is not available. Recent attempts to use molecular techniques have demonstrated that these will be able to provide sufficient data to produce a phylogeny.

Although DNA sequences show the promise of resolving this long-standing problem, two issues remain: Most, if not all, museum specimens are not suitable for these techniques, so that the material that is the basis for the present debate will not be part of the solution, unless the same localities are revisited and recollected. Secondly, many parts of Africa where *Hyperolius viridiflavus* is likely to occur have never had adequate collections made. The taxonomic confusion in the *Hyperolius viridiflavus* complex is likely to remain until detailed fieldwork and extensive laboratory analyses can be completed.

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THE MALE NUPTIAL CHARACTERISTICS OF *ARTHROLEPTIDES MARTIENSSENI* NEIDEN, AN ENDEMIC TORRENT FROG FROM TANZANIA'S EASTERN ARC MOUNTAINS

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Recent field work in the Udzungwa Mountains of Tanzania has resulted in the discovery of the hitherto unreported nuptial characters of male *Arthroleptides martiensseni* which are described and illustrated in this contribution. These newly described characters are compared with the nuptial characters of male *Arthroleptides dutoiti*, a supposedly extinct species from Mount Elgon (Kenya) as well as with members of the West African genus *Petropedetes*, which exhibit some remarkable morphological and ecological similarities with *Arthroleptides martiensseni*.

INTRODUCTION

The montane rainforests of Cameroon and Tanzania contain a remarkable diversity of anurans, characterized by a high degree of endemism (Perret, 1966; Howell, 1993; Lawson, 1993). Two genera, *Petropedetes* (Reichenow, 1874) and *Arthroleptides* (Neiden, 1910) contain species that are highly specialized for living on wet cliff faces, often in and immediately adjacent to waterfalls and cascades. These adaptations include tadpoles that have evolved a distinctive suite of morphological characters that allow for their development on mossy, wet rock faces, as well as a distinctive "T-shaped" terminal expansion of the digits in adults, which allow these frogs to adhere to the slippery rock surfaces as described by Boulenger (1905) and Loveridge (1925).

Reichenow (1874) described the genus based on *Petropedetes cameronensis* sp. nov. from "Bimbia in the Cameroon foothills." Boulenger (1887) described *Cornufer (=Petropedetes) johnstoni* from "Rio del Rey, Cameroons District." This description was based on a single, sub-adult male (Amiet, 1983). Subsequently Boulenger (1900) placed *Cornufer johnstoni* in the genus *Petropedetes*. Bocage (1895) described *Tympanoceros (=Petropedetes) newtonii*, from "Fernaõ do Pó" (=Fernando Po), noting that the large tympanum was *"surmonté tout près de son bord supérieur par un tubercule cylindrique."* Boulenger (1905) described *P. natator* from "Sierra Leone at 800 feet" and *P. palmipes* from "Efulen, South Cameroon." Ahl (1924) described *P. obscurus* from "Tscharra Dana-Fluss" in Kenya. *P. obscurus* was synonymized with *P. cameronensis* by Perret (1984) who found the two species morphologically indistinguishable, and considered Ahl's Kenya locality data to be erroneous. Amiet (1973) described *P. perreti* from "Nsoung, 1400-1500 m., Cameroon" and *P. parkeri* (Amiet, 1983) from "d'Atolo (région de Mamfé), Cameroon." Six of the eight species of the genus *Petropedetes* occur in the western and southern portions of Cameroon, including Fernando Po. *P. cameronensis* has also been collected in eastern Nigeria, and *P. newtoni* in Equatorial Guinea, which indicates a remarkable center of speciation in the region of the Bight of Biafra (Perret, 1984).

Neiden (1910) described the genus *Arthroleptides* based on *Arthroleptides martiensseni* sp. nov. from "Amani, Deutschostafrika", now Tanzania. This species has a restricted distribution, endemic to the Eastern Arc Mountains of Tanzania, reported from the East and West Usambaras, the Ulugurus, and the Udzungwas (Howell, 1993). Neiden considered *Arthroleptides* to be intermediate between *Petropedetes*, with which it shared the distinctive "T-shaped" terminal phalanges and *Arthroleptis*, with which it shared the absence of vomerine teeth and the reduction of webbing between the toes. The second member of the genus, *A. dutoiti* was described by Loveridge (1935) from two adults (male and female) and an immature specimen from the eastern slopes of Mt. Elgon, Kenya. This species is considered extinct by Baillie & Groombridge (1996). *Petropedetes* and *Arthroleptides* share many morphological and ecological similarities. However, certain members of the genus *Petropedetes* possess a suite of morphological characters unique to breeding males. These include enlarged femoral glands, the presence of a bony, metacarpal projection, tympanic and brachial hypertrophy, and most distinctive, the presence of a nipple-like projection on the tympanum. Perret (1984) summarized the characters of breeding male *Petropedetes*, reporting that the tympanic projection occurred in four of the seven recognized species (i.e. *perreti*, *newtoni*, *parkeri*, and *johnstoni*). Perret also noted that the location of the projection varied by species, from the centre of the tympanum to the upper

margin. An examination of Perret's data also shows that these four species have larger tympani than the three species (i.e. *cameronensis*, *natator* and *palmipes*) that lack tympanic projections. All four species that have tympanic projections also possess a bony, metacarpal projection; however, this character is also shared with *P. palmipes*.

Considerable confusion exists in the literature as to the structure of the tympanic projection, undoubtedly the most distinctive sexually dimorphic character of male *Petropedetes*. This was illustrated by Noble (1931) and by Duellman & Trueb (1994). These authors concluded that the projection was the columella thrust through the eardrum, covered by dermis. Duellman & Trueb (1994) stated that this projection would diminish the vibratory capacity of the tympanum, thereby reducing its sensitivity to high frequencies. However, du Toit (1943) in his anatomical monograph on *Petropedetes* stated that "*Petropedetes* is unique in possessing a seasonal secondary sexual characteristic in the form of a so-called tympanic papilla. The latter, which Noble (1931) mistook for the *pars externa plectri* thrust through the drum is in reality a thickened portion of the outer dermal part of the tympanic membrane." He further explains the structure of the papilla to be quite complex, consisting of epidermal and cutis components, with a concentration of glands, collagenous fibres in its basal portion, a subepidermal pigment layer that is better developed in the papilla than other portions of the tympanum, as well as a superficial cornified layer that is produced into tiny, wart-like asperities. This paper reports the discovery of an equivalent suite of nuptial characteristics in breeding male *Arthroleptides martiensseni*, and discusses the taxonomic relationships between *Petropedetes* and *Arthroleptides* in the light of this new discovery.

MATERIALS AND METHODS

In December 1995, an intensive, multi-taxa biodiversity inventory of two sites in the Udzungwa Mountains National Park was conducted at the beginning of the several-month long rainy season. Nine days were spent at a single site at the foot of the Udzungwa Escarpment, along the Mwaya River at 350 m. This locality included a mixture of riverine forest, miombo, and disturbed edge and agricultural habitats near Man'gula village. Nine days were spent at a single upland site (1100-1200 m) along the upper reaches of the Njokomoni River, in primary, undisturbed rainforest. Sampling was conducted in a systematic, repeatable manner. Herpetological samples were collected using 200 meter-long drift fences, with 20-litre buckets (ca. 0.5 m depth) placed at 5 m intervals, and were checked in the morning and evening each day. Hundreds of litter-dwelling amphibians and reptiles, as well as invertebrates and small mammals, were collected using this technique. These were counted to gain a crude measure of relative abundance and, with the exception of a small percentage that were preserved, released. Additional data were collected by conducting time-constrained transect searches, along trails and river-edge, both during the day and at night; by opportunistic examination of fallen trees and rotten logs; and by making several excavations, to exhume fossorial species.

Specimens were collected to document the diversity of species, and whenever possible, the range in variation, sexual dimorphism, and life history stages of each species of amphibian and reptile found. These voucher specimens were anaesthetized, individually labelled, and fixed in formalin. Tissue samples for DNA analysis were collected prior to fixation, and stored in ethyl alcohol. Colour photographs were taken of selected specimens prior to preservation and tape recordings of frog calls were made. All specimens were exported to the American Museum of Natural History (AMNH) for follow-up identification and study. Preparation of this paper required examining specimens of *Petropedetes* and *Arthroleptides* deposited in both the AMNH and the Natural History Museum (London). As preserved frogs are quite pliable, all measurements reported on Tables 1 and 2 were made with calipers and rounded to the nearest 0.5 mm. Great care was exercised to be consistent in measuring technique, i.e. the application of pressure to the specimen from the caliper's jaws. Unless otherwise noted, the sex of all specimens examined was verified by internal examination through a slit cut through the abdomen wall.

RESULTS AND DISCUSSION

On 8 December 1995, a series of eleven frogs (AMNH 151340-50) was collected at night by the author and A. M. Nikundiwe in primary rainforest, perched on large boulders in cascades on the Njokomoni River, near where the river drops off of the edge of the Udzungwa Escarpment in Tanzania's Udzungwa Mountains National Park, 7°48'57"S, 36°51'15"E, 1100 m. That afternoon the dry weather had broken with an exceptionally heavy thunderstorm. Two of the largest individuals of this series were mature males (AMNH 151342-43). They were quite remarkable in that they possessed several of the distinctive breeding characteristics of male *Petropedetes*, including the unique tympanic projection which was located between the center and upper rim of the tympanum, as well as tympanic and brachial hypertrophy, and a large, metacarpal knob. Although we remained at this location until 16 December, these large adult frogs were found only once, on this single night that had been preceded by heavy rains.

Juveniles and sub-adults were found consistently along the Njokomoni River, up to its headwaters at 1200 m. During the day they remained hidden under stones along the river bank; at night they were active on the forest floor and in the river, and one was found perched in a small tree 1 m above the ground. Several voucher specimens were collected along the length of the river (AMNH 151351-55). A single sub-adult

TABLE 1. Measurements/nuptial characters of *Arthroleptides martiensseni* from the Eastern Arc Mountains of Tanzania. 0, not present; 1, weakly developed; 2, strongly developed; ?, data not available. AMNH, American Museum of Natural History, New York; BM, Natural History Museum, London; KMH, Kim Howell field series (to be deposited at the Natural History Museum, London).

(1) MALES

Museum No.	Length (mm)	Papilla	Metacarpal knob	Tympanic hypertrophy	Brachial hypertrophy	Chin+ gular spines	Femoral glands	Month collected
AMNH 151342	73	2	2	2	2	2	not visible	12
AMNH 37281	58.5	2	2	2	2	2	? visible	09
AMNH 151343	55	2	2	2	2	0	not visible	12
KMH 13571	52	1	2	1	0	0	not visible	07
BM 1982.543	43	0	?	0	0	0	not visible	01
AMNH 151347	42	0/1	0/1	0/1	0	0	not visible	12
AMNH 151339	41.5	0	0/1	0	0	0	not visible	12
BM 1994.794	39	0	?	0/1	0	0	not visible	?
BM 1980.363	38	0	?	1	0	0	not visible	10
BM 1994.647	37.5	0	?	0/1	0	0	not visible	10
BM 1974.57	37	0	?	1	0	0	not visible	10
BM 1994.795	36	0	?	1	0	0	not visible	?
BM 1974.58	33.5	0	?	1	0	0	not visible	10

(2) FEMALES

Museum No.	Length (mm)	Papilla	Metacarpal knob	Tympanic hypertrophy	Brachial hypertrophy	Chin+ gular spines	Femoral glands	Month collected
BM 1986.483	60	0	?	0/1	0	0	not visible	10
AMNH 151341	58	0/1	1	1	0	0	not visible	12
BM 1980.199	58	0	?	1	0	0	not visible	11
AMNH 151344	57	0	1	1	0	0	not visible	12
AMNH 151345	57	0	1	1	0	0	not visible	12
BM 1982.542	55.5	0	?	1	0	0	not visible	01
BM 1994.719	50	0	?	0/1	0	0	not visible	?
AMNH 151340	42.5	0/1	0/1	0/1	0	0	not visible	12
AMNH 151346	39.5	0/1	0/1	0/1	0	0	not visible	12
AMNH 151355	39	0	0	0/1	0	0	not visible	12

specimen was collected at a lowland site, in riverine forest, at the foot of the Udzungwa Escarpment, in the Udzungwa Mountains National Park, Man'gula Camp Site No. 3 on the Mwaya River, 7°50'51"S, 36°53'00"E, 350 m (AMNH 151339).

Initially, these specimens were identified as *Petropedetes* based on the morphology of the breeding males. However, upon comparison with specimens at the Natural History Museum (London) it became apparent that these were referable to *Arthroleptides martiensseni*. In his anatomical study of *Arthroleptides dutoiti*, du Toit (1938) examined a presumably mature male of 25 mm (the type specimen is a gravid 31 mm female) and although making note of some unusual features, such as the "*annulus tympanicus* has an additional ventromedially directed process not met with in other forms", makes no reference to any tympanic projections. How had such a distinctive anatomical feature of *Arthroleptides martiensseni* escaped detection for nearly a century? Examination of the extensive Tanzanian holdings of the Natural History Museum began to shed some light on this puzzle. Juvenile and sub-adult *Arthroleptides* were well represented in this collection, as were adult females, but large males were absent (see Table 1).

The largest male in the Natural History Museum collection was an uncatalogued specimen, K.M. Howell Field Number 13571, collected during the early dry season on 29 July 1996 in the Mtai Forest Reserve, Tanzania (38°46'E, 4°51'S), measuring 52 mm snout-urostyle length. The left tympanum was slightly hypertrophic with a tiny tympanic projection. The right tympanum showed no signs of hypertrophy or tympanic projection. The secondary sexual characteristics of this specimen were so subtle, they could easily be overlooked. This contrasts markedly with AMNH 151343, measuring 55 mm snout-urostyle length, but collected at the beginning of the rainy season. This specimen had well-defined tympanic papillae and hypertrophied tympani surrounded by a raised ring of

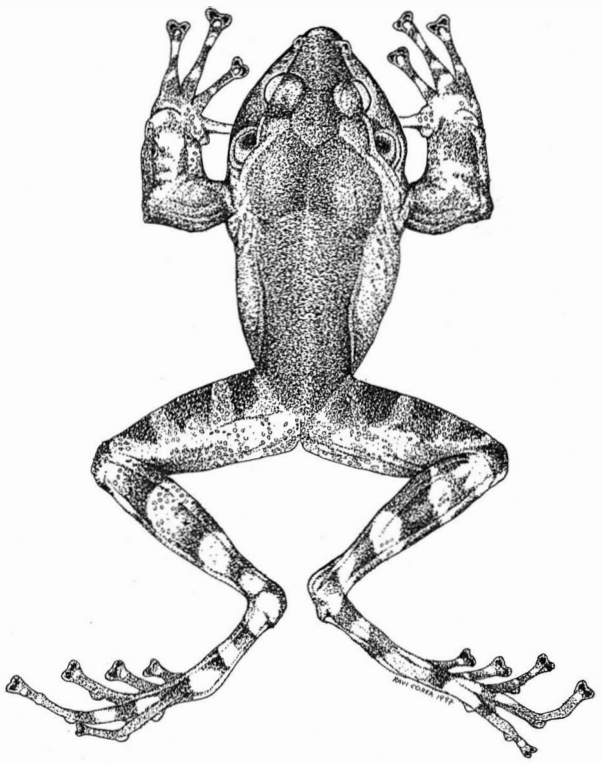

FIG. 1. Dorsal view of AMNH 151342 (M.W. Klemens Field No. 11003), *Arthroleptides martiensseni*, illustrating the distinctive nuptial characteristics of the breeding male. Note the variation in shape of the tympanic papilla. (x 0.75).

knobs. Brachial hypertrophy was also well-developed in this specimen as was the metacarpal knob.

The most spectacular development of these secondary sexual characteristics are found on AMNH 151342 (Figs. 1 & 2). This specimen measures 73 mm snout-urostyle length, far larger than any specimen reported in the literature. It has well-developed, strongly protruding tympanic papillae; the papilla on the right tympanum has a curved surface, whereas the papilla on the left tympanum is distinctly squared-off. The tympani are hypertrophied, both surrounded by a ring of knobs. There are well-defined spines on the chin and throat and the forelimbs exhibit marked brachial hypertrophy. There is a strongly protruding metacarpal knob. Another specimen, a 58.5 mm male collected by Arthur Loveridge in the Uluguru Mountains on 19 September 1926 (AMNH 37281) exhibits all the distinctive characteristics of AMNH 151342 but, due to the extremely soft preservation, accompanied by some minor tissue

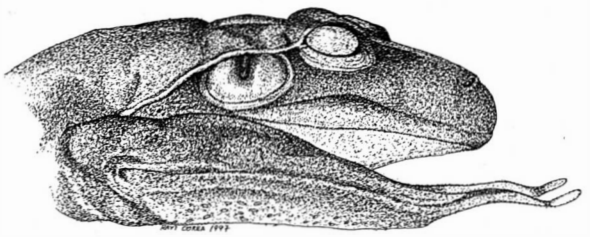

FIG. 2. Lateral view of AMNH 151342 (M.W.Klemens Field No. 11003), *Arthroleptides martiensseni*, illustrating the squared-off tympanic papilla referred to in the text. (x 1.5).

deterioration, the presence of what appear to be a pair of deeply imbedded femoral glands were noted.

I also examined a series of eight *Arthroleptides dutoiti* collected by R. Keith on 6 April 1962 (except AMNH 68677 collected on 17 May 1962) along the Suan River, NE Mt. Elgon, Kenya, 7000 feet (AMNH 68670-77). Two of these were quite small and presumed to be juveniles. The remaining six specimens could be divided into two morphologically distinct groups. As this species is presumed to be extinct, and these specimens irreplaceable, their sex was not verified by dissection. Three frogs (AMNH 68672-73, 68675) range from 23.5-25.5 mm snout-urostyle length. They possess none of the distinctive breeding characteristics of male *A. martiensseni*, however all three of these specimens possessed large, conspicuous femoral glands (see Table 2) and are presumed to be males. The remaining three specimens (AMNH 68670-71, 68677) are larger (i.e. 30-31.25 mm snout-urostyle length). They do not possess any of the distinctive breeding characteristics of male *A. martiensseni*, nor any trace of femoral glands and are presumed to be females. The ventral colouration varies markedly between these two groups. The females have a strongly marbled violet/black and grey/white ventral pattern, which is consistently uniform from head to thigh. The males have a weaker pattern, strong on the thighs, but dissipating on the venter, and becoming very weak in the gular region. Loveridge (1935) made no reference to the presence of enlarged femoral glands on his single male paratype. Possibly he overlooked these glands, but as Loveridge had a keen eye, I suspect that it is more likely that the male paratype was not in full breeding condition. Loveridge's male paratype measured 25 mm and the type, a gravid female, measured 31 mm.

TABLE 2. Measurements of presumed male *Arthroleptides dutoiti* from Mt. Elgon, Kenya. All measurements in mm.

Museum Number	Length	Right thigh	Right femoral gland	Left thigh	Left femoral gland
AMNH 68675	23.5	13.5	6.5	13.5	7.0
AMNH 68672	25.5	12.5	6.0	12.5	6.5
AMNH 68673	25.5	13.0	6.5	13.5	7.0

Both these specimens, as well as a 10.5 mm juvenile paratype, were collected on 8 January 1934. Loveridge noted differences in ventral colouration which do not agree with my observations on the AMNH series, stating that "below and posterior aspect of thighs light violet brown slightly flecked with white in the type, rather more abundantly flecked and mottled in the paratypes."

CONCLUSIONS

Mature *Arthroleptides martiensseni* males possess many of the same secondary sexual characters that were considered unique to *Petropedetes*. These include the presence of a tympanic papilla, brachial and tympanic hypertrophy, an enlarged metacarpal knob, and spines on the chin and gular region. In fact, *Arthroleptides martiensseni* bears more than a superficial resemblance to *Petropedetes parkeri* as described by Amiet (1983). Breeding males of both species are characterized by rudimentary digital webbing; larger body size than females; gular granulations topped with a tiny, dark spine; cutaneous spinosity, especially in the area between the tympanum and the forelimbs; marked brachial hypertrophy; large tympanum with papilla located toward the top of the tympanic rim; metacarpal knob; and reduced (possibly absent in the case of *A. martiensseni*) femoral glands. Breeding male *A. dutoiti* do not resemble *A. martiensseni*. However, they share, with breeding males of several species of *Petropedetes*, a prominent, enlarged femoral gland.

The marked similarities in both morphology and ecology of these two genera of specialized frogs, their distribution in the western and extreme eastern portions of the equatorial African rainforest bloc, and the apparent absence of any similar taxa in between raises many interesting biogeographical and evolutionary questions which remain to be resolved. Although it is premature to extrapolate from these data, a systematic analysis of the relationships between the various constituent taxa of *Petropedetes* and *Arthroleptides* is fertile ground for future investigation. These frogs may all be referable to *Petropedetes*, with *parkeri* and *martiensseni* sharing a close relationship, i.e. they may be sister-taxa. *Arthroleptides dutoiti* closely resembles *Petropedetes cameronensis*, warranting a re-evaluation of Perret's (1984) synonymy of *Petropedetes obscurus* and re-examination of Ahl's syntypes, as Perret may not have considered the possibility that Ahl's specimens were *Arthroleptides* (this possibility only became apparent after I had returned Ahl's syntypes to Berlin). The need for additional herpetological inventory across the African rainforest bloc should be a high priority, not only for basic scientific knowledge, but to develop a scientifically-informed strategy for designating priority areas for conservation within this vast expanse of rainforest, second in size only to Amazonia. Possibly the "missing link" that will bridge the gap between the torrent frogs of west and east Africa awaits discovery behind a waterfall on an as-of-yet unexplored inselberg in the Congo basin.

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A MOLECULAR PHYLOGENETIC STUDY OF THE OLD WORLD TREEFROG FAMILY RHACOPHORIDAE

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A phylogenetic tree is presented for the Old World treefrog family Rhacophoridae and other ranoid frogs that that have an Africa-Madagascar-Asia distribution. The tree was inferred from parts of the mitochondrial ribosomal 12S and 16S genes and the $tRNA^{val}$ gene sequences with the Microhylidae as outgroup. The tree indicates that the rhacophorids are a monophyletic group composed of a Madagascar clade and an Asian-African clade. When endemic Madagascar mantellids were added to the tree, they also were part of the Madagascar rhacophorid clade, but the support for this assignment is weak. *Tomopterna labrosa*, a ranid endemic to Madagascar, appears more closely related to the Madagascar rhacophorids than it does to the ranids included in the analysis. Support for this relationship is strong enough to merit reinvestigation of the morphology and extension of the molecular data set.

INTRODUCTION

Ever since spending a sabbatical year in Africa in 1972, CMR has had an interest in the African reedfrog family Hyperoliidae, first in genetic and developmental problems (Richards, 1976, 1981, 1982; Richards & Schiotz, 1977; Richards, Carlson & Rogers, 1975; Richards, Carlson & Connelly, 1977) and more recently in the molecular systematics of the family (Richards & Moore, 1996). Richards & Moore (1996) showed that the 3' portion of the 12S mtDNA plus the $tRNA^{val}$ (a total of 606 nucleotides) were able to ascertain with some certainty the relationships among the genera of the family Hyperoliidae. This led to an investigation of the other Old World family of treefrogs, the Rhacophoridae, which has a single genus, *Chiromantis*, in Africa, two genera (*Boophis* and the monotypic *Aglyptodactylus*) in Madagascar, while the remaining seven genera are distributed across Asia. It also led to an interest in other African frogs which share this distribution. These land masses, so disconnected today, had a long shared history before the breaking up of Pangea.

Work in progress shows that the same amount of sequence data that resolved the Hyperoliidae is not enough to resolve the relationships among the Asian and African rhacophorids. The deep branches of the tree are very short, so that there are not enough differences in the Asian groups to distinguish among genera with confidence. Consequently, a 475 bp sement of 16S rDNA was added to the data set. The combined data set (1081bp) gave rise to a strongly supported tree for the Rhacophoridae and some related Madagascar species.

The species sequenced (Table 1) included representatives of all rhacophorid genera except *Nyctixalus* and *Theloderma*. Mantellids were also included because Liem (1970) included them in his morphological study of the Rhacophoridae, and because their taxonomic and phylogenetic relations to other families are disputed (see discussion).

MATERIALS AND METHODS

GENE AMPLIFICATION AND SEQUENCING

The isolation of DNA from frozen and alcohol-fixed specimens followed standard procedures (Richards & Moore, 1996). All DNA sequencing was done from Polymerase Chain Reaction (PCR) products. Standard protocols of Kocher, Thomas, Meyer, Edwards, Paabo, Villablanca & Wilson (1989) were used with minor modifications for the specific genes being amplified (Richards & Moore, 1996). The primers listed in Richards & Moore (1996) were used to amplify a 606bp segment of 12S rDNA and $tRNA^{val}$; primers 16L8 (Hedges, 1994) and 16H9 (Ruvinsky & Maxson, 1996) were used to amplify a 475bp region of the 16S gene.

Other modifications in the Kocher protocol were directed at reducing the chance of amplifying contaminant sequences (see Derr, Davis, Wooley & Wharton, 1992; Hackett, Griffiths, Bates & Klein, 1995; Moore & DeFilippis, 1997 for discussion). Aerosol resistant pipette tips were used in all procedures involving isolation and PCR. DNA isolation and PCR mixes were exposed to short-wave UV light to destroy any contaminant DNA before adding DNA template (Cimino *et al.*, 1990; Moore & DeFilippis, 1997). Where possible, two specimens of each species were sequenced to assure that the sequences are indeed the target species. Where only a single specimen was available, the gene was sequenced in both directions.

All PCR products were cleaned using WizardTM PCR Preps (Promega) and quantified before running sequencing reactions. Sequencing was done both on a Pharmacia ALF and an ABI automated sequencer. For the ALF, cycle-sequencing reactions were run on PCR products using fluorescein-labeled primers and Thermo Sequenase kits (Amersham).

TABLE 1. List of species sequenced. Museums: UMMZ, University of Michigan Museum of Zoology; USNM, U. S. National Museum; AMNH, American Museum of Natural History; FMNH, Field Museum of Natural History; TTV, Texas Tech University.

Species name	Museum No.	Location
Rhacophoridae		
Aglyptodactylus madagascariensis	UMMZ 37571	Madagascar
Boophis erythrodactylus	USNM 336403	Madagascar
Boophis tephraeomystax	USNM 59146	Madagascar
Chiromantis xerampelina	UMMZ 210197	Kenya
Chiromantis sp.	AMNH A153250	Tanzania
Chirixalus eiffingeri	UMMZ 190578	Taiwan
Chirixalus idiooticus	UMMZ 5732	Taiwan
Philautus mjobergi	FMNH 18084	Malaysia
Philautus petersi	FMNH 18164	Malaysia
Polypadetes megacephala	UMMZ 189969	Taiwan
Polypadetes leucomystax	USNM 498993	Philippines
Rhacophorus moltrechti	UMMZ 190564	Taiwan
Rhacophorus arboreus	TTV-R-11748	Japan
Buergeria japonica	UMMZ 190060	Taiwan
Buergeria robusta	UMMZ 189974	Taiwan
Mantellinae		
Mantidactylus grandidieri	UMMZ 42628	Madagascar
Mantella sp.	UMMZ 46479	Madagascar
Ranidae		
Arthroleptides martiensseni	AMNH A151339	Tanzania
Ptychadena mascareniensis	UMMZ 213491	Madagascar
Tomopterna labrosa	UMMZ 43541	Madagascar
Microhylidae		
Probreviceps macrodactylus loveridgei	AMNH A153248	Tanzania
Scaphiophryne gottlebei	UMMZ 53423	Madagascar
Scaphiophryne breviceps	UMMZ 53430	Madagascar

ALIGNMENT AND TREE CONSTRUCTION

The sequences were aligned by eye using the computer program ESEE (version 3, Cabot & Breckenbach, 1993) with careful attention to the stem and loop secondary structure of the RNA molecule as explained in detail in Kjer (1995) and Richards & Moore (1996). Hypervariable regions whose alignments were uncertain were omitted from the analysis because homology of the bases was not assured. Of the 1081 bases in the combined sequences, 913 were used in the analysis. Sequences have been submitted to Genbank (Accession numbers AF 026341-79).

Test version 4.0d55 of PAUP*, written by David L. Swofford was used to construct a Neighbor Joining tree using the gamma distribution in conjunction with the Tamura-Nei divergence model. Both transitions and tranversions were used. Ribosomal sequences have different rates of change at different positions (Van de Peer, Jansen, De Rijk & De Wachter, 1997). A gamma distibution is most appropriate for this kind of data (Gu, Fu & Li, 1995; Yang, 1996; Hillis, Moritz & Mable, 1996). A value of $\alpha = 0.4$ was estimated by Greg Spicer for this data set using PAUP*. Bootstrap values are for 500 replicates. The tree created in Paup* was imported into TREEVIEW (Page, 1996) and edited in Microsoft Power Point 4.0.

OUTGROUP SELECTION

Liem (1970), Drewes (1984) and Channing (1989) provide arguments for the selection of the Ranidae as the most appropriate outgroup for the Rhacophoridae. The Madagascan species *Tomopterna labrosa* (subfamily Raninae) and the African *Arthroleptides martiensseni* (subfamily Petropetedinae) were selected to sequence, because Africa has been postulated as the site of the major radiation of ranids (Duellman & Trueb, 1986) and to increase the species diversity of the outgroup, as suggested by Smith (1994). *Tomopterna labrosa* is endemic to Madagascar; its closest relatives are species of the same genus in Africa and two species in India-Sri Lanka. The subfamily Petropedetinae is found only in sub-Saharan Africa. We originally in-

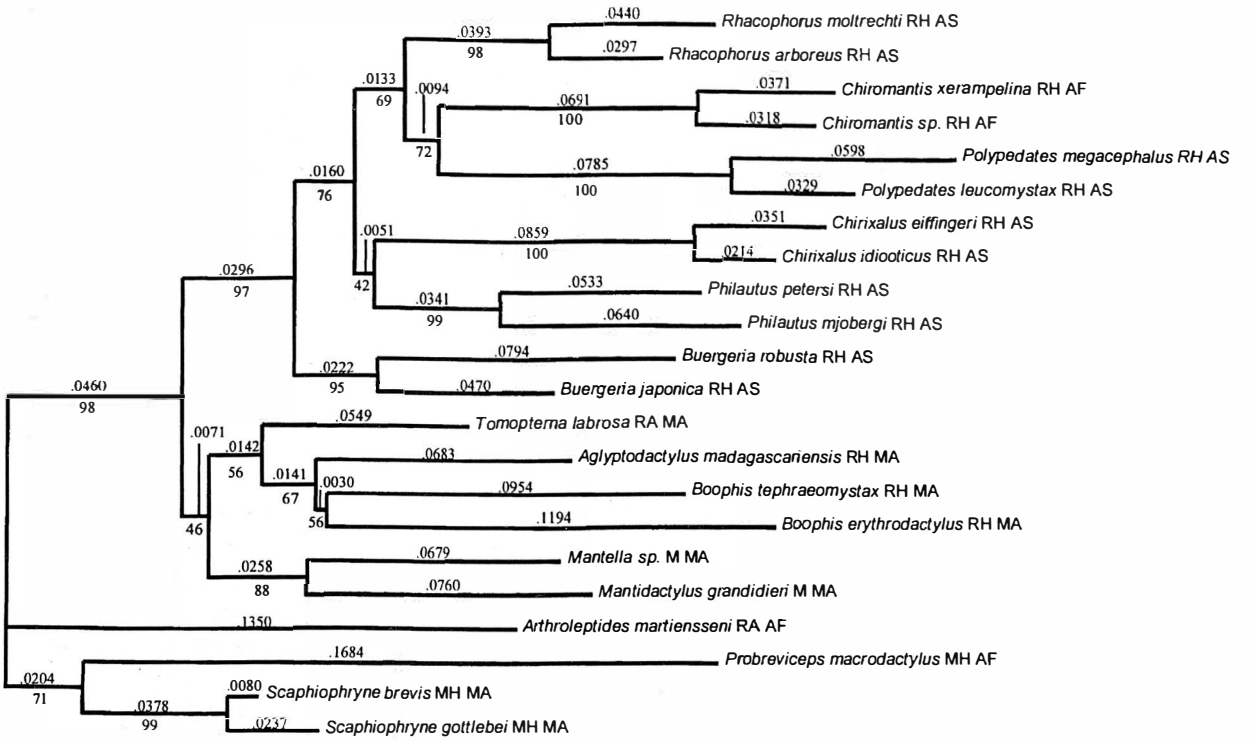

FIG. 1. Neighbor Joining tree for the species in Table 1. Families: RH, Rhacophoridae; M, Mantellidae; RA, Ranidae; MH, Microhylidae. Continent: AF, Africa; MA, Madagascar; AS, Asia. Numbers above a line are distances; below, the bootstrap value for 500 bootstraps.

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RESULTS

The Neighbor Joining phylogenetic tree constructed from these data, using the criteria described in materials and methods, is shown in Fig. 1. When the ranid *Arthroleptides* and three microhylids are used as outgroup species, the Rhacophoridae and associated Madagascar genera form a strongly supported clade. This clade, in turn, is composed of a strongly supported Asian-African clade and a more weakly supported (bootstrap value 46) clade that includes the Madagascar rhacophorids, the mantellids and the "ranid" *Tomopterna* (see below and discussion). If a tree is constructed for the rhacophorids alone (without the mantellids and *Tomopterna*), the bootstrap values for the African and Asian clade and a Madagascar clade are 97 and 95, respectively (data not shown). Clearly, the family Rhacophoridae consists of at least two distinct clades.

It is also clear that the African *Chiromantis* is very closely related to the Asian genera. The bootstrap support for the Asian *Polypedates* as sister group to *Chiromantis* is 72. These two genera form a clade with *Rhacophorus,* and this clade is sister to a clade including *Chirixalus* and *Philautus,* with *Buergeria* as the basal genus to all the other Asian and African species. Bootstrap support for an African-Asian clade is a very strong 97%.

In the tree in Fig. 1, *Tomopterna labrosa* is sister group to the Madagascar rhacophorids, but with only modest bootstrap support and does not group with the ranid *Arthroleptides* as would be expected. Using the branch swapping facility of MacClade (Maddison & Maddison, 1992), making *Tomopterna* sister group to the mantellids gave an equally parsimonious tree, but making it sister group to *Arthroleptides* produced a tree that is 21 steps longer. Thus, the most parsimonius tree supports the placement of *Tomopterna labrosa* in the Rhacophoridae.

The mantellids are the sister group to the Madagascar rhacophorids. However, the support for this position is also weak, and using the branch swapping facility of MacClade to make the mantellids sister group to the entire rhacophorid clade results in a tree that is only two steps longer, an insignificant difference. However, making the mantellid branch sister group to the Ranidae or the Microhylidae results in a tree that is 18-21 steps longer.

In the course of this work, it was discovered that the branch length of *Ptychadena* was extraordinarily long.

TABLE 1. List of species sequenced. Museums: UMMZ, University of Michigan Museum of Zoology; USNM, U. S. National Museum; AMNH, American Museum of Natural History; FMNH, Field Museum of Natural History; TTV, Texas Tech University.

Species name	Museum No.	Location
Rhacophoridae		
Aglyptodactylus madagascariensis	UMMZ 37571	Madagascar
Boophis erythrodactylus	USNM 336403	Madagascar
Boophis tephraeomystax	USNM 59146	Madagascar
Chiromantis xerampelina	UMMZ 210197	Kenya
Chiromantis sp.	AMNH A153250	Tanzania
Chirixalus eiffingeri	UMMZ 190578	Taiwan
Chirixalus idiooticus	UMMZ 5732	Taiwan
Philautus mjobergi	FMNH 18084	Malaysia
Philautus petersi	FMNH 18164	Malaysia
Polypadetes megacephala	UMMZ 189969	Taiwan
Polypadetes leucomystax	USNM 498993	Philippines
Rhacophorus moltrechti	UMMZ 190564	Taiwan
Rhacophorus arboreus	TTV-R-11748	Japan
Buergeria japonica	UMMZ 190060	Taiwan
Buergeria robusta	UMMZ 189974	Taiwan
Mantellinae		
Mantidactylus grandidieri	UMMZ 42628	Madagascar
Mantella sp.	UMMZ 46479	Madagascar
Ranidae		
Arthroleptides martiensseni	AMNH A151339	Tanzania
Ptychadena mascareniensis	UMMZ 213491	Madagascar
Tomopterna labrosa	UMMZ 43541	Madagascar
Microhylidae		
Probreviceps macrodactylus loveridgei	AMNH A153248	Tanzania
Scaphiophryne gottlebei	UMMZ 53423	Madagascar
Scaphiophryne breviceps	UMMZ 53430	Madagascar

ALIGNMENT AND TREE CONSTRUCTION

The sequences were aligned by eye using the computer program ESEE (version 3, Cabot & Breckenbach, 1993) with careful attention to the stem and loop secondary structure of the RNA molecule as explained in detail in Kjer (1995) and Richards & Moore (1996). Hypervariable regions whose alignments were uncertain were omitted from the analysis because homology of the bases was not assured. Of the 1081 bases in the combined sequences, 913 were used in the analysis. Sequences have been submitted to Genbank (Accession numbers AF 026341-79).

Test version 4.0d55 of PAUP*, written by David L. Swofford was used to construct a Neighbor Joining tree using the gamma distribution in conjunction with the Tamura-Nei divergence model. Both transitions and tranversions were used. Ribosomal sequences have different rates of change at different positions (Van de Peer, Jansen, De Rijk & De Wachter, 1997). A gamma distibution is most appropriate for this kind of data (Gu, Fu & Li, 1995; Yang, 1996; Hillis, Moritz & Mable, 1996). A value of $\alpha = 0.4$ was estimated by Greg Spicer for this data set using PAUP*. Bootstrap values are for 500 replicates. The tree created in Paup* was imported into TREEVIEW (Page, 1996) and edited in Microsoft Power Point 4.0.

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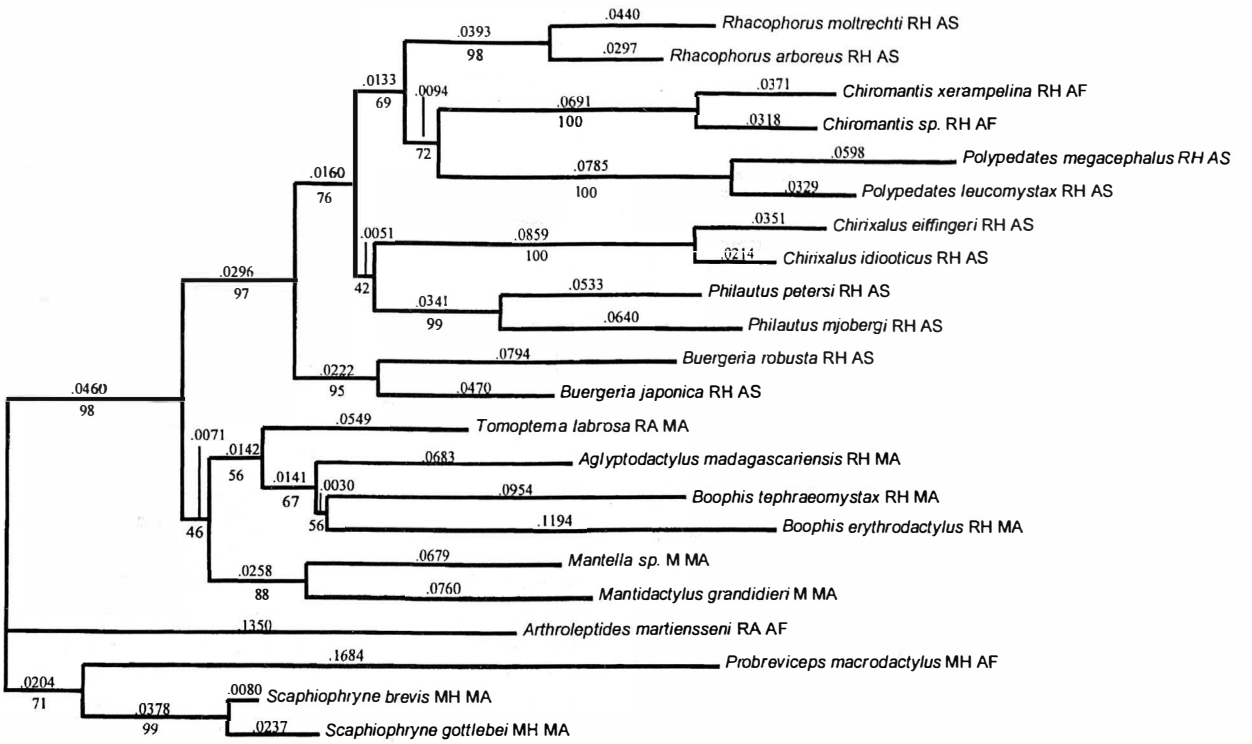

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In the course of this work, it was discovered that the branch length of *Ptychadena* was extraordinarily long.

This is an indication of many differences between it and even its closest relatives. Surprisingly, in the 12S segment it has nine autapomorphies in characters that are invariant in some 90 ranoid frogs we have sequenced. In addition to these many differences, there is a 2bp insertion in the loop between stems 38 and 39 (positions 183-184 in the alignment), a 16bp insertion in the loop between stem 45' and stem 47 (positions 304-319) and the loop region between the 12S gene and the adjacent tRNAval gene has been reduced to a single nucleotide (position 533). See Richards & Moore (1996) for loop numbers. These are not artifacts of sequencing or of misidentification of specimens as two individuals from different localities in Madagascar and one from mainland Africa were sequenced (see Table 1), and they all contained these same insertions and deletions. Since we felt that there was something unusual about these long branch sequences, they were removed from the analysis (see discussion). The 16S sequence showed no such anomalies.

DISCUSSION

The results presented above reflect the rhacophorid relationships suggested by morphology. Liem (1970), in his morphological study, found the African *Chiromantis* among the Asian rhacophorids and *Buergeria* at the base of the African and Asian clade, but the level of resolution of the Asian genera was not great. These relationships are now corroborated at the molecular level. However, Liem placed *Chiromantis* near *Buergeria* at the base of the clade while this study shows *Polypedetes* as the closest relative. Channing's reanalysis of Liem's data (Channing, 1989) presents a somewhat different phylogenetic interpretation of the data where he finds *Buergeria* at the base of all the rhacophorids including *Mantidactylus*, a conclusion not supported by the molecular data. Channing also moved *Chiromantis* to a position higher in the Asian clade rather than at the base. This placement is supported by the molecular data.

Liem also included *Mantidactylus* in his study and found, as here, that it was related to the Madagascar rhacophorids, *Aglyptodactylus* and *Boophis* with all three outside the Asian and African clade. The relationships of the mantellids to other groups has long been disputed. They have variously been assigned to the Ranidae (Blommers-Schlosser, 1979; Frost, 1985; Duellman & Trueb, 1986), to the Rhacophoridae (Liem, 1970; Channing, 1989) and to their own family, the Mantellidae (Blommers-Schlosser & Blanc, 1991; Duellman, 1993). This study suggests that the mantellids belong to the Rhacophoridae and not the Ranidae.

It is not surprising that the Madagascan rhacophorid species are differentiated from the Asian ones, given the biogeographic history of the land masses involved. Madagascar and India separated 88 million years ago (Storey, Mahoney, Saunders, Duncan, Kelley & Coffin, 1995; Storey, 1995) and the species have been evolving separately for a very long time. After separation from Madagascar, India floated northward until it joined the Asian continent. The progenitors of the Asian rhacophorids then spread out over a vast continent with empty treefrog niches, so it can be speculated that they speciated quite rapidly, a speculation that is perhaps supported by the short branch lengths deep in the tree. The fact that the African *Chiromantis* is placed deep in the Asian rhacophorid clade would suggest that the progenitor stock of this genus did not remain behind in Africa when India-Madagascar broke away but rather that it was originally an Asian genus that migrated westward overland and thence entered Africa.

Tomopterna is a genus with the same geographic distribution as the Rhacophoridae: Africa, Madagascar and Asia. It is a burrowing form with the morphological adaptations suited to a fossorial life and it seems slightly fanciful for it to be even remotely related to treefrogs with their own suite of morphological adaptations that enable them to climb so well. Nevertheless, the molecular data thus far available are strong enough to be an indication that this matter needs clarification at both the molecular and morphological levels. At present, there are just four molecular character states linking *Tomopterna* to the Madagascar rhacophorids. More sequence data, either additional ribosomal sequence or sequence from another gene, are needed to expand the data presented here. The morphology should also be examined even more closely to determine whether fossorial adaptations are obscuring true phylogenetic relationships. Channing's group (personal communication) has sequenced the same 12S region in South African *Tomopterna* species, and these do show a close relation to the Ranidae.

Details about the sequence of *Ptychadena* are included here to point out that the peculiarities of the 12S sequence that make it so very different from all the other ranoids sequenced may prove to be exceedingly valuable characters in resolving the phylogeny of the Ranidae. The Ranidae is an extremely large and diverse family with a world-wide distribution. The only attempt at creating a phylogeny was the morphological study of Clarke (1981) who worked only with the African members of the subfamily Raninae. Other mitochondrial sequence peculiarities have been found in the genus *Rana* (Macey, Larson, Ananjeva, Fang & Papenfuss, 1997; Yoneyama, 1987). In *Rana catesbeiana* and *Rana limnocharis*, four of the mitochondrial tRNA's have been rearranged relative to the common vertebrate mitochondrial gene order found in species as diverse as fish, *Xenopus* and mammals (Macey *et al.*, 1997), but the order among these four genes differs in the two species.

Alternately, since the *Ptychadena* sequence is very different from that of other ranoid frogs, the possiblity must be entertained that it is a mitochondrial sequence that has been transposed to the nucleus (Zhang & Hewitt, 1996). This could explain the large number of nucleotide changes in positions that are conserved in the mitochondria of almost all ranoids. If this were a nonfunctional nuclear pseudogene evolving at a differ-

ent rate and under different constraints, such conserved positions could be free to change. In either case, this sequence can be used as a phylogenetic marker provided it is compared with truly orthologous sequences.

Species groups that have radiated rapidly and consequently have very short branches in the deep portions of the tree are going to require longer sequences of ribosomal genes or sequence from additional genes to resolve the phylogenetic relationships in the Rhacophoridae and to establish firmly the phylogenetic position of the mantellids with some degree of confidence. If not, another gene, probably a nuclear protein gene, will be selected in an effort to gather sufficient data for firm resolution.

NOTE ADDED IN PROOF

At the Third World Congress of Herpetology in Prague (August 1997), Glaw, Vences & Böhme reported two new species of *Aglyptodactylus*. An analysis using 18 phylogenetically informative characters showed a closer relationship between *Aglyptodactylus* and *Tomopterna* than between *Aglyptodactylus* and *Boophis*, the other rhacophorid on Madagascar. On the basis of this, they propose removing *Aglyptodactylus* from the Rhacophoridae and placing it in the Ranidae despite the presence of intercalary phalangeal elements in *Aglyptodactylus* which are unknown in ranines (Glaw, Vences & Böhme, in press).

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HITHERTO UNDESCRIBED ULTRASTUCTURAL FEATURES IN THE EPIDERMIS OF TWO AFRICAN AMPHIBIANS

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The epidermis of *Hyperolius nitidulus* (Peters, 1875) (Hyperoliidae) consists of three strata: stratum corneum, stratum granulosum and stratum germinativum. The stratum corneum of both *H. nitidulus* and *Hemisus marmoratus* (Peters, 1854) (Hemisiidae), contains 1-2 replacement layers. The granular cells of *H. nitidulus* are arranged in 2-3 rows. No granular cells typical of the stratum granulosum could be seen in *H. marmoratus*. This is a unique situation for any amphibian. Very large germinative cells were observed in both anurans. In *H. nitidulus* a unique, long and slender 'pillar cell' is situated under the stratum corneum, extending through the stratum germinativum, and the basement membrane deep into the dermis. This cell contains abundant tonofilaments, and appears to function as a pillar suppporting the frequently moulting stratum corneum.

INTRODUCTION

The ultrastructure of the amphibian epidermis cells has been described in about 12 anuran species. No epidermis of an African amphibian has previously been studied. A number of epidermal cell types have been described (Fox, 1986; Warburg *et al.*, 1994). Among these are cells, typical only of the larval stages, which ultimately disappear; others first appear near the completion of metamorphic climax, and persist in the juvenile and adult stages (Warburg & Lewinson, 1977; Rosenberg & Warburg, 1978, 1992, 1993, 1995; Warburg *et al.*, 1994).

Hyperolius nitidulus (D & B) (Hyperoliidae) is a unique frog in many ways (Linsenmair, 1998). It is a short-lived frog in which only the juveniles aestivate successfully, by clinging to vegetation exposed to the harsh ambient conditions persisting during the dry season. Two phases can be recognized in this frog: the wet season phase and the dry season phase. These phases differ in both their water balance and dermal skin structure (Geise & Linsenmair, 1986, 1988; Kobelt & Linsenmair, 1986, 1992). The dry season frog has a thin layer of desiccated mucus sealing the body surface and thus reducing water loss (Geise & Linsenmair, 1986). The main barrier against desiccation seems to be the stratum corneum (Geise & Linsenmair, 1988). Moreover, the dermis contains several layers of iridiophore filled with purine platelets that are partly arranged parallel to the surface and thus cause a high reflection (up to 70%) of the sun's radiation in the visible and infrared spectrum (Kobelt & Linsenmair, 1986, 1992).

In contrast, *H. marmoratus* is a long-lived, fossorial frog that rarely ventures onto the surface, and then only under very humid conditions. There is little published information on this frog (it is presently studied by one of us: KEL).

In the present study we describe some of the novel ultrastructural features in the ventral epidermis of these two African amphibian species: *H. nitidulus* and *Hemisus marmoratus* (Hemisiidae).

MATERIALS AND METHODS

Animals were collected in the Ivory Coast (by one of us: KEL) and dissected shortly after capture. Between capture and dissection they were kept under humid conditions and fed on either flies (*Hyperolius*) or ants (*Hemisus*). Pieces of ventral epidermis of three adult *H. nitidulus* and *H. marmoratus* were examined histologically and ultrastructurally using light and transmission electron microscopy. The methods are described in greater detail in Warburg *et al.*, (1994).

For light microscopy, tissues were fixed in Bouin's fluid for 24 hrs. Sections were stained with haematoxylin and eosin. Epon sections 2 μm thick, were stained with toluidin blue.

For Transmission Electron Microscopy (TEM), pieces of skin were fixed cold in 3% glutaraldehyde in 0.05 M cacodylate buffer for 2-4 hrs, and then rinsed in buffer containing 8% sucrose. Post-fixation was in 1% OsO_4 containing 1.5% potassium ferrocyanide for 1 hr, and dehydrated in graded ethanols. Finally, the material was embedded in Epon 812. Unstained sections 70-90A thick were mounted on copper grids 300 mesh, and were examined in a Jeol 100 B (TEM) operated at 60 KV.

RESULTS

In *H. nitidulus*, under the upper layer of the stratum corneum there are 2-3 replacement layers composed of flattened, tightly packed stratum corneum cells (Fig.1A,B). In *H. marmoratus*, the epidermis appears to consist only of a stratum corneum with wide intercellular spaces in the ventral epidermis (Fig. 3A,B), and

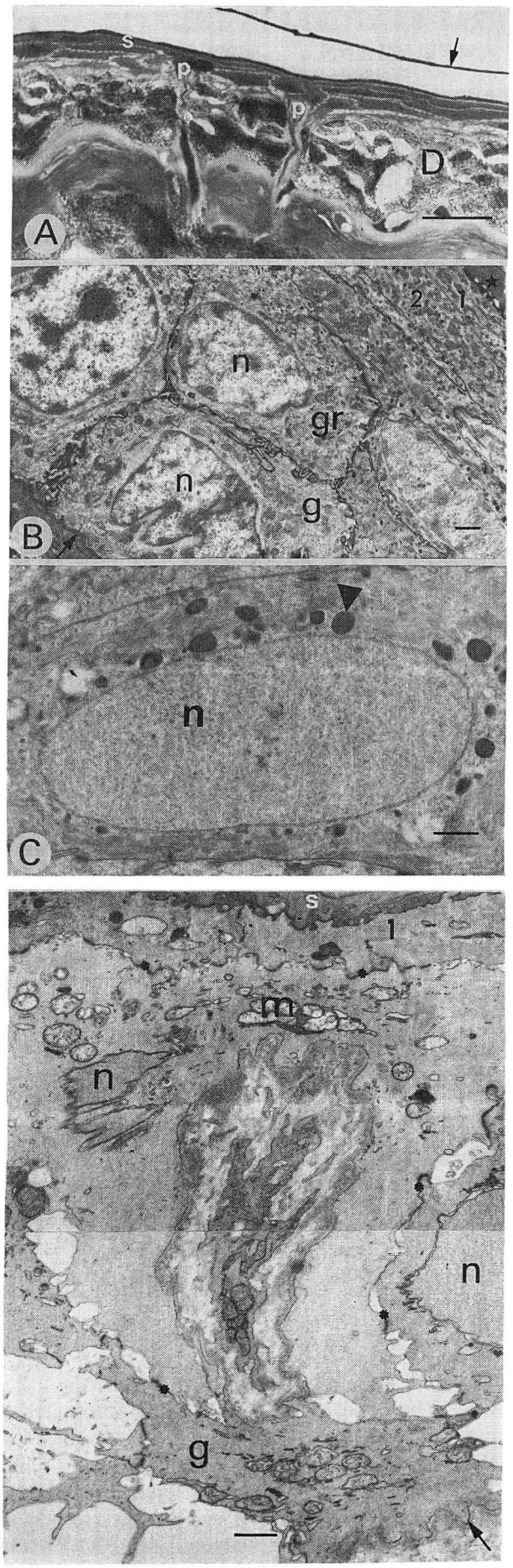

FIG. 1 (TOP LEFT). Sections through the epidermis of *Hyperolius nitidulus*. A, section through the entire depth of the ventral epidermis to the dermis (D) viewed in light microscope. Note the long pillar cells (p) situated perpendicular to the surface (arrow indicates moult) and stratum corneum (s), penetrating through the basement layer into the dermis (D)(x 420; scale bar=10 μm). B, same as in A but an ultramicrograph. Note the several stratum corneum layers (asterisk,1,2), the granular cells (gr), and the large germinative cells (g) situated on the basement layer (arrows) (n-nucleus) (x 6060; scale bar=1μm). C, granular cell containing granules viewed in EM (arrowhead; n-nucleus) (x 10 500; scale bar=1 μm).

FIG. 2 (BOTTOM LEFT). The large, slender pillar cell located perpendicular to the stratum corneum (s,1) and the basement layer (arrow), containing a nucleus (n) and mitochondria (m), is separated from neighbouring cells through tight junctions (flowers). A germinative cell (g) is situated on the basement layer (arrow) (x 9000; scale bar=1 μm).

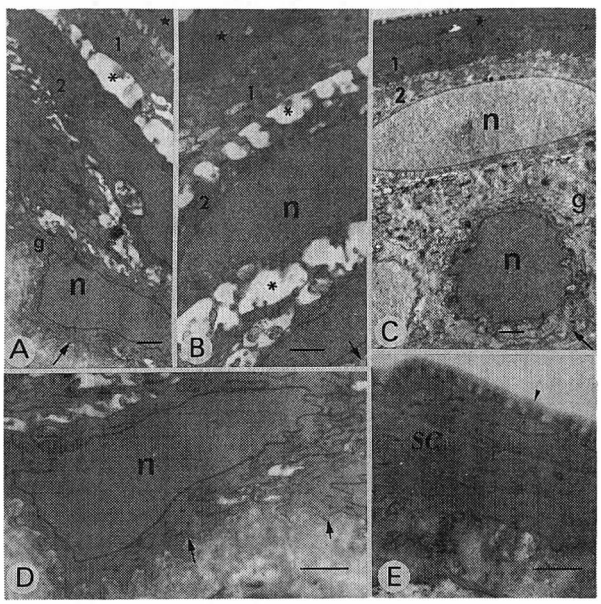

FIG. 3 (ABOVE). Sections through the ventral (A,B,D) and dorsal (C,E) epidermis of *Hemisus marmoratus*. A, section through the entire width of the ventral epidermis from the stratum corneum (asterisk), through its replacement layers (1,2) separated from each other by a wide intercellular space (flower), to a stratum germinativum cell (g) situated on the basement layer (arrow; n, nucleus). (x 7500; scale bar=1 μm). B, as in A but enlarged. The stratum corneum (asterisk) and its two replacement layers (1,2) separated from each other and from the stratum germinativum by wide intercellular spaces (flowers). Arrow indicates the basement layer (x 10 500; scale bar=1 μm). C, section through the entire width of the dorsal epidermis. Note the compact layers of stratum corneum (asterisk,1,2), and the stratum germinativum cell (g) situated on the basement layer (arrow; n-nucleus) (x 7500; scale bar=1 μm). D, germinative cell situated on the basement membranes (arrows; n-nucleus). Note the abundance of tonofillaments (x 15000; scale bar=1 μm). E, enlarged section through the dorsal stratum cornem (SC) showing the microvilli covered by fuzz (arrowhead) (x 15 000; scale bar= 1 μm).

tightly packed cells in its dorsal epidermis (Fig.3C,E), as well as a stratum germinativum (Fig. 3D). The stratum granulosum appears to be missing in this frog (Fig.3A-C). This is the first time that a stratum granulosum could not be found in an amphibian. On the other hand, in *H. nitidulus* the stratum granulosum, which is situated under the stratum corneum, is composed of 1-2 rows of large, oval-shaped cells containing large granules around the large oval nucleus (Fig. 1B,C).

The stratum germinativum in both species is composed of large cells, fully packed with tonofilaments and mitochondria in a perinuclear arrangement, which are situated on the basement membrane (Figs. 1.B; 3 A-D).

In *H. nitidulus*, a unique new cell type was seen for the first time. This is a very long, slender, pillar-shaped cell, situated under the stratum corneum, with its root-shaped base penetrating through both the stratum germinativum and the basement membrane deep into the dermis (Figs. 1A; 2). This cell contains numerous tonofilaments, thus possibly indicating its function as a supporting cell. We suggest naming this cell "pillar cell" because of its pillar-like shape and its vertical position in the tissue, indicative of a putative function in supporting the stratum corneum. It seems to be unique to *H. nitidulus*, and was never previously observed in the epidermis of any other amphibian species.

DISCUSSION

Epidermal cell layers of amphibians increase in number until the completion of metamorphosis (Rosenberg & Warburg, 1978; Robinson & Heintzelman, 1987; Warburg *et al.*, 1994). Thus, in the juvenile *Pelobates syriacus* there are three epidermal cell layers, whereas in the adult between three and four layers. The epidermis of the two African anurans studied here differs in the number of layers. The epidermis of *H. marmoratum* appears to be composed of only two layers.

At metamorphic climax, the process of cell flattening and keratinization reaches its peak, culmunating in the formation of the stratum corneum (Budtz, 1977; Warburg & Lewinson, 1977). There are a number of stratum corneum replacement layers found in *Hyperolius*. These may be related to the high frequency of moulting taking place in this species (Kobelt & Linsenmair, 1986). The replacement layer of the stratum corneum contains mucous granules (Fox, 1977). The presence of granules in epidermal surface cells appears to be a feature of the keratinization process (Budtz & Larsen, 1975). We have observed in *H. nitidulus* an ouststanding richness in granules in these cells.

In the stratum granulosum of *Hyperolius*, the granular cells are most abundant. In *Rana ridibunda* these granules were apparently situated closer to the stratum corneum, possibly related to the process of keratinization, whereas in *Bufo calamita* they were found in deeper layers, possibly indicating a role in water conservation (Navas *et al.*, 1980).

The large pillar cells described here for the first time in *Hyperolius*, may play a role in supporting the epidermis of this unique frog during its frequent moultings. These frequent and rather costly moultings are a means of preventing the formation of cocoon-like structures found in some other xeric-inhabiting arboreal frogs (Warburg, 1997). The reason could be that the formation of a cocoon will impede this frog's free functioning during the dry season (Linsenmair, unpublished).

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COMPARISON OF MOTILITY PATTERNS OF SPERM ASPIRATED FROM AMPLECTANT PAIRS OF *XENOPUS LAEVIS*, BY ELECTRO-EJACULATION AND FROM THE TESTES

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Studies on anuran spermatozoa have been restricted to the use of testicular sperm. Although these studies have been effective in creating baseline data, the motility parameters of ejaculate spermatozoa could present a clearer picture of the reproductive physiology. Methods were devised to aspirate sperm from amplectant males and collect the ejaculate from males. Sperm motion was then studied quantitatively and the various sperm motion parameters compared. Further research is necessary to determine whether Wolffian duct and cloacal secretions play a role in altering sperm motion characteristics of anuran testicular spermatozoa.

INTRODUCTION

Most studies regarding amphibian sperm physiology are concerned with testicular sperm. Anurans do not possess epididymides and previous researchers such as Poirier & Spink (1971) and Reed & Stanley (1972) have assumed that sperm maturation is completed in the testes. Evaluation of the sperm motion characteristics indicated that the anuran spermatozoa are adapted for fertilization in their respective fertilization environments (Wilson, 1994). Studies pertaining to the fertilizing capacity and motility of anuran spermatozoa should be undertaken on ejaculate sperm to effectively evaluate the motility parameters.

Xenopus laevis is widely used as a laboratory model for anatomical and physiological studies and earlier researchers laid the basis for studies of the reproductive biology. *X. laevis* is regarded as one of the more primitive aquatic breeders (Passmore & Carruthers, 1979) and the data assembled from these toads can be used in research of more advanced anurans. Wolf & Hedrick (1971) used *X. laevis* to investigate the viability and *in vitro* fertilization of the gametes. Grey, Wolf & Hedrick (1974) enhanced our knowledge on fertilization in *Xenopus* by investigating the fertilization envelope. Wolf (1974) developed a method to recover cortical granules which prevent polyspermic fertilization from *Xenopus* eggs. Spannhof, Fiedler, Schlisio & Jürss (1976) investigated osmotic and ionic regulation in *X. laevis*. A low osmolality is necessary for the initiation of sperm motility in *X. laevis* (Inoda & Morisawa, 1987).

Anuran reproductive tracts undergo characteristic seasonal changes with regard to the size of the testes and reproductive accessories (Mann & Lutwak-Mann, 1963). In this study we attempted to simulate these changes via peptide injections with human chorionic gonadotrophin (hCG). *X. laevis* toads were injected to go into amplexus and the ejaculate sperm collected. An electro-ejaculation technique was also used to collect sperm from the cloacae of the males.

The purpose of our study was therefore to develop techniques and present data pertaining to the motility characteristics of ejaculate *Xenopus* spermatozoa. The research included the following:

(1) Techniques were devised to aspirate and collect spermatozoa from amplectant males, electro-ejaculated males, or from dissected testicular tissue.

(2) The swimming characteristics and percentage motility of the spermatozoa obtained via the various techniques were then compared.

(3) Determination of the osmolality and pH of the medium in which the spermatozoa are ejaculated. Osmolalities of the cloacal fluid and sperm suspensions were also determined.

MATERIALS AND METHODS

Xenopus laevis adults were obtained from a breeding farm in Cape Town. Twelve females and fifteen males were used. They were kept in a steel tank at temperatures ranging from 18° to 25°C. The water in the tank was changed every second day. The animals were fed every morning between 10.00 and 11.00 hr with fish food pellets.

ASPIRATION OF SPERM

Male and female toads were kept apart in separate fish tanks with the water at a constant 23°C for 24 hours prior to the injections. A disposable syringe with a bent needle, was used to administer the hCG (pregnyl) as described by Brown (1970). Three pairs of toads were used each time for sperm aspiration. The female toads were given a primer injection of 250 i.u. of pregnyl. The males were given a primer of 150 i.u. Males and females were kept apart at a constant temperature and after 48 hrs the booster injections were administered. Females were given a booster of 300 i.u. and males 200

i.u. The injections were administered subcutaneously in the dorsal lymph sacs. The toads were then randomly paired off and placed in the two fish tanks.

Different dosages of pregnyl were also compared. Higher dosages in females (up to 350 i.u. as primer) induced them to deposit eggs within an hour of the primer injections. It was found that with dosages as high as 300 i.u. (booster injection) in males, amplexus was induced, but throughout amplexus no sperm was ejaculated.

Collection of sperm from amplectant males in the tanks. Males started their mating calls from the water approximately one hour after the primer injections. The nuptial pads on the hands of the males could be discerned approximately five hours after the primer. Within an hour after the booster injections the toads went into amplexus. *Xenopus* has the primitive inguinal clasp and the toads could stay in amplexus for up to 30 hrs.

During the time of amplexus the toads were closely observed for changes in their movements. The females exhibit a jerky swimming movement when they are ready to deposit their eggs. The male bends his back so as to bring his cloaca nearer to that of the female. The distance from the cloaca of the male to that of the female normally ranges from 19.6 to 27.4 mm. When the female goes into her egg-laying swimming routine, the male brings his cloaca to within 5 mm of that of the female. The eggs were released by the female, passed along the abdomen of the male and induced the male to ejaculate. This ejaculate was collected with a Gilson pipetman pipette.

Collection of sperm from amplectant males in chamber or bag. We devised a chamber, which we called the Lewis chamber, to house an amplectant pair of toads. It was made of perspex with holes in the casing to provide air. The chamber was 30 cm deep, 30cm long and 20 cm wide with a double bottom. The top part of the bottom of the chamber sloped inwards into a funnel-like structure so that the sperm and fluids could run down to the true bottom from where the sperm were collected via a pipette. This was done to collect pure ejaculate without the water as collected in the fish tank. The Lewis chamber yielded poor results as the holes in the casing caused the chamber to have the same effects as a desiccator, dehydrating the toads.

Another method used to obtain pure ejaculate, was to put the amplectant pairs in sealed A4 size plastic bags. This method yielded better results than the Lewis chamber. The ejaculate obtained by this method, however, contained mucous from the toads. It is possible that the effect of the sealed bags cause stress in the animals.

Electro-ejaculation. Male toads were injected with 250 i.u. pregnyl 12 to 24 hrs before sperm collection. They were then anaesthetized with MS222 and the abdominal cavity exposed. A stimulating electrode was connected to a Harvard stimulator using multiple pulses (50 Hz at 6 to 8 Volts). The testes and Wolffian ducts were stimulated for approximately 10 seconds at a time. The best results, pertaining to amount of sperm aspirated, were obtained when the testes were stimulated. Spermatozoa were subsequently collected with a Gilson pipetman pipette from the cloaca.

All the spermatozoa sampled were suspended in a 10% Ham's F10 solution. This is a nutrient medium with an osmolality of approximately 300 mOsm/kg. Different dilutions of Ham's F10 were used to test the effect of osmotic concentrations on the motility of the spermatozoa (Wilson, 1994). It was found that the vigour of sperm motility and the percentage motile sperm decreased rapidly in suspensions with an osmolality higher than 50 mOsm/kg and no sperm motion could be detected in suspensions higher than 200 mOsm/kg. These results are in line with the findings of Inoda & Morisawa (1987), and Bernardini, Andrietti, Camatini & Cosson (1988), that effective sperm motion of these anurans is initiated by low osmolalities. Ham's F10, diluted in distilled water to 10%, had an osmolality of 30 mOsm/kg and this suspension was found to be ideal for studying anuran sperm motion (Wilson, 1994).

After spermatozoa were collected by electro-ejaculation, the testes were removed and spermatozoa aspirated from them by squashing them between microslides.

Assessment of the Motility Patterns

Five µl of sperm were suspended in 5 ml 10% Ham's F10 solution in a petri dish by placing the sperm in the centre of the solution in the petri dish. Sperm were allowed to swim out towards the periphery of the dish. This technique we called the swim out technique. One ml of suspension was then collected from the periphery of the petri dish and placed in a sperm chamber. The sperm chamber is made from perspex and is 8 cm in length and 1 cm thick. It has a cavity in the centre approximately 8 mm deep. The suspension in the cavity is then covered with a coverslip which can be screwed in position.

The sperm chamber was placed on an inverted Zeiss microscope (ICM 405) using the 16X phase contrast objective. Every 15 min for up to 5 hrs the swimming patterns of the spermatozoa were videotaped. At each videotaping session at least four different fields were taped. The swimming patterns of at least 75 spermatozoa of each specimen were videotaped at the different time intervals. Davis & Katz (1989) proposed that between 50 to 200 spermatozoa be used for computer-aided sperm analysis. Analysis of the sperm swimming patterns was done with the Sperm Motility Quantifier (SMQ) (Wirsam Scientific, Pty LTD).

The SMQ is a Computer Assisted Sperm Motility Analysis (CASMA) system, developed by Van der Horst (1992). Seventeen motility parameters as well as sperm density measurements can be assessed by this system in both the automated and manual modes. The videotaped swimming patterns of the spermatozoa were played back via a VCR through the SMQ card in a 486 computer, to a TV monitor.

TABLE 1. Interpretation of the sperm motility parameters (Van der Horst, 1992).

Parameter	Abbreviation	Explanation
Curvilinear velocity	VCL	The time average velocity of the sperm head on its actual (precise) path. Expressed in μm/s.
Straightline velocity	VSL	The time average velocity of the sperm head projected along the straight line between its first and final detected positions. Expressed in μm/s.
Average Path velocity	VAP	The time average velocity of the sperm head projected along its spatial average trajectory. Expressed in μm/s.
Linearity	LIN	Ratio of projected length to total length of the curvilinear trajectory. Expressed as %.
Dance	DNC	Space occupied by the sperm head path during 1 sec. Product of VCL and mnALH. Expressed in $\mu m^2/s$.
Percentage Motile Sperm	PM	Percent motility of sperm population. Represents all forms of motility.

Video images were captured at 3 Hz (Frameskip 4). This means that the image was captured every tenth of a second. Sperm motion was taped for 8 seconds at a time (As *Xenopus* sperm are slow-moving it was possible to keep individual sperm in focus over this time interval - about 12 spermatozoa were visible per field). Automatic evaluation of the motility parameters was then performed. Data on the motility patterns were stored as text files (.DXP and .PXP) and imported to a spreadsheet for further analysis.

OSMOLALITY AND PH MEASUREMENTS

Osmolality measurements were made on a Wescor 5500 Vapor Pressure Osmometer using Wescor sample discs type SS-033 and only required a sample of 7 to 8 il. The pH readings were done on a Beckman Zeromatic IV pH meter and on a pH M80 portable pH meter No. 64R53N46.

Measurements were done on the following suspensions to determine the osmolalities and pH of the micro-fertilization environment into which the spermatozoa are deposited: (1) eggs and mucous in the plastic bag; (2) fluid extracted from the cloaca of the female; (3) eggs and tank water; (4) tank water; (5) eggs and fluid in the Lewis chamber

RESULTS

The motility characteristics of the spermatozoa aspirated by the methods described were analysed and statistically compared. For the purposes of this study the following parameters were statistically compared: VCL; VSL; LIN; VAP; DNC; and PM (Abbreviations as described in Table 1). Table 2 and Figs. 1 and 2 detail the differences and similarities of the various motility parameters of the sperm aspirated via the different methods.

Fig. 1 indicates the high velocities of sperm from the amplectant males. It is clear from the graph that these values differ significantly from values obtained from testicular sperm. The velocities of the sperm aspirated via electro-ejaculation and sperm from the testes after electro-ejaculation, are more or less similar.

Multivariate analyses were performed using star symbol plot analyses (Fig. 2). This method analyses data by presenting them as rays in a star symbol. The lowest value of a particular parameter is calculated and expressed as a ray 10% the length of the highest value. All the data present in a single set of star symbols are

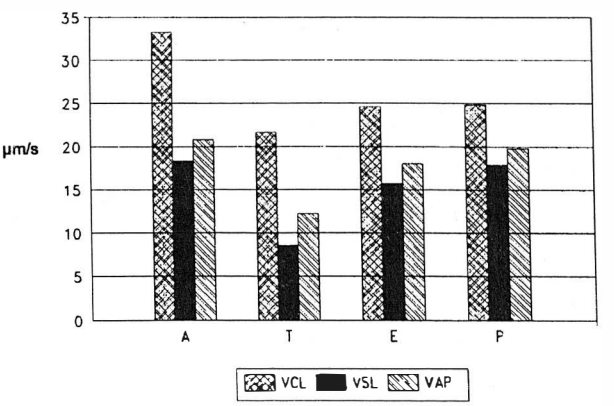

FIG. 1. Comparison of the velocities of the sperm aspirated by different methods.

TABLE 2. Data on the motility parameters of sperm aspirated by different methods. A, sperm obtained from amplectant males; T, testicular sperm (toads not subjected to pregnyl injections); E, sperm obtained via electro-ejaculation technique; P, sperm obtained from testes after pregnyl injections and electro-ejaculation. Values shown are means±SD.

	A	T	E	P
VCL (μm/s)	33.2±12.6	21.7±7.7	24.6±10.2	24.8±6.6
VSL (μm/s)	18.3±13.2	8.6±3.5	15.7±9.9	17.9±6.7
LIN (%)	49.7±21.3	41.3±14.3	60.6±19.4	70.7±16.5
VAP (μm/s)	20.8±12.7	12.2±3.7	18.0±9.8	19.8±6.1
DNC (μm²/s)	61.8±40.8	62.4±63.5	30.0±23.4	28.9±16.7
PM (%)	27.5±2.3	12.9±4.6	48.3±3.4	15.1±2.8

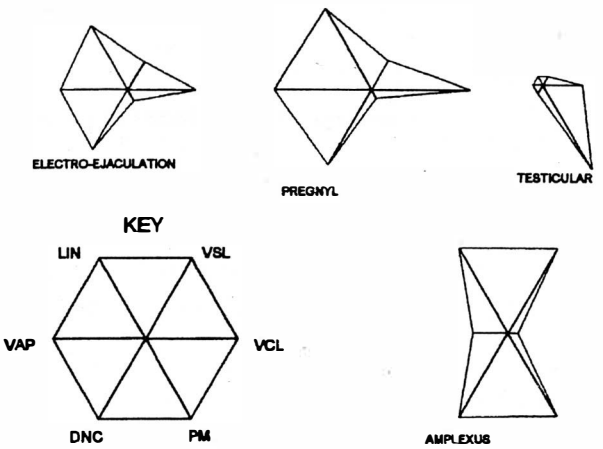

FIG. 2. Star symbol plot analysis of motion parameters of sperm aspirated by different methods.

therefore compared. These star symbols were used as a multivariate test to graphically compare patterns in sperm motility parameters. The star symbol plots indicate similar patterns of the electro-ejaculate and testicular sperm after hCG (pregnyl) injections. The star symbol plot reflecting the values for sperm from amplectant males displays the high values of these spermatozoa, whilst the plot for testicular sperm reflects their low overall values.

The osmolalities of the different solutions and pH values are recorded in Table 3.

DISCUSSION

It is problematic to obtain an ejaculate from submammalian vertebrates and invertebrates. Anuran spermatozoa are thought to mature in the testes (Poirier & Spink, 1971; Reed & Stanley, 1972) because of the lack of epididymides. To date, the reproductive biology of frogs and toads has primarily involved investigations on testicular sperm. This study was undertaken to determine whether viable sperm can be obtained by using different techniques to aspirate sperm via an ejaculate. Sperm motility parameters were used as criteria to ascertain the viability of ejaculate sperm.

Ham's F10 as medium to assess sperm motility was found to be ideal (Wilson, 1994). Bernardini, Andrietti *et al.* (1988) found the duration of *Xenopus* sperm motility to be less than 10 mins. We still found motile sperm in the 10% Ham's F10 suspension after 5 hrs of swim out (Wilson, 1994).

Xenopus has a very low percentage of motile testicular sperm. This is in accord with the findings of Van der Horst (1976). The percentage motile sperm aspirated from the testes after pregnyl injections and electro-ejaculation showed no significant difference with "normally aspirated" testicular sperm. The higher percentages of motile sperm in the electro-ejaculate and ejaculate from the amplectant males (Table 2) differed significantly from testicular sperm ($P< 0.01$). These differences may indicate that maturation changes take place in the reproductive ducts during ejaculation. In the testes, the spermatozoa are neatly packaged and embedded in Sertoli cells (Wilson, 1994). Maturation is not yet completed and this may possibly be one of the reasons for the low percentage of motile testicular spermatozoa. Another factor may be the low percentage of normal spermatozoa in the testis of the toad (Wilson, 1994).

TABLE 3. Data on the osmolalities and pH values of the suspensions (Mean±SD; range)

SUSPENSION	OSMOLALITY (mOsm)	pH
Tank water	0±1; 39 - 41	7.85±0.13; 7.62 - 7.96
Eggs and tank water	45±6; 41 - 63	7.15±0.06; 7.07 - 7.23
Fluid in plastic bag	112±6; 99 - 126	7.94±0.19; 7.72 - 8.21
Fluid in Lewis chamber	72±1; 71 - 73	7.10±0.08; 7.01 - 7.22
Female cloacal fluid	49±7; 38 - 56	7.94±0.07; 7.82 - 7.99

More significant, however, were the differences in the motility parameters. Analysis of these parameters indicated that the spermatozoa obtained from the amplectant pairs had significantly higher velocities (VCL, VSL and VAP) than sperm obtained from the testes ($P< 0.01$). Bernardini, Belgiojoso & Camatini (1988) concluded that the respiration rate of *Xenopus* spermatozoa is not affected by their status of motility. They used testicular spermatozoa and a comparative study of spermatozoa obtained from males in amplexus would be extremely valuable.

It is clear from Table 2 and Fig. 1 that the spermatozoa obtained from the amplectant males differed significantly from those obtained from the testes for most of the motion parameters. A high value of DNC may be an indication of the viability of the sperm to fertilize ($P<0.01$). Penetration of the jelly layers surrounding the eggs involves chemical and mechanical means. Spermatozoa normally exhibit a star-spin movement (lashing about of the sperm heads) in the vicinity of the eggs (Yanagimachi, 1988). This is a mechanical movement which helps with the penetration of the egg capsules. A high DNC value could be indicative of this hyperactivation. Normally aspirated testicular spermatozoa and spermatozoa from amplectant males exhibited the highest DNC values (Table 2). The high DNC value of the spermatozoa from the amplectant males could be explained in terms of the mechanisms necessary to penetrate the egg capsules.

Spermatozoa obtained via electro-ejaculation and from the testes of the electro-ejaculated toads displayed significantly higher LIN than those obtained via other means ($P< 0.01$). The stimulation of the testes may be instrumental in the sperm having a high LIN. It did not seem that the pregnyl injections played a role in the spermatozoon linearity as the amplectant males were given pregnyl injections as well. The linearity of testicular spermatozoa and spermatozoa aspirated from amplectant males differed significantly from those of the electro-ejaculated sperm and sperm from testes after pregnyl injections and electro-ejaculation.

It was clear that the motility parameters of the electro-ejaculate spermatozoa and spermatozoa from testes after pregnyl injections, exhibited more similarities. Their pattern of motion also showed similarities. This is portrayed in the star symbol plot (Fig. 2). This could be ascribed to the fact that the spermatozoa analysed were obtained from the same specimens. The small star symbol representing motility parameters of testicular sperm is an indication that these parameters were almost consistently lower than those of the others.

Inoda & Morisawa (1987) in comparing freshwater fishes and anurans in relation to the environment in which reproduction takes place, found that hypo-osmolality stimulated sperm motility in *Xenopus*. Bernardini, Andrietti *et al.* (1988) found anuran sperm motility to be inhibited at osmolarities higher than 200 mOsm/l. The results as reflected in Table 3 indicate the low osmolality of the aqueous environment in which the spermatozoa are released. Suspensions with a low osmolality, such as Ham's F10 (10%) and Toad Zwartkops solutions (Van der Horst, 1986) were found to be ideal solutions in which to initiate sperm motility.

A pH ranging from 7.1 to 7.9 is crucial for fertilization in anurans (Miceli, Fernández, Mansilla & Cabada, 1987; Bernardini, Andrietti *et al.*, 1988; Díaz Fontdevila, Bloj & Cabada, 1991). We found the pH values of the different media in which the spermatozoa were released, to fall within this range.

The results of our study indicated that motile sperm can be obtained from testes, electro-ejaculate, and from amplectant males. Differences in the various sperm motility parameters may reflect physiological differences between sperm from the testes and the ejaculate. It may be an indication that the Wolffian ducts and cloacal secretions play a part in the final maturation phases of the spermatozoa. The higher velocities of the sperm from amplectant males suggested that amplexus is a major stimulus in bringing the spermatozoa to maximum fertilizing capacity. Further analyses regarding the physiology of ejaculate sperm in the anurans are required in order to ascertain the functions of the reproductive tracts and cloacal secretions in the maturation of spermatozoa.

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DIVERSIFICATION IN NORTH-WEST AFRICAN WATER FROGS: MOLECULAR AND MORPHOLOGICAL EVIDENCE

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We have assessed the consistency of allozyme and morphometric data sets in discriminating water frogs at inter- and intraspecific level. Twenty allozyme loci and 14 morphometric characters were used in a study on Iberian and North African water frogs. The results from the morphometric analysis, using PCA, confirmed the interspecific differences between *Rana perezi* from the Iberian Peninsula and *Rana saharica* from North-west Africa previously detected by allozyme analysis. Allozyme and morphometric data were also consistent in discerning between Algerian and Moroccan populations of *R. saharica*, pointing to the presence of at least two subspecies in the Maghreb: *R. saharica saharica* from Algeria and *R. saharica riodeoroi* from Morocco. A possible paleobiogeographical scenario of the divergence between the two groups is discussed.

INTRODUCTION

The taxonomy of Palearctic water frogs has been disputed in recent years following the work by Berger (1968,1973,1977). This has yielded a new view of the classification of the group (Dubois & Ohler, 1994*a*) and in some cases created a debate over nomenclature (Dubois, 1991; Hotz, Uzzell, Beerli & Guex, 1996). The main reason for this complexity stems from the capacity of several species to hybridize. The reproductive mechanism used by some of these hybridizing species has largely added to the confusion. This mechanism, known as hybridogenesis, yields fertile hybrid progeny which are capable of hemiclonal reproduction after excluding one of the parental genomes (Schultz, 1969; Graf & Polls-Pellaz, 1989). However, it seems evident that these hybridogenetic hybrids only occur in certain areas of the water frog distribution, and they all carry the genome of *R. ridibunda* (Hotz, Mancino, Bucci-Innocenti, Ragghianti, Berger, & Uzzell, 1985), capable of inducing exclusion. The most widespread hybrid is *R. esculenta*, which arises from hybridization between the Mendelian species *R. lessonae* and *R. ridibunda*. The distributions of the other hybrids such as *R. grafi* or *R. hispanica* are constrained to smaller areas (Graf &Polls-Pellaz, 1989; Crochet, Dubois, Ohler & Tunner, 1995).

It is only recently that the taxonomic status of the remaining Mendelian water frog species is beginning to be understood, especially in the case of the Aegean (Beerli, Hotz, Tunner, Heppich & Uzzell, 1994) and Middle East water frogs (Schneider, Sinsch & Nevo, 1992). The Maghrebian region has posed problems after Hemmer, Konrad & Bachman (1980) indicated the presence of a hybrid complex in North Africa and Steinwarz & Schneider (1991) extended the range of *R. perezi* beyond the Iberian Peninsula into Morocco, Algeria and Tunisia. Both propositions seem unlikely in the light of the latest findings based on allozyme differentiation (Arano, Llorente, Herrero & Sanchíz, 1994; Beerli,1994; Buckley, Arano, Herrero, Llorente & Esteban, 1994), which confirm that hybridogenetic populations are not found beyond the north-east of the Iberian Peninsula and that the range of *R. perezi* does not extend to the north of Africa. On the contrary, the species present in the north of Africa would be *Rana saharica*. The differences between *Rana perezi* and *Rana saharica* are further supported by data on larval morphology (Llorente, Arano, Carretero, García-Paris, Herrero & Esteban, 1996).

However, *Rana saharica* is more diversified than originally thought. This is what the allozyme studies by Buckley *et al.* (1994) disclosed in a preliminary survey comparing Moroccan and Algerian water frog populations, suggesting that a different subspecies should be attributed to each country. Nevertheless, differentiation at a molecular level did not seem complemented by clearly discriminating morphological characters. Hence, the purpose of this paper is to make a comparative study at molecular and morphological levels, trying to assess the consistency of both approaches in discriminating at species (*R. perezi* and *R. saharica*) and subspecies level (Moroccan and Algerian *R. saharica*). A parallel aim of the study is to find consistent morphological characters which can be used to differentiate these taxa in the field.

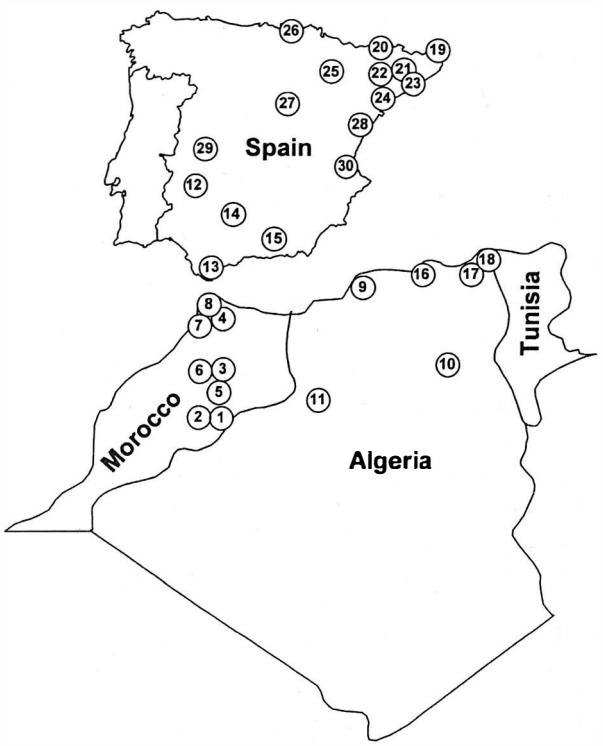

FIG. 1 Localities used in the allozyme and/or morphometric analyses. Allozyme and morphometric analysis: 1 Maadid, 2 Krair, 3 Annoceur, 4 Souk el Arba des beni Hassan, 5:N'Zala, 6 Dayed Aaoua, 7 Larache, 8 Zinat, 9 Chiffa River, 10 Tegernina, 11 Namous, 12 Mérida, 13 Tarifa, 14 Córdoba, 15 Nerja. Morphometric analysis only (sample size in brackets): 16 Djurjura (4), 17 El Tarf (4), 18 Tabarka (3), 19 Port Bou (5), 20 Pont de Suert (2), 21 Moianés (3), 22 Lleida (3), 23 Barcelona (6), 24 Tarragona (6), 25 Huesca (2), 26 Euskadi (2), 27 Soria (2), 28 Castellón (12), 29 Cáceres (6), 30 Valencia (6).(Although Tabarka lies in Tunisia, on the limit with the Algerian border, we refer to it as "Algerian" in the text to avoid semantic confusion.)

MATERIALS AND METHODS

Fig. 1 shows the localities used in both the electrophoretic and morphometric studies. In the case of the electrophoretical survey, samples of heart, liver, muscle and stomach were removed in the field, after animals were anaesthetised with MS222 (Sandoz), frozen and stored at -70°C. Tissues were later homogenized, and centrifuged, and the supernatant was used on standard horizontal starch gel electrophoresis (see Buckley *et al.*, 1994, for details on electrophoretic conditions). A total of 20 presumptive loci were examined: aspartate aminotransferase (AAT, EC 2.6.1.1.), alcohol-deshydrogenase (ADH, EC 1.1.1.1), adenylate-kinase (AK, EC 2.7.4.3), esterases (EST, EC 3.1.1.-.), glucosephosphate-isomerase (GPI, EC 5.3.1.9), glucose-6-phosphate-dehydrogenase (G6PD, 1.1.1.49), alpha-glycerophosphate-deshydrogenase (G3PDH, EC 1.1.1.8), isocitrate-deshydrogenase (IDH, EC 1.1.1.42), lactate-deshydrogenase (LDH, EC1.1.1.27), malate-deshydrogenase (MDH, EC 1.1.1.37), mannosephosphate-isomerase (MPI, EC 5.3.1.8), Peptidase-C (with leucine-alanine as substrate, PEP-C, EC 3.4.13.*), peptidase-D (with phenyl-proline as substrate, PEP-D, EC 3.4.13.9), phosphogluconate deshydrogenase (6PGDH, EC 1.1.1.44), phosphoglucomutase (PGM, EC 2.7.5.1) and superoxyde-dismutase (SOD, EC 1.15.1.1.). Allele frequencies were used in the computation of Nei's (1978) and Rogers' (1972) genetic distances by means of the BIOSYS-1 (Swofford & Selander, 1981) program. UPGMA and distance Wagner dendrograms were constructed using the distance coefficients.

Only adults were used in the morphological analysis. The measurements used were: LCC: body length (head-urostyle); MA: forelimb length; MP: hindlimb length; F: femur length; TM: length of metatarsal tuberculum; T: tibial-fibular length; P: foot length; ASE: head width; IO: interocular distance; DN: distance between nasal apertures; NO: eye-nasal aperture distance; O: eye diameter; OT: eye-tympanum distance; T: tympanum diameter.

A univariate analysis was carried out on the distribution of each variable. The presence of intersexual or interspecific differences was contrasted by means of a two-way ANCOVA (main factors: species and sex), using LCC as covariate. Logarithmic transformed data were used in the analyses. A canonical Principal Components Analysis (PCA) was used to study the variation and divergence within the morphometric characters. The variables used for this analysis were obtained from a previous factorial PCA. Using more representative variables, a discriminant function was obtained that was capable of differentiating between *R. perezi* and *R. saharica* and between the two *R. saharica* types. These functions were estimated using raw values in order to facilitate their use in the field. Morphometric relationships were corroborated by means of Mahalanobis' distances calculated between each group centroids.

RESULTS

The results of the allozyme analysis are shown in Table 1, where the allele frequencies for all the populations examined are given. Diagnostic loci for *R. perezi* and *R. saharica* were pointed out in Buckley *et al.* (1994). In the case of the north African species, Sod is a diagnostic locus for the Moroccan populations while Pep-D is diagnostic for those from Algeria (Table 1). These latter populations are also characterized by several exclusive alleles such as Est-1 a, G3pdh d, Idh-1 a, Ldh-A c, Ldh-B g and Mdh d and e. These differences explain the genetic distances found between Moroccan and Algerian populations (Buckley *et al.*, 1994 and Table 2). The Distance Wagner tree obtained using Rogers' genetic distances and *R. ridibunda* from Greece as an outgroup (Fig. 2a), shows two clearly distinct groups corresponding to *Rana perezi* and *Rana saharica*. However, within the latter grouping there are two distinct clusters corresponding to the Moroccan and Algerian populations. All distance/clustering method combinations yielded equal results, with only slight differences in the resolution of the internal branches of the Moroccan group.

TABLE 1. Allele frequencies across populations. Locality numbers correspond to those in Fig. 1. (N)= number of individuals

	POPULATIONS														
	Morocco									Algeria		Spain			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
LOCI															
AAT															
(N)	19	45	6	10	17	8	29	9	2	4	6	5	5	9	3
A	-	-	-	-	.029	-	-	-	-	-	-	-	-	-	-
B	1.000	1.000	1.000	1.000	.971	1.000	1.000	1.000	1.000	1.000	1.000	.700	.600	.667	.667
C	-	-	-	-	-	-	-	-	-	-	-	.300	.400	.333	.333
ADH-1															
(N)	13	40	10	7	13	8	9	9	2	4	6	5	6	8	5
A	.038	.038	-	.286	-	.062	-	-	-	-	-	.200	-	-	-
B	.962	.962	1.000	.714	1.000	.938	1.000	1.000	1.000	1.000	1.000	.800	1.000	1.000	1.000
ADH-2															
(N)	10	38	10	2	10	8	9	7	2	1	1	4	6	7	5
A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	-	.132	-	-	.200	-	-	.143	-	-	-	1.000	1.000	1.000	1.000
C	1.000	.868	1.000	1.000	.800	1.000	1.000	.857	1.000	1.000	1.000	-	-	-	-
AK															
(N)	29	5	5	9	7	3	16	3	1	4	5	3	5	7	1
A	.018	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	.948	1.000	1.000	1.000	1.000	1.000	.906	1.000	1.000	1.000	.900	1.000	.800	1.000	1.000
C	-	-	-	-	-	-	.063	-	-	-	.100	-	.200	-	-
D	.034	-	-	-	-	-	.031	-	-	-	-	-	-	-	-
EST-1															
(N)	16	17	3	8	11	1	3	2	2	4	6	4	3	4	3
A	-	-	-	-	-	-	-	-	-	.500	-	-	-	-	-
B	.781	.882	.833	.250	.591	-	.667	1.000	.250	.500	.250	-	-	-	-
C	-	-	-	-	.045	-	-	-	.500	-	-	.750	1.000	.750	.667
D	.219	.118	.167	.750	.364	1.000	.333	-	.250	-	.750	.250	-	.250	.333
EST-2															
(N)	17	13	15	10	4	7	22	8	2	4	5	5	6	6	5
B	.853	.346	.200	.450	.375	.357	.909	1.000	-	.500	-	.200	.750	.417	.800
C	.147	.615	.767	.550	.625	.643	.091	-	1.000	.500	1.000	.500	.250	.333	.200
D	-	.038	.033	-	-	-	-	-	-	-	-	.300	-	-	-
E	-	-	-	-	-	-	-	-	-	-	-	-	-	.250	-
G6PD															
(N)	19	5	5	7	9	7	4	3	2	1	1	5	6	5	1
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
G3PDH															
(N)	22	45	11	9	17	8	25	9	2	4	6	5	6	9	5
B	-	-	.273	-	-	-	.020	-	-	-	-	-	-	-	-
C	1.000	1.000	.727	1.000	1.000	1.000	.980	1.000	1.000	.875	1.000	1.000	1.000	1.000	1.000
D	-	-	-	-	-	-	-	-	-	.125	-	-	-	-	-
GPI															
(N)	23	37	18	10	17	7	18	9	2	3	5	5	6	9	5
A	-	-	-	-	-	-	.028	-	-	-	-	-	-	-	-
B	.804	.986	1.000	1.000	1.000	1.000	.972	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
C	.196	.014	-	-	-	-	-	-	-	-	-	-	-	-	-
IDH-1															
(N)	22	36	17	10	17	8	27	9	2	4	6	5	6	9	5
A	-	-	-	-	-	-	-	-	.500	-	.583	-	-	-	-
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.500	1.000	.417	1.000	1.000	1.000	1.000
IDH-2															
(N)	22	41	16	10	17	8	29	9	2	4	5	5	5	9	5
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.900	.900	1.000	.700
B	-	-	-	-	-	-	-	-	-	-	-	.100	.100	-	.300
LDH-A															
(N)	21	29	13	7	6	8	24	6	2	4	6	5	5	8	5
A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	.119	.155	-	-	-	-	-	-	-	-	-	1.000	1.000	1.000	1.000
C	-	-	-	-	-	-	-	-	.500	-	-	-	-	-	-
D	.881	.845	1.000	1.000	1.000	1.000	1.000	1.000	.500	1.000	1.000	-	-	-	-
LDH-B															
(N)	37	45	18	10	19	8	29	8	2	4	6	5	6	9	5
A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E	.081	.200	-	-	-	-	-	-	-	-	-	1.000	1.000	1.000	1.000
G	-	-	-	-	-	-	-	-	.500	-	-	-	-	-	-
H	.919	.800	1.000	1.000	1.000	1.000	1.000	1.000	.500	1.000	1.000	-	-	-	-
MDH															
(N)	27	44	18	10	19	8	29	9	2	4	6	5	6	9	5
A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.100
B	.056	.023	-	-	.105	-	-	-	.500	-	.167	-	-	-	-
C	.944	.977	1.000	1.000	.895	1.000	1.000	1.000	.500	.625	.583	1.000	1.000	1.000	.900
D	-	-	-	-	-	-	-	-	-	.375	-	-	-	-	-
E	-	-	-	-	-	-	-	-	-	-	.250	-	-	-	-
MPI															
(N	21	44	15	9	17	7	29	9	2	4	6	5	5	9	5
A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	-	-	-	-	-	-	-	-	-	1.000	.417	.900	.800	.611	1.000
C	-	.011	-	-	-	-	-	-	-	-	-	.100	.200	.389	-
D	.048	.045	.067	-	.118	-	.086	-	.750	-	.500	-	-	-	-
E	.952	.943	.933	1.000	.882	1.000	.914	1.000	.250	-	.083	-	-	-	-
PEP-C															
(N)	14	27	5	8	8	4	17	3	1	3	2	2	5	7	5
A	.786	.833	1.000	1.000	.562	1.000	.941	1.000	1.000	-	.250	.250	.800	.429	.800
B	-	.130	-	-	.063	-	.059	-	-	1.000	.750	-	.200	.571	.200
C	.214	.037	-	-	.375	-	-	-	-	-	-	.750	-	-	-
PEP-D															
(N)	13	22	11	4	9	2	8	9	2	4	3	1	6	2	3
A	-	-	-	-	-	-	-	-	1.000	1.000	1.000	-	-	-	-
C	1.000	1.000	1.000	1.000	.944	1.000	1.000	1.000	-	-	-	1.000	1.000	1.000	1.000
D	-	-	-	-	.056	-	-	-	-	-	-	-	-	-	-
6PGDH															
(N)	17	38	15	10	19	8	23	9	2	4	5	3	5	6	4
A	-	-	-	-	-	-	-	-	-	-	-	.500	.500	.500	.500
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	-	-	-
C	-	-	-	-	-	-	-	-	-	-	-	.500	.500	.500	.500
PGM															
(N)	26	41	18	10	17	8	27	5	2	4	6	5	6	9	5
A	-	.146	.167	-	.147	-	.019	.100	-	-	.083	-	-	-	-
B	1.000	.854	.833	1.000	.853	1.000	.981	.900	1.000	1.000	.917	1.000	1.000	1.000	1.000
SOD-1															
(N)	20	46	13	10	17	6	12	4	2	4	6	5	6	9	5
A	-	-	-	-	-	-	-	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	-	-	-	-	-	-

TABLE 2. Mean genetic distances between groups of populations: Nei: Nei's (1972) genetic distance; Nei*: Nei's (1978) unbiased genetic distance; Rogers: Rogers' modified genetic distance; Cavalli: Cavalli-Sforza & Edwards arc distance; Cavalli*: Cavalli-Sforza & Edwards chord distance.

	NEI	NEI*	ROGERS	CAVALLI	CAVALLI*
Morocco	0.03	0.026	0.161	0.177	0.173
Algeria	0.131	0.100	0.190	0.352	0.335
Spain	0.034	0.015	0.167	0.183	0.183
Morocco-Algeria	0.231	0.224	0.425	0.432	0.403
Morocco-Spain	0.453	0.441	0.570	0.592	0.541
Algeria-Spain	0.453	0.437	0.553	0.586	0.539

TABLE 3. Results of factorial Principal Components Analysis.

	Factor 1	Factor 2	Factor 3
LOGLCC	0.96653	-0.172026	-0.011013
LOGMA	0.94673	-0.154758	-0.129836
LOGMP	0.95507	-0.208568	-0.122346
LOGF	0.97469	-0.041224	-0.054902
LOGT	0.95505	-0.219451	-0.083720
LOGP	0.94251	-0.177952	-0.142242
LOGASE	0.96283	-0.052926	-0.017892
LOGIO	0.86543	0.405423	0.064863
LOGDN	0.82380	0.289684	0.071100
LOGNO	0.86353	0.239011	0.246404
LOGO	0.84143	0.360570	-0.219881
LOGOT	0.79587	-0.236919	0.506620
LOGTI	0.91366	0.076275	-0.011488
Expl. vara.	10.76965	0.678739	0.437644
Prp. total	0.82843	0.052211	0.033665

TABLE 4. Standardized coefficients for canonical variables.

Variable	Root 1	Root 2
Factor 1	0.133	0.238
Factor 2	1.105	0.022
Factor 3	0.317	-0.122
Factor 4	0.261	0.410
Factor 5	-0.007	-0.301
Factor 6	0.240	-0.150
Factor 7	0.056	0.329
Factor 8	0.138	-0.584
Factor 9	-0.062	0.421
Factor 10	0.113	-0.369
Factor 11	-0.053	-0.462
Factor 12	0.327	-0.129
Factor 13	0.059	-0.124
Eigenvalue	4.128	0.430
Cum. prop.	0.906	1.000

TABLE 5. Discriminant prediction at interspecific level. %: Percentage of correctly classified individuals.

Species	%	*R. perezi*	*R. saharica*
Rana perezi	100.0	58	0
Rana saharica	94.7	2	36
Total	97.92	60	36

TABLE 6. Discriminant prediction at intraspecific level. %: Percentage of correctly classified individuals. *Rs*M: *Rana saharica* from Morocco; *Rs*A: *Rana saharica* from Algeria.

Species	%	*Rs*M	*Rs*A
*Rs*M	92.6	25	2
*Rs*A	81.8	2	9
Total	89.47	27	11

Due to the low number of individuals per locality, especially in the case of the Algerian sample, populations were pooled into three groups, Iberian Peninsula, Morocco and Algeria, for the morphometric study. This pooling was also supported by the results of the allozyme analysis. Previous to the populational morphometric analysis, an ANOVA showed no differences in the LCC between sexes of each species. Likewise, a two-way MANCOVA (sex-species), carried out for *Rana perezi* and *R. saharica*, did not show significant differences between sexes (Wilks λ =0.863, P=0.255) nor for the sex-species interaction (Wilks λ = 0.873, P=0.317), although it did show significant differences between species (Wilks λ =0.265, P<0.001). These differences were significant at P<0.05 for variables F, ASE, DN, NO, O and TI. The same type of analysis for *R. perezi* and the two *R. saharica* varieties showed similar results (Wilks λ =0.895, P=0.515; Wilks λ =0.772, P=0.364; Wilks λ =0.218 P<0.001), differences being significant at P<0.05 for variables MP, F, T, P, ASE, IO, DN, NO, O and TI. Since intersexual differences were not significant, the sexes were pooled for the remaining analyses.

Using the first three factors, factorial PCA explains 91.43% of the variance (Table 3). The factors extracted from this analysis have been used in a canonical PCA on the 13 morphological variables considered. The first factor was not eliminated, since the ANOVA using LCC of the three OTUs did not show significant differences ($F_{2,90}$=2.096, P=0.129). Results show that each group (*R. perezi* -*Rp*-, Moroccan *R.saharica* -*Rs*M- and Algerian *R.saharica* -*Rs*A-) is significantly different from the others, as indicated by the F statistics associated with Mahalanobis' distances (*Rp*-*Rs*M 21.04, P<0.0001; *Rp*-*Rs*A 9.91, P<0,0001; *Rs*M-*Rs*A, 2.57 P=0.005). Subsequent classifications using this same

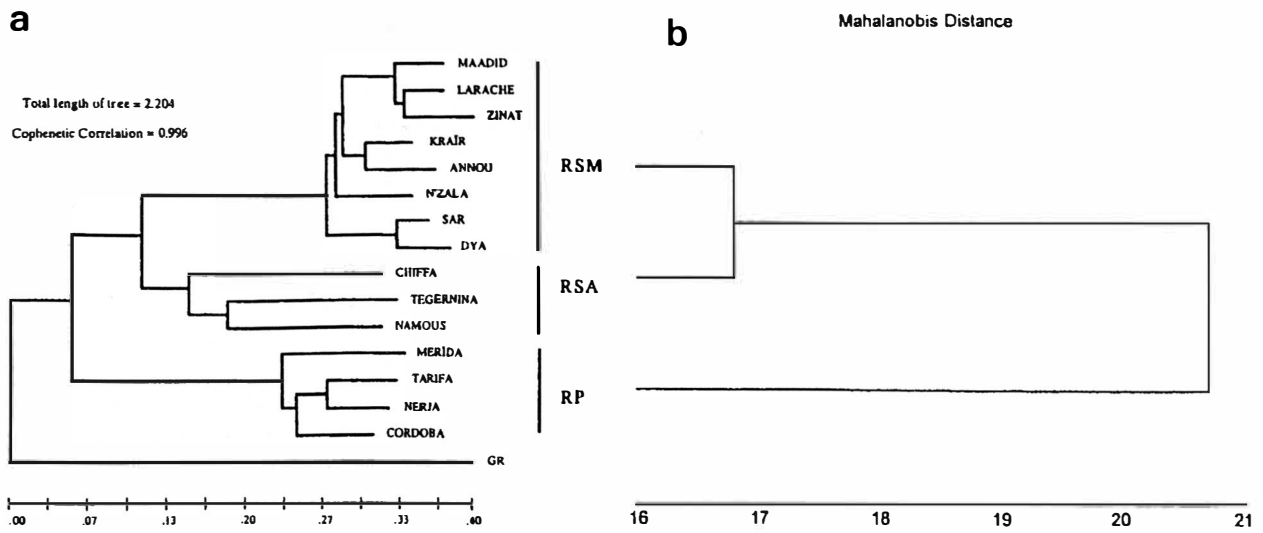

FIG. 2. (a) Wagner Distance tree built using modified Rogers' genetic distances. Outgroup corresponds to Greece (GR); (b) UPGMA cluster using Mahalanobis' distance.

TABLE 7. Discriminant function coefficients for the inter- and intraspecific analyses. ASE, head width; IO, interocular distance; LCC, body length (head-urostyle); O, eye diameter; P, foot length.

Variables	*R. perezi*	*R. saharica*	*Rs*M	*Rs*A
ASE	-	-	-1.205	-2.272
IO	2.662	7.661	-	-
LCC	-0.045	-0.942	1.055	0.307
O	3.436	7.050	-	-
P	-	-	0.783	2.144
Coefficient	-19.012	-31.375	-34.833	-32.125

distance were correct in 91.39% of the cases (79.17-98.28%). Errors were mainly at the intraspecific level (*Rs*M vs. *Rs*A). The two canonical axes explain a variance of 90.56% and of 9.44% respectively with values of 4.13 and 0.43 (Table 4). Fig. 3 shows the graphic representation of the canonical analysis with a 95% confidence interval. Standardized coefficients for both canonical variables are shown in Table 4. Root 1 of the canonical analysis clearly separates the two species *R. perezi* and *R. saharica*, while root 2 separates the Moroccan and Algerian populations. The characters involved in root 1 are components for shape of the head. Hence, both *R. perezi* and *R. saharica*, would differ in IO, O and DN, larger in *R. saharica* than in *R. perezi*. The axis (root 2) separates the two groups of *R. saharica* with less resolution. The characters involved, DN, MA, ASE, T and P, are larger in Morocco than in the Algerian group of *R. saharica*. The UPGMA cluster for the three groups RP, *Rs*M and *Rs*A using Mahalanobis' distances (Fig. 2b) is consistent with that obtained using Rogers' genetic distances (Fig. 2a).

A discriminant analysis among species using non-transformed variables yields a function with variables

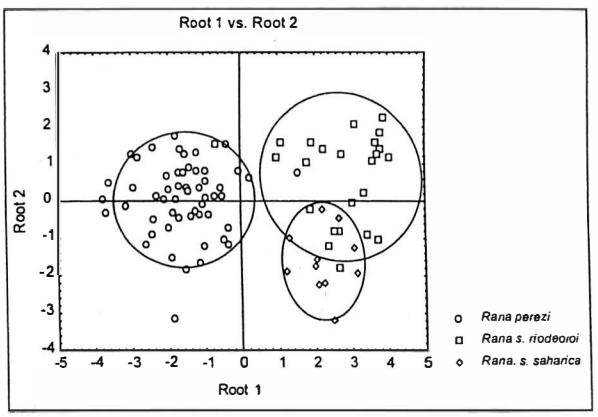

FIG. 3. Graphic representation of the canonical PCA for the three groups considering the two roots. Circles indicate the confidence intervals at 95% from each group centroid.

LCC, IO and O, which classifies 97.92% of the cases correctly (Table 5). A second function was obtained for the North-west African populations using variables LCC, P and ASE with 89.47% correct classifications (Table 6). Coefficients for both discriminant functions are shown in Table 7.

The length of the metatarsal tuberculum (TM) has been used traditionally as a discriminant character for Central European water frog species. However, in our study this character shared neither inter- nor intraspecific differences (ANCOVAinter: F=1.688, P=0.195; ANCOVAintra: F=0.7171, P=0.400). For this reason, it was excluded from subsequent analyses.

DISCUSSION

Buckley *et al.* (1994) proposed that new analyses were necessary to identify all the different forms of north African water frogs. As we previously pointed out, recent studies in the Middle East (Schneider *et al.*,

1992) and the Aegean islands (Beerli *et al.*, 1994) have revealed new species where only *R. ridibunda* was thought to be present. These findings were based on non-morphological characters. A combination of several character sets is needed to detect hidden differentiation in this group, since morphology alone is not conclusive enough to distinguish among different water frog taxa.

Despite claims that molecular and morphological data can be in conflict, many systematists are currently understanding the value of multidisciplinary studies (Larson, 1989). Some data sets are useful to unravel the phylogenetic relationships among closely related species, whereas others are more suitable when dealing with species in a larger temporal scale.

Our results offer two kinds of data which are not in conflict since clear genetic differentiation is congruent with morphological differences. Allozyme data suggest the taxonomic separation of Algerian and Moroccan water frogs at subspecific level. This view is supported by clear differences in loci Sod and Pep-D, as well as by the genetic distances obtained (DN*=0.224). According to Avise & Aquadro (1982), distance values of 0.2-0.5 can be found at both intra- and interspecific level.

When multivariate techniques such as PCA are used with size and shape components, morphological data confirm the taxonomic differentiation between *R. perezi* and *R. saharica*. Likewise, these analyses clearly discriminate between the Algerian and Moroccan water frogs. Based on both data sets, we propose a subspecific status for each *R. saharica* group.

We searched into the nomenclature history of North African water frogs in order to avoid further confusion when assigning names to the new subspecies. In 1913 Boulenger (Hartert, 1913) described *R. esculenta* var. *saharica* from the Saharan oases in the South of Algeria. More recently, Salvador & Peris (1975) described *R. ridibunda riodeoroi*, its type locality being Rio de Oro (western Sahara). In the latest revisions of the group, Dubois & Ohler (1994*a*; 1994*b*) and Salvador (1996) did not consider *R. ridibunda riodeoroi* a valid subspecies but as a synonym of *R. saharica*. This implies that *R. saharica* is considered by these authors as the only valid name for the North African water frogs. Despite this, the differences found between Algerian and Moroccan populations indicate that they should be considered distinct subspecies and be named as such. Hence, their formal nomenclatural denomination should be *Rana saharica saharica* for the Algerian populations and *Rana saharica riodeoroi* for the Moroccan ones. However, these denominations should be used cautiously until similar studies are carried out on the type localities. According to Bons & Geniez (1996), southern Moroccan populations would be different from Northern ones, and possibly more similar to the Algerian *Rana*. It is therefore possible that more differentiation is still to be detected within *R. saharica*.

A widely accepted hypothesis is that the separation between *R. perezi* and *R. saharica* can be related to the separation of the Iberian Peninsula from the north of Africa due to the Strait of Gibraltar opening (Busack, 1986). The south of the Iberian Peninsula and the north of Africa were part of the Betic-Riffian plate, which was separated from both continents. During the Messinian several factors led to shifting of the three plates (African, Iberian and Betic-Riffian) together, the sea connections became interrupted and the subsequent salinity crisis caused the Mediterranean desiccation (Hsü *et al.*, 1977). The collapse of the Betic-Riffian arch connected both continents, and allowed the contact between their respective faunas. The later and final opening of the Gibraltar Strait during the Early Pliocene contributed to interrupted gene flow between the two water frog groups leading to a speciation process, where the ancestral groups for *R. perezi* and *R. saharica* would have inhabited the Iberian Peninsula and the north of Africa respectively.

Presumably, the two taxa presently found in Morocco and Algeria would have evolved from the *R. saharica* ancestral pool. According to our data (Buckley, Arano, Herrero & Llorente, 1996) this would have taken place approximately 2 my ago in the period between the upper Pliocene and the Pleistocene. Again the separation can be related to palaeogeographical events, although the case is not as strong as with the Gibraltar Strait opening. According to Weijermars (1988), the coast lines of both the Iberian Peninsula and the North of Africa did not acquire their present configuration until the beginning of the Pleistocene (2 my). Until then, the sea continued to flood land masses which had emerged during the collision of the three plates (Fig. 4). The former Betic and Riffian channels, that had connected the Atlantic and the Mediterranean before the collapse of the Gibraltar arch, were partially flooded, contributing to the isolation of the Betic and Riffian blocks from both continents. In the case of the Riffian block, the connection between the Atlantic and the Mediterranean was never re-established.

The fact that the Riffian block was isolated to a certain extent from the African continent through the

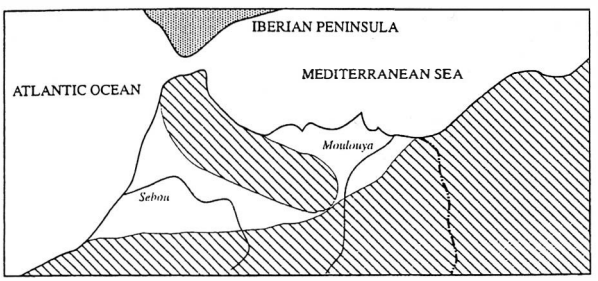

FIG. 4. Map of the approximate coastline of the northwestern corner of Africa during the Plio-Pleistocene boundary (approx. 2 my ago) (following Weijermars, 1988; Benson *et al.*, 1992). Diagonal shading represents emerged land. The discontinuous line represents the current Algerian-Moroccan border.

marine transgression could be related to the isolation of both taxa. This transgression would have lasted long enough to contribute to the differentiation of the Riffian population into *R. saharica riodeoroi* and the continental one into *R. saharica saharica*. Once the continents acquired their present topography during the Pleistocene, each taxon would have dispersed towards the south, reaching their present distribution. Studies on population structure of *R. saharica riodeoroi* (Buckley *et al.*, 1996) suggest that the expansions could have followed a pattern of extinction and recolonization cycles, linked to climatic conditions.

Although the distribution limits of both taxa are still to be established, we are inclined to consider the River Moulouya basin as a cause of discontinuity between them. This river appears to be a natural barrier preventing gene flow among many other species of amphibians and reptiles from North Africa (Lanza, Nascetti, Capula & Bullini, 1986; Mateo, 1990). More recently, Steinfarz, Joger & Barrio (in prep.) have found further evidence of gene flow interruption in two groups of urodeles: *Pleurodeles waltl* (Morocco)/ *P. poireti* (Algeria/Tunisia) and between the western and eastern subspecies of *Salamandra algira*. Although it seems unlikely that a river could act as a barrier for amphibians, it is necessary to bear in mind that the courses of North African rivers would have become established after the Plio-Pleistocene, following previous sea introgressions (Doadrio, 1994). In the case of the Moulouya river its course corresponds to the Betic-Riffian channel and the Pliocene marine transgression zones. Interestingly, the area between the Moulouya valley and the Algerian border, represents the most arid coastal strip in Mediterranean Northwest Africa, and has been named the "Moulouya steppe" by Bons (1960). Hence, the present river can be considered as the reflection of a previous palaeogeographical barrier that is actively interrupting gene flow between amphibian populations and contributing to the morphological and genetic differentiation processes observed.

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7. Line drawings and photographs are numbered in sequence in arabic numerals, e.g. FIG. 1. Colour photographs can only be included at cost to the author. If an illustration has more than one part each should be identified as (a), (b), etc. The orientation and name of the first author should be indicated on the back. They should be supplied camera-ready for uniform reduction of one-half on A4 size paper. Line drawings should be drawn and fully labelled in Indian ink, dry-print lettering or laser printed. Illustrations produced using other types of computer printer are not usually of suitable quality. A metric scale must be inserted in micrographs etc. Legends for illustrations should be typed on a separate sheet.

8. References in the text should be given as in the following examples: "Smith (1964) stated —"; "—as observed by Smith & Jones (1963)." "—as previously observed (Smith, 1963; Jones, 1964; Smith & Jones, 1965)". For three or more authors, the complete reference should be given at the first mention, e.g. (Smith, Jones & Brown, 1972), and *et al.* used thereafter (Smith *et al.*, 1972). For the list of references the full title or standard abbreviations of the journal should be given. The following examples will serve to illustrate the style and presentation used by the Journal.

Bellairs, A. d'A. (1957). *Reptiles*. London: Hutchinson.

Boycott, B. B. & Robins, M. W. (1961). The care of young red-eared terrapins (*Pseudemys scripta elegans*) in the laboratory. *British Journal of Herpetology* **2**, 206–210.

Dunson, W. A. (1969*a*). Reptilian salt glands. In *Exocrine glands*, 83–101. Botelho, S. Y., Brooks, F. P. and Shelley, W. B. (Eds). Philadelphia: University of Pennsylvania Press.

Dunson, W. A. (1969*b*). Electrolyte excretion by the salt gland of the Galapagos marine iguana. *American J. Physiol.* **216**, 995–1002.

9. Final acceptance of a paper will depend upon the production by the author of a typescript, illustrations and computer diskette ready for the press. However, every assistance will be given to amateur herpetologists to prepare papers for publication.

10. Proofs should be returned to the Editor by return of post. Alterations should be kept to the correction of errors; more extensive alterations will be charged to the author.

11. Twenty-five offprints and one complimentary copy of the Journal are provided free of charge. Further copies (minimum of twenty-five) may be purchased provided that they are ordered at the time the proofs are returned.

12. All submissions are liable to assessment by the editorial board for ethical considerations, and publication may be refused on the recommendation of this committee. Contributors may therefore need to justify killing or the use of other animal procedures, if these have been involved in the execution of the work. Likewise, work that has involved the collection of endangered species or disturbance to their habitat(s) will require full justification.

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