SALAMANDRA	45	3	129-146	Rheinbach, 20 August 2009	ISSN 0036-3375

Diversity of Ridged Frogs (Ptychadenidae: *Ptychadena*) in the easternmost remnant of the Guineo-Congolian rain forest: an analysis using morphology, bioacoustics and molecular genetics

Beryl A. Bwong, Robert Chira, Susanne Schick, Michael Veith & Stefan Lötters

Abstract. We studied taxonomic diversity of Ridged Frogs, genus *Ptychadena*, in the Kakamega Forest, western Kenya. It is the easternmost relict of a former continuous Guineo-Congolian rain forest belt which continuously existed in the past. Based on morphometrical and non-morphometrical morphology adult characters and using information obtained from male advertisement calls as well as sequences of the mitochondrial 16S rRNA gene, we distinguish five syntopic species. Standardized diagnoses with 35 characters are provided for each. In the absence of a taxonomic revision of the genus *Ptychadena* and because we did not use comparative material from elsewhere, we only provisionally allocate species names to the taxa identified by us and discuss available names on the basis of comparisons with bioacoustic and molecular data from other regions. The power of characters defining *Ptychadena* species and the value of different methodical approaches are discussed, especially important for rapid and easy field identification for conservation purposes. Furthermore, we provide evidence that *Ptychadena* has independently colonised the Kakamega Forest from or via the Congo basin, as well as East and South Africa.

Key words. Amphibia, Anura, Ptychadenidae, advertisement calls, DNA barcoding, Kakamega Forest, Kenya, syntopy.

Introduction

The Kakamega Forest in western Kenya (Fig. 1) is believed to be the easternmost remnant of a former continuous forest belt stretching from West to East Africa during cooler past periods (Kokwaro 1988, Wagner et al. 2008). Situated in Western Province (00°10' to 00°21' N; 34°47' to 34°58' E, ca. 1520-1680 m above sea level), roughly 200 km northwest of Nairobi, the Kakamega Forest belongs to the Lake Victoria water catchments. Vegetation is composed of lowland rainforest and Afroalpine species. Climate is tropical humid with pronounced rain fall in April/May and August/September (Mutangah et al. 1992).

The Kakamega Forest is considered a refuge for many plant and animal species. This is relevant to conservation aspects, since the forest's size constantly shrinks due to human impact making it a conservation priority area (Fishpool & Evans 2001). We have focused on the species diversity of Ridged Frogs, genus *Ptychadena* Boulenger, 1917 (Ptychadenidae), in this forest. These anurans are common across sub-Saharan Africa, Madagascar and some smaller Oceanic islands. Currently, 49 species are recognized (Frost, 2008), which often occur syntopically (e.g. Rödel 2000, Channing & Howell 2006).

There have been several efforts towards an understanding of *Ptychadena* systematics (e.g. Guibé & Lamotte 1957, 1958, 1960, Lamotte 1967, Perret 1979, 1981, 1987, 1994, 1996, Amiet 1989, Poynton & Broadley 1985, Largen 1997, 2000). However, despite these, the genus cannot be considered well understood, as the majority of the suggested species remains improperly defined. Standardized diagnostic schemes have been applied by a few previous workers and for a limited numbers of species only (e.g. Guibé

& Lamotte 1957, Perret 1979, Poynton & Broadley 1985). As a result, several of the suggested taxa are difficult to distinguish and their geographic ranges remain unknown. Moreover, based on such a weak basis, it is difficult to follow the placement of certain nominal species into the synonymy of others. Especially since a molecular study by Vences et al. (2004) uncovered the existence of species complexes within Ridged Frogs it has become clear that additional efforts are needed towards a better understanding of *Ptychadena* systematics.

We are optimistic that the existing gaps can be filled. Not only because we have just entered the new era of DNA barcoding (e.g. Vences et al. 2004, 2005) but also because *Ptychadena* species display various external features which should allow for proper diagnoses when applied in a standardized way. In addition, due to the common syntopic occurrence of many Ridged Frog species, male advertisement calls are a useful tool to distinguishing them (e.g. Passmore 1977, Rödel 2000, Channing & Howell 2006).

Analyzing data from all three methods, we conclude that five *Ptychadena* species syntopically occur in the Kakamega Forest (Fig. 2). It is our purpose to here provide standardized diagnostic schemes for these and, in accordance with previous authors listed above, to identify characters clearly providing evidence for specific distinctness within the genus *Ptychadena.* In expectance of comprehensive revisionary action and because we have neither included specimens from elsewhere nor type material, we only provisionally apply names here. Apart from this, a molecular analysis allowed permitted preliminary conclusions about the possible colonisation of the Kakamega Forest by *Ptychadena* species.

Materials and methods

A total of 130 adult *Ptychadena* specimens from the Kakamega Forest (from Buyangu Hill area and Isecheno, Fig. 1), deposited at

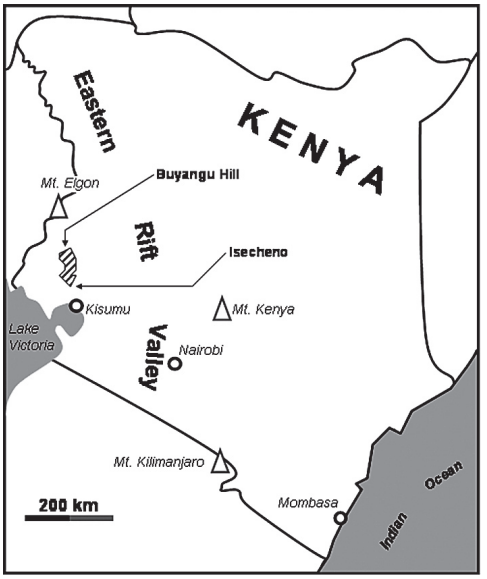


Fig. 1. Location of the Kakamega Forest (shaded) with the Buyangu Hill and Isecheno.

National Museums of Kenya (NMK), Nairobi, were used for morphometrical and morphological studies (Appendix 1). Sex was determined through presence (as in the 93 males) versus absence of lateral vocal openings. Additional specimens used for bioacoustic and molecular studies are deposited at NMK and ZFMK (Zoologisches Forschungsmuseum Alexander Koenig, Bonn). Collection numbers for these additional specimens are mentioned throughout the text.

Following Guibé & Lamotte (1957, 1958, 1960), Lamotte (1967), Perret (1979, 1981, 1987, 1994, 1996), Amiet (1989), Largen (1997, 2000), Poynton & Broadley (1985), 10 morphometrical and 25 non-morphometrical morphology characters of adults were identified to be useful in defining and diagnosing *Ptychadena* taxa. Using dial callipers, the 10 body measurements were taken to the nearest 0.1 mm: (1) snout-vent length (SVL); (2) foot length from the proximal edge of the heel to the tip of Toe IV (FL); (3) tibia length (TL); (4) head width at broadest (HW); (5) head length from angle of jaw to tip of snout

(HL); (6) distance from tip of snout to anterior corner of nostril (SN); (7) distance from anterior corner of eye to posterior nostril (EN); (8) horizontal tympanum diameter (TD); (9) horizontal eye diameter (ED); (10) inter-narial distance (ID). Fifteen non-morphometric morphology characters were coded as present or absent: (11) row of tubercles on metatarsus; (12) ridges on legs; (13) ridges on lateral sides of body; (14) warts on legs; (15) warts on lateral sides; (16) light tibial line; (17) light bands on posterior face of femur; (18) dark bands on posterior face of femur; (19) dark mottling on posterior face of thigh; (20) pale triangle on dorsal snout; (21) whitish spots on lower lip; (22) whitish ring (at least in part) around tympanum; (23) green or light brown median dorsal band; (24) outermost dorsal ridge coloured whitish; (25) outer metatarsal tubercle. Four non-morphometrical morphology characters were coded as follows: if dark bands on femurs present, are they continuous or discontinuous from knee to knee (in species diagnoses below added to (18) if applicable); if outer metatarsal tubercle present, is it smaller or larger than inner (in species diagnoses below added to (25) if applicable); (26) canthus rostralis from eye to nostril concave versus straight; (27) nostrils visible versus invisible from above. In addition, we studied (28) whether the external vocal openings in males are situated above, at level of or below arm insertion (i.e. conditions A-C of Perret 1979), we counted (29) the number of tubercles on Toe IV, (30) the total number of dorsal ridges and (31) how many of these are short only (i.e. reduced to dorsolateral or the sacral ridges; Perret 1979); (32) we identified the area on the head where the dorsal ridges develop from, i.e. behind, at level of or before eyes (i.e. conditions A-C of Perret 1979) and (33) took the foot webbing formula following the manner described by Glaw & Vences (1994).

The 10 morphometrics and 13 of the 15 present and absence morphological characters were computed in Principal Component Analyses (PCAs) using Statistica 6.0 (StatSoft). Characters (14) and (21) showed no variation and were hence neglected in PCAs but can be useful when comparing to other than the Kakamega Forest *Ptychadena* species. All morphometrics, excluding SVL, were freed from the effects of body size by regressing them against SVL. The resulting residuals were then used as the variables for analysis since they are normally distributed and hence suitable for multivariate analysis. Principal components with eigenvalues > 1 were extracted and submitted to an orthogonal VARIMAX-rotation. This procedure attempts to simplify the columns of the factor matrix by making all values close to 0 or 1. Factor scores resulting from the analysis were plotted in a scatter plot to better show the relationships between species studied. The first factor score was plotted against the second factor score (x and y axis, respectively) and differences of the studied pre-grouped species determined by the various clusters formed. Morphometric and non-morphometrical morphology data were analysed separately as well as in combination.

Advertisement calls (for definition see Heyer et al. 1990) of 11 specimens allocatable to different taxa (see below) were recorded at temporary puddles in September 2001 and April-July 2004. For recording a Sony WM D6C tape recorder, a Sennheiser Me-80 directional microphone and metal cassettes were used. Air temperature at 1.0 m above ground was obtained from a Greisinger GFTH 95 digital thermometer immediately after recording. Call recordings were sampled at a rate of 2205 Hz and 16-bit resolution and analyzed with Cool Edit 96 (Syntrillium) on a PC. Frequency information was obtained through Fast Fourier Transformation (width 512 points). Time scales of the figured spectrograms and waveforms were chosen to allow for a maximum display of call characteristics. Spectral settings in figures are Hanning window function with 256-band resolution. In definition of 'call' and 'note' we follow Heyer et al. (1990). For vocalisation data and voucher specimens see Table 1.

Tab. 1. Characteristics of advertisement calls of *Ptychadena* species from the Kakamega Forest (compare Fig. 5). In the first three rows the mean is followed by one standard deviation and the range in parentheses. Voucher specimens (SVL in mm) are: species 2 – NMK A/3840/4 (50.6); species 3 – NMK A/4222 (39.8), two vouchers not collected; species 4 – NMK A/3955/1 (32.4); species 5 – NMK A/4219/1 (47.8), A/4219/1 (48.3), A/4219/3 (49.7), A/4223 (50.3), A/4233/1 (53.6), A/4233/2 (47.7).

parameter	species 2 N = 1 (9 calls)	species 3 N = 3 (35 calls)	species 4 N = 1 (6 calls)	species 5 N = 6 (65 calls)
call length (ms)	231.5 ± 15.8 (200-290)	218.2 ± 4.8 (216-248)	221.3 ± 14.6 (202-229)	291.9 ± 16.2 (263-333)
number of notes per call (N)	12.3 ± 1.3 (10-14)	12 ± 0.8 (11-13)	35.3 ± 2.3 (34-38)	6.3 ± 1.5 (5-9)
number of notes per second (N)	42.2 ± 4.5 (34.5-48.3)	54.9 ± 2.7 (52.6-59.1)	159.6 ± 81.3 (145.9-148.5)	21.6 ± 2.1 (19.6-25.0)
frequency range (Hz)	1300-4000	1400-4500	1000-4000	500-3000
dominant frequencies (Hz)	ca. 1600 and 3400	ca. 1800-2000 and 3600-3800	ca. 1000-2000 and 3400	ca. 860-1000 and 2200-2800
temperatures during recording (°C)	18.0	15.9, 19.4, 20.6	unknown	14.0, 16.9, 17.6, 17.9, 18.0, 18.4

Tissue from four specimens belonging to different taxa (see below), i.e. clipped toes in ca. 98 % ethanol, was used to sequence a ca. 500 base pair (bp) fragment of the mitochondrial 16S rRNA gene as it may serve as a universal marker for amphibian DNA taxonomy or DNA barcoding (Vences et al. 2005). For methods and standard primers applied see Vences et al. (2004). Sequences of species are deposited in GenBank (http://www.ncbi.nlm.nih.gov; Benson et al. 2004). All obtained sequences were verified as *Ptychadena* DNA by standard nucleotide-nucleotide BLAST search in GenBank. Sequences were aligned using Mega 3.1 (Kumar et al. 2004) and for uncorrected p-distances (Tab. 2) executed in PAUP*4b10 (Swofford 2001). Bayesian inference was performed using Mr.Bayes 3.1. (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). The GTR model (GTR + I + G) was used; model parameters were estimated by Mr.Bayes. Two simultaneous and completely independent analyses were initiated with random starting trees. In total, 3,000,000 generations with four independent Markov Chains were started while sampling every 100th tree. The first 3,000 trees (burnin) were excluded from the 50 % majority rule consensus tree (Fig. 4). Sequences of species from different localities were included in the present study as well as different *Ptychadena* samples available at GenBank. Representatives of the families Pyxicephalidae and Dicroglossidae were used as outgroups: *Amietia angolensis* (Bocage, 1866), *Hoplobatrachus occipitalis* (Günther, 1858).

Diagnoses of species from the Kakamega Forest cover 35 aspects using characters (1) to (33) plus information from advertisement calls (34) and DNA sequencing (35). We included non-variable characters (14) and (21) in order to provide a standardised diagnostic scheme applicable to the entire genus *Ptychadena.*

For the discussion of available names, we used information summarized by Frost (2008).

Species account

Species 1 "anchietae" (Fig. 2a)

Diagnosis: (1) SVL 42.6 ± 6.7 (22.9-60.9 with the smallest being males and the largest being females); (2) FL 34.2 ± 5.4 (29.6-48.2); (3) TL 27.2 ± 2.3 (25.2-31.8); (4) HW 12.7 ± 1.2 (10.8-14.6); (5) HL 14.2 ± 0.8 (13.3-15.7); (6)

Tab. 2. Uncorrected p-distances for the mitochondrial 16S rRNA gene data set of four *Ptychadena* species from the Kakamega Forest. Voucher specimens and GenBank accession numbers: species 1: NMK A/3845, AY517609; species 2: NMK A/3840/1, AY517599, species 4: voucher not maintained, DQ071575; species 5: NMK 517608, AY517608.

	species 1	species 2	species 4
species 2	0.17157		
species 4	0.2174	0.16636	
species 5	0.08946	0.18493	0.22349

SN 3.7 ± 0.4 (3.4-4.0); (7) EN 4.1 ± 0.4 (3.8-4.7); (8) TD 3.4 ± 0.3 (3.0-3.9); (9) ED 4.9 ± 0.3 (4.4-5.4); (10) ID 3.5 ± 0.3 (3.0-3.8); (11) rows of tubercles on metatarsus absent; (12) leg ridges present; (13) ridges on lateral sides of body absent; (14) warts on legs absent (15) warts on lateral sides absent; (16) light tibial line absent; (17) light bands on posterior face of femur present; (18) dark bands on posterior face of femur present, discontinuous from knee to knee; (19) dark mottling on posterior face of thigh absent; (20) pale triangle on dorsal snout present; (21) whitish spots on lower lip present; (22) complete whitish ring around tympanum present; (23) green or light brown median dorsal band absent; (24) outermost dorsal ridge whitish; (25) outer metatarsal tubercle absent; (26) canthus rostralis straight; (27) nostrils visible from above; (28) vocal openings in males below arm insertion (condition C); (29) three tubercles on Toe IV; (30) 6-8 dorsal ridges; (31) if 8, two are short sacral ridges only; (32) dorsal ridges develop from behind the eyes (condition A); (33) foot webbing formula 1e(0) 2i/e(0) 3i/e(0) 4i/e(1) 5i(0); (34) no call data available; (35) for sequence of a 494 bp fragment of the mitochondrial 16S rRNA gene see GenBank (accession number: AY517609).

Species 1 is similar to species 5 but usually smaller and with shorter FL and TL and with more extensive foot webbing (Fig. 3). Further, it can be distinguished through presence (versus absence in species 5) of light and dark bands on posterior face of femur, as well as absence (versus presence in species 5) of dark mottling on posterior face of thigh. In species 2-4 leg ridges are absent, a light tibial line is present (each inverse in species 1) and foot webbing is less extensive, and in species 4 the dark bands on the posterior face of the femur are continuous from knee to knee (while discontinuous in species 1). Species 2 and 4 do not have the vocal openings in males below arm insertion (i.e. above or at level of arm insertion). Species 3 has an outer metatarsal tubercle (absent in species 1). Species 1 is the only taxon, except species 5, in which a green or light brown median dorsal band absent (i.e. present in species 2-4).

Taxonomic comments: Species 1 is referable to *P. anchietae* (Bocage, 1868 "1867") as it was termed by Schick et al. (2005). Originally described from Angola, this species is suggested to display a distribution from southern Africa north to Eritrea (e.g. Channing & Howell 2006). We will neither rule out either (i) that *P. anchietae* in fact displays such a large geographical range nor (ii) that different taxa are involved. An argument for the former is the exceptional environmental adaptability of many Ridged Frog species (e.g. Rödel 2000). An argument for the latter is that Vences et al. (2004) uncovered that cryptic diversity occurs in the genus.

Species 1 is genetically similar to all *P. anchietae* sensu lato studied by us from different localities in East and South Africa (Angolan samples were not available). In a phylogeny by Vences et al. (2004), as in our own phylogeny (Fig.4), all *P. anchietae* sensu lato formed a well supported monophylum, at least suggesting that different geographic samples of *P. anchietae* constitute an evolutionary unit. Due to limited genetic differentiation, one could treat them as conspecifics

Fig. 2. Representatives of *Ptychadena* species from the Kakamega Forest: (a) species 1, 3, 4 in preservative (NMK A/3845, A/4222 A/3955/2) and (b) species 2, 5 in live (not collected).

(Fig. 4). However, in order to clarify if the name *P. anchietae* sensu stricto is applicable to this assemblage, information on specimens from the type locality or its vicinity is required.

Four names, currently in the synonymy of *P. anchietae,* from the species' northern range are available: *P. abyssinica* (Peters, 1881) from Eritrea, *P. gondokorensis* (Werner, 1908 "1907") from Sudan, *P. aberae* (Ahl, 1925 "1923") from Ethiopia, *P. migiurtina* (Scortecci, 1933) from Somalia. It remains to be studied if one of these may be applicable to species 1 instead of *P. anchietae* sensu stricto.

Species 2 "mascareniensis" (Figs. 2b)

Diagnosis: (1) SVL 48.4 ± 4.7 (34.1-61.1 with the smallest being males and the largest being females); (2) FL 41.5 ± 4.1 (28.9-50.4); (3) TL 30.5 ± 3.6 (20.4-39.3); (4) HW 13.5 ± 1.4 (11.3-17.5); (5) HL 15.5 ± 1.5 (12.2-19.8); (6) SN 3.7 ± 0.5 (2.8-4.9); (7) EN 5.3 ± 0.5 (3.5-6.4); (8) TD 4.1 ± 0.4 (3.4-4.9); (9) ED 5.3 ± 0.5 (4.0-6.1); (10) ID 3.8 ± 0.4 (4.5-2.3); (11) rows of tubercles on metatarsus absent; (12) leg ridges absent; (13) ridges on lateral sides of body absent; (14) warts on legs absent; (15) warts on lateral sides present or absent; (16) light tibial line present or absent; (17) light bands on posterior face of femur present; (18) dark bands on posterior face of femur present, continuous or discontinuous from knee to knee; (19) dark mottling on posterior face of thigh absent; (20) pale triangle on dorsal snout absent; (21) whitish spots on lower lip present; (22) incomplete whitish ring around tympanum present; (23) green or light brown median dorsal band present; (24) outermost dorsal ridge whitish; (25) outer metatarsal tubercle absent; (26) canthus rostralis straight; (27) nostrils visible from above; (28) vocal openings in males above arm insertion (condition A); (29) three tubercles on Toe IV; (30) 6-8 dorsal ridges; (31) if 8, two are short dorsolateral ridges only; (33) foot webbing formula 1e(1) 2i/e(1-2) 3i/e(1.5-2.5) 4i/e(2-3) 5i(1-2); (34) number of notes per second ranges 34.5-48.3 (mean 42.2 ± 4.5), dominant frequencies are at ca. 1600 and 3400 Hz (Fig.

5a); (35) for sequence of a 531 bp fragment of the mitochondrial 16S rRNA gene see GenBank (accession number: AY517599).

Species 2 is similar to species 4 but usually with larger SVL, FL and TL. The two can be distinguished through the presence (versus absence in species 4) of a whitish ring around the tympanum; further, in species 2 the vocal openings in males are above (not at level of as in species 4) arm insertion. Species 3 also shares several characters with species 2 but can be distinguished on the basis of absence (versus presence in species 2) of light and dark bands on posterior face of femur as well as presence (versus absence) of dark mottling on posterior face of thigh and outer metatarsal tubercle. Species 1 and 5 display more extensive foot webbing than species 2 (Fig. 3) and in both species leg ridges and a pale triangle on dorsal snout are present while a light tibial line is absent (each inverse in species 2). Species 2 is the only taxon in which the vocal openings in males are above arm insertion (below or at level of arm insertion in all other species).

Taxonomic comments: Species 2 is an unidentified taxon in the species complex behind the name *P. mascareniensis* (Duméril & Bibron, 1841). As advocated by Vences et al. (2004), two taxa of this complex occur in Kenyan territory, one of which is known from the Kakamega Forest (i.e. haplotype D sensu Vences et al. 2004). In the phylogeny by Vences et al. (2004), involving numerous *P. mascareniensis* sensu lato, species 2 was sister to specimens from Cameroon and West Africa – not with those from central Kenya, or elsewhere in East Africa. Our results support their findings. Figure 4 suggests that species 2 belongs to an East African/central African clade which is sister to a West African clade rather than any other *P. mascareniensis* sensu lato. Due to their comparatively limited differentiation, one may consider both to represent two lineages of a single species.

Our advertisement call data exhibit overlap in most parameters with voice recordings of *P. mascareniensis* sensu lato from Cameroon, South Africa and Tanzania, as summarized by Rödel (2000) as well as data provided by Channing & Howell (2006) for East Africa. However, all these recordings displayed a lower dominant frequency than specimens recorded by us, and those from Tanzania have remarkably shorter call length than frogs studied by us (120 ms versus 200-290 ms). We conclude that too few data are available for detailed analysis and species discrimination within *P. mascareniensis* sensu lato.

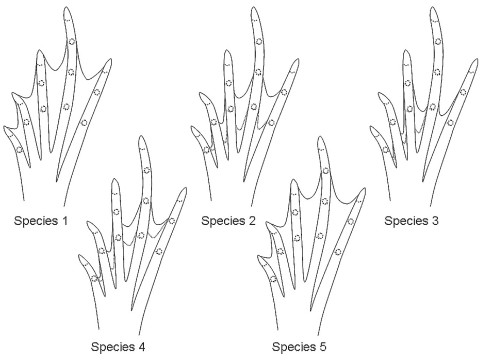


Fig. 3. Generalised *Ptychadena* foot (not to scale) showing extent of webbing and maximum variation (i.e. stippled versus solid line) in the five species from the Kakamega Forest.

As already discussed by Vences et al. (2004) the name *P. venusta* (Werner, 1908 "1907") is perhaps applicable to species 2. If this name has to be referred to haplotype A of Vences et al. (2004), species 2 likely represents an undescribed species.

Species 3 "porosissima" (Fig. 2a)

Diagnosis: (1) SVL 41.7 ± 4.6 (38.1-47.6 with the smallest being males and the largest being females); (2) FL 30.5 ± 6.0 (22.8-38.7) (3) TL 26.7 ± 2.6 (23.3-29.7); (4) HW 13.3 ± 1.2 (11.6-15.1); (5) HL 13.8 ± 1.2 (11.5-14.8); (6) SN 3.4 ± 0.4 (2.5-3.7); (7) EN 3.8 ± 0.2 (3.5-4.4); (8) TD 3.1 ± 0.3 (2.6-3.6); (9) ED 4.4 ± 0.3 (4.2-5.8); (10) ID 3.7 ± 0.6 (3.2-4.2); (11) rows of tubercles on metatarsus absent; (12)

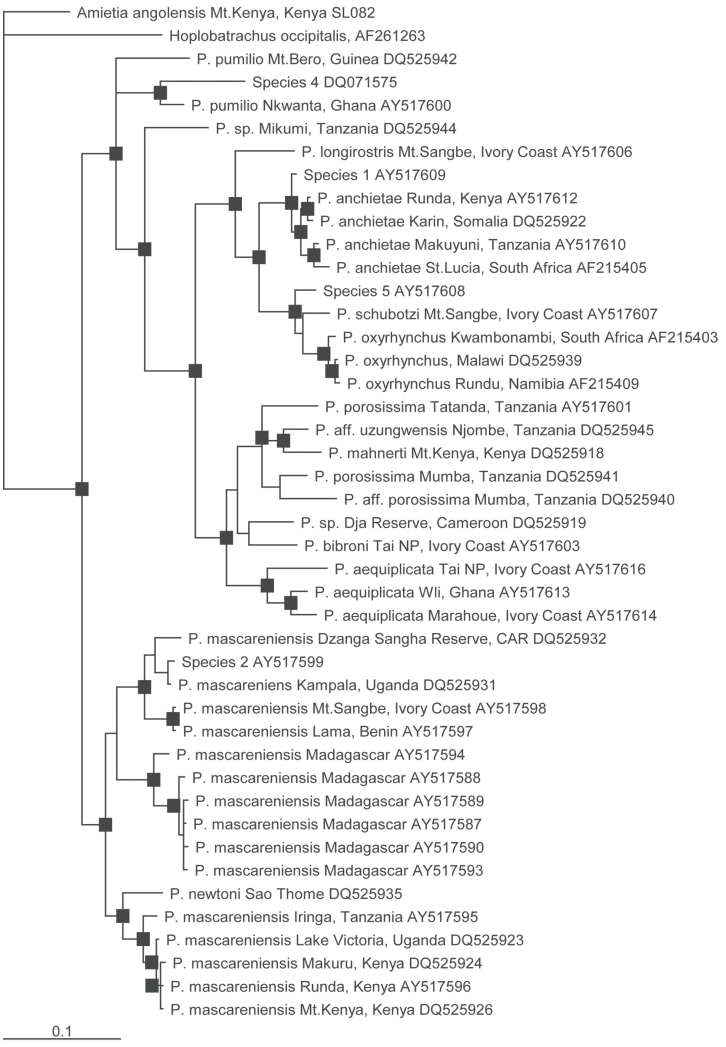


Fig. 4. Bayesian phylogram of *Ptychadena* haplotypes sequenced for a fragment of the mitochondrial 16S rRNA gene, including species 1, 2, 4, 5 and samples taken from GenBank (with accession numbers and origin, CAR = Central African Republic). Filled squares on branches indicate Bayesian posterior probabilities = 95.

leg ridges absent; (13) ridges on lateral sides of body absent; (14) warts on legs absent (15) warts on lateral sides absent; (16) light tibial line present; (17) light bands on posterior face of femur absent; (18) dark bands on posterior face of femur absent; (19) dark mottling on posterior face of thigh present; (20) pale triangle on dorsal snout absent; (21) whitish spots on lower lip present; (22) complete whitish ring around tympanum present; (23) green or light brown median dorsal band present; (24) outermost dorsal ridge whitish; (25) outer metatarsal tubercle present, larger than inner; (26) canthus rostralis straight; (27) nostrils visible from above; (28) vocal openings in males below arm insertion (con-

dition C); (29) three tubercles on Toe IV; (30) 6-8 dorsal ridges; (31) if 8, two are short dorsolateral ridges only; (32) dorsal ridges develop from behind the eyes (condition A); (33) foot webbing formula 1e(2) 2i/e(2-3) 3i/e(1-3) 4i/e(3) 5i(1); (34) number of notes per second ranges 52.6-59.1 (mean 54.9 ± 2.7), dominant frequencies are at ca. 1800-2000 and 3600-3800 Hz (Fig. 5b); (35) no genetic data available.

Species 3 is similar to species 2 and 4 but differs from them through absence (versus presence) of light and dark bands on posterior face of femur. Moreover, species 4 is usually smaller than species 3 and in species 3, the vocal openings in males are below arm insertion (versus above in species 2 and at level of arm insertion in species 4). Species 1 and 5 are distinguishable from species 3 through presence of leg ridges and a pale triangle on dorsal snout, absence of light tibial line (inverse in species 3) and more extensive foot webbing (Fig. 3). Species 1 and 5 also have larger FL and TL. Species 3 is the only taxon which has an outer metatarsal tubercle (i.e. absent in all other species).

Taxonomic comments: Species 3 is allocable to *P. porosissima* (Steindachner, 1867) as done by Schick et al. (2005). This species was originally described from Angola and is suggested to encompass a similarly large geographical range as *P. anchietae* (e.g. Channing & Howell 2006); see species 1. For the same reason as in this species one has to act with caution in *P. porosissima*. Two other names have been proposed, *P. loveridgei* Laurent, 1954 from Ruanda and *P. poyntoni* Guibé, 1960 from South Africa, the former of which may be applicable to species 3 if more than one species is involved.

Vocalisations studied by us are similar but longer (call length = 200 ms versus 216-248 ms) than those described by Passmore (1977) for nominal *P. porosissima* from South Africa. It is likely to us that this is due to intraspecific variation or different individual motivation rather than the two are not conspecifics.

Species 4 "taenioscelis" (Fig. 2a)

Diagnosis: (1) SVL 35.2 ± 7.1 (29.0-45.5 with the smallest being males and the largest being females); (2) FL 30.4 ± 6.1 (25.2-39.4); (3) TL 22.3 ± 4.2 (18.1-28.7); (4) HW 10.2 ± 1.7 (8.1-12.8); (5) HL 11.8 ± 2.3 (8.6-15.5); (6) SN 2.8 ± 0.4 (2.3-3.4); (7) EN 3.7 ± 0.6 (3.3-4.7); (8) TD 3.1 ± 0.6 (2.7-4.0); (9) ED 4.1 ± 0.5 (3.5-5.2); (10) ID 2.4 ± 0.7 (1.7-3.2); (11) rows of tubercles on metatarsus absent; (12) leg ridges absent; (13) ridges on lateral sides of body absent; (14) warts on legs absent; (15) warts on lateral sides absent; (16) light tibial line present; (17) light bands on posterior face of femur present; (18) dark bands on posterior face of femur present, continuous from knee to knee; (19) dark mottling on posterior face of thigh absent; (20) pale triangle on dorsal snout present; (21) whitish spots on lower lip present; (22) whitish ring around tympanum absent; (23) green or light brown median dorsal band present; (24) outermost dorsal ridge whitish; (25) outer metatarsal tubercle absent; (26) canthus rostralis straight; (27) nostrils visible from above; (28) vocal openings in males at level of arm insertion (condition B); (29) three tubercles on Toe IV; (30) 6-8 dorsal ridges; (31) if 8, two are short dorsolateral ridges only; (32) dorsal ridges develop from behind the eyes (condition A); (33) foot webbing formula 1e(1) 2i/e(1-2) 3i/e(1.5-2) 4i/e(2-3) 5i(1); (34) number of notes per second ranges 145.9-148.5 (mean 159.6 ± 81.3), dominant frequencies are at ca. 1000-2000 and 3400 Hz (Fig. 5c); (35) for sequence of a 482 bp fragment of the mitochondrial 16S rRNA gene see GenBank (accession number: DQ071575).

Species 4 is similar to species 2 but usually smaller. The two can be distinguished through absence (versus presence in species 2) of a whitish ring around tympanum. Species 3 also shares several characters with species 4 but differs through absence (versus presence in species 4) of light and dark bands on posterior face of femur and presence (versus absence) of dark mottling on posterior

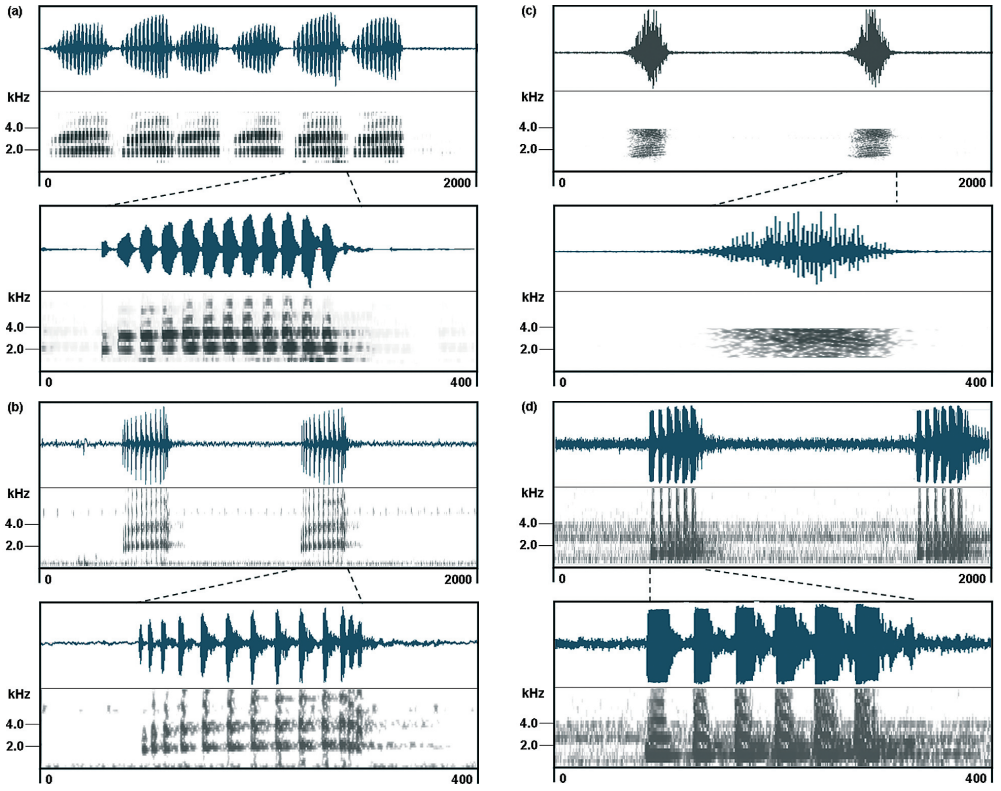


Fig. 5. Oscillograms and sound spectrograms (each a 2000 and 400 ms section) of advertisement calls of four species from the Kakamega Forest (a: species 2, NMK A/3840/4 at 18.0°C; b: species 3, NMK A/4222 at 19.4°C; c: species 4, NMK A/3955/1 at unknown temperature; d: species 5, A/4233/2 at 18.4°C). For vocalisation details see Table 1.

face of thigh and outer metatarsal tubercle. Species 1 and 5 differ from species 4 through more extensive foot webbing (Fig. 3) and having leg ridges present and light tibial line absent (each inverse in species 4). Moreover, in species 1 the dark bands on the posterior face of the femur are discontinuous from knee to knee (while continuous in species 4). Species 4 is the smallest species, the only one lacking a whitish ring around tympanum (present in all other species) and having the vocal openings in males at level of arm insertion (below or above arm insertion in all other species).

Taxonomic comments: Species 4 can be referred to *P. taenioscelis* Laurent, 1954 which has been described from Democratic Republic of Congo (Schick et al. 2005). Biogeographic relationships between the Kakamega Forest and Central Africa are not well known and in part difficult to understand (Schick et al. 2005; own unpubl. data). As a result, the applicability of this name to species 4 remains to be resolved. An alternative name, currently a junior synonym of *P. taenioscelis,* could be *P. smithi* Guibé, 1960, which has been described from South Africa.

Species 4 is genetically similar but well differentiated to material from Guinea and Ghana allocated to *P. pumilio* (Boulenger, 1920). In a phylogeny by Measey et al. (2007) as well as in our Figure 4 the Ghanaian sample and species 4 comprise a well supported clade, while the Guinean sample is apparently sister to both (with weak support, howev-

er). This requires further studies on the relationship between East and West African material including their taxonomic identity. So far, samples from elsewhere determined as *P. taenioscelis* are not available.

Advertisement calls of nominal *P. taenioscelis* from South Africa described by Passmore (1976) are similar to those studied by us; differences are noted in the dominant frequency (i.e. 2800-3500 Hz versus two dominant frequencies, one of considerably lower, species 4; Tab. 1) and in the number of notes per call (termed 'pulses' by the author), i.e. 22-25 versus 34-38 in species 4. We consider both as possibly conspecific and suggest that vocalisation differences may be the result of different recording or analysis methods, intraspecific variation or different individual motivation.

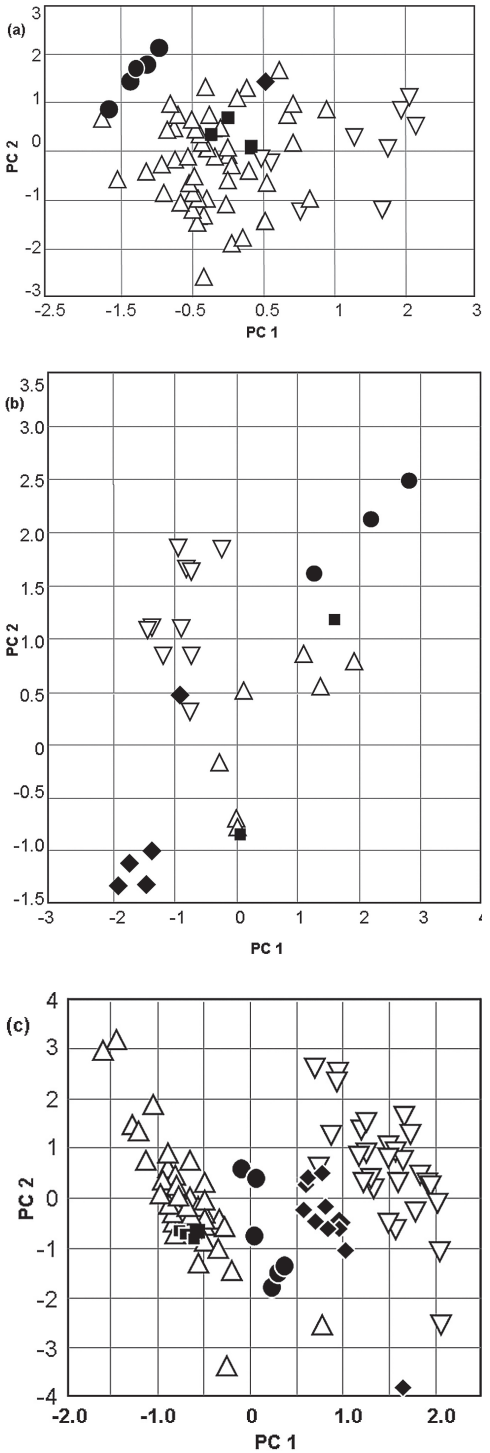
Species 5 "oxyrhynchus" (Figs. 2b)

Diagnosis: (1) SVL 50.4 ± 4.0 (38.1-59.7 with the smallest being males and the largest being females); (2) FL 40.8 ± 4.3 (26.8-48.3); (3) TL 35.2 ± 2.8 (27.5-41.3); (4) HW 14.9 ± 1.2 (11.5-17.9); (5) HL 19.6 ± 14.7 (14.4-17.0); (6) SN 4.9 ± 0.4 (3.9-5.8); (7) EN 5.11 ± 0.5 (4.3-6.5); (8) TD 4.5 ± 0.4 (3.4-5.4); (9) ED 5.9 ± 0.4 (4.2-7.2); (10) ID 3.9 ± 0.4 (3.1-4.8); (11) rows of tubercles on metatarsus absent; (12) leg ridges present; (13) ridges on lateral sides of body present (14) warts on legs absent (15) warts on lateral sides absent; (16) light tibial line absent; (17) light bands on posterior face of femur absent; (18) dark bands on posterior face of femur absent; (19) dark mottling on posterior face of thigh present; (20) pale triangle on dorsal snout present; (21) whitish spots on lower lip present; (22) complete whitish ring around tympanum present; (23) light brown median dorsal band absent; (24) outermost dorsal ridge whitish or ridge absent; (25) outer metatarsal tubercle absent; (26) canthus rostralis straight; (27) nostrils visible from above; (28) vocal openings in males below arm insertion (condition C); (29) three tubercles on Toe IV; (30) 6-8 dorsal ridges; (31) if 8, two are short sacral ridges only; (32) dorsal ridges develop from between eyes (condition B); (33) foot webbing formula 1e(0) 2i/e(0) 3i/e(0) 4i/e(1) 5i(0); (34) number of notes per second ranges 19.5-25.0 (21.6 ± 2.1), dominant frequencies are ca. 860-1000 and 2200-2800 Hz (Fig. 5d); (35) for sequence of a 448 bp fragment of the mitochondrial 16S rRNA gene see GenBank (accession number: AY517608).

Species 5 is similar to species 1 and is distinguishable from it through usually larger FL and TL, absence (versus presence) of light and dark bands on posterior face of femur, presence (versus absence) of dark mottling on posterior face of thigh and less extensive foot webbing (Fig. 3). Species 2-4 each have less extensive foot webbing and lack leg ridges and possess a light tibial line (each inverse in species 5). Moreover, species 2 and 4 do not have the vocal openings in males below arm insertion (i.e. above or at level of arm insertion); species 4 is also smaller. Species 3 and 5 are the only taxa lacking light and dark bands on posterior face of femur and having dark mottling on posterior face of thigh; the two can be distinguished on the basis of presence versus absence (in species 5) of an outer metatarsal tubercle. Species 5 is the largest species displaying the longest legs as indicated by SVL, FL and TL.

Taxonomic comments: Species 5 is referable to *P. oxyrhynchus* (Smith, 1849) as it was treated by Schick et al. (2005). Originally described from South Africa this species is suggested to occur in circum-forest sub-Saharan Africa (e.g. Channing & Howell 2006). We feel unable to judge (i) if *P. oxyrhynchus* actually covers such a large geographical range or (ii) if different taxa are involved. Since both is known in Ridged Frogs (see under species 1), it remains to be studied whether species 5 indeed is conspecific with *P. oxyrhynchus* sensu stricto.

Vocalisation information given for species 5 in Table 1 differs from that described by Rödel (2000) for West African and by



Channing & Howell (2006) for East African *P. oxyrhynchus* sensu lato. West African advertisement calls are shorter (call length = 200 ms versus 263-333 ms in species 5) at higher dominant frequencies (starting at ca. 1300 and 2500 Hz versus 860-1000 and 2200-2800 Hz in species 5) and contain more notes (termed 'pulses' by the author), e.g. 24 per call as in Rödel's (2000) illustrated sound spectrogram (versus 5-9 in species 5). In contrast, advertisement call data given by Channing & Howell (2006) are longer (call length > 400 ms versus < 333 ms in species 5) and contain a similarly high number of pulses per call (= 10 versus 5-9 in species 5) while the dominant frequencies are similar to those in species 5. Advertisement calls of nominal *P. oxyrhynchus* from South Africa available from Passmore (1977) are similar to those described by Channing & Howell (2006) from eastern Africa. Although information on advertisement calls is still limited, our comparison leads us to the conclusion that perhaps different taxa are involved.

Molecular phylogenies by Vences et al. (2004) and Measey et al. (2007) placed species 5 (as *P.* aff. *schubotzi*) in the proximity of specimens from Ivory Coast allocated to *P. schubotzi* (Sternfeld, 1917) (a species originally described from Central African Republic) and specimens from southern Africa allocated to *P. oxyrhynchus*. Similar results were obtained by us but there was little support for the arrangement of samples (Fig. 4). This may support our conclusion drawn from vocalization comparisons, i.e. that different species are hidden behind the name

Fig. 6. Scatter plots showing results of PCAs of (a) 10 morphometrical characters, (b) 13 non-morphometrical morphology characters (presence versus absence) and (c) both combined of *Ptychadena* species from the Kakamega Forest (n = 130): species 1 = solid diamond, species 2 = triangle, species 3 = solid dot, species 4 = solid square, species 5 = inverse triangle (note that symbols can overlay). For characters included and their definitions see material and methods section. Factor loadings are provided in Appendix 2.

P. oxyrhynchus. One of the following names, currently treated as junior synonyms of *P. oxyrhynchus,* is perhaps applicable: *P. bistrigatus* (Werner, 1894) from an unknown locality, *P. hailiensis* (Meek, 1897) from Somalia, *P. gribinguensis* (Angel, 1922) from Central African Republic.

Comparisons among *Ptychadena* species from Kakamega Forest

We above provided diagnostic characters for five species. Although diagnostic, not all have equal power to define reproductively separated units. We here investigate characters which define taxa. Also we discuss in how far different methodical approaches help to distinguish species.

Morphology

Although the five species defined herein differ in many of the morphometrical and non-morphometrical morphology characters studied, they are in part similar to each other (Fig. 2). To better understand the values of different external body features for *Ptychadena* species discrimination in the Kakamega Forest, we ran PCAs using morphometrics and presence versus absence non-morphometrical morphology data. Regarding the morphological characters not included in PCAs only the foot webbing formula was variable among species, best distinguishing species 1 and 5 from species 2 to 4 (Fig. 3).

A PCA performed for the 10 morphometrics alone revealed that all five species are close to each other (Fig. 6a). Only on the left and right peripheries of this clump species 3 and 5 became slightly evident. In contrast, a PCA considering the 13 non-morphometrical morphology characters (Fig. 6b) supported four groups, three of which were well separated (species 1, 3 and 5), while the other two composed of species 2 and 4 showed some overlap. Combining morphometrics with non-morphometrical morphology resulted in similar grouping (Fig. 6c). For factor loadings of principal components and explained variance see Appendix 2. Most characters used were informative.

A PCA using morphometry alone was not informative. Using non-morphometrical morphology both alone and in combination with morphometry only species 2 and 4 could not be discriminated through PCAs. This may be explained by the fact that they indeed look similar (Fig. 2) and the limited number of specimens available for species 4 (see Appendix 1). Nevertheless, we have no doubt in the validity of these species since advertisement call and molecular data well support them (see below).

Advertisement calls

As shown in Figure 5 and Table 1, advertisement call information is available for four species. Following morphometrical and non-morphometrical morphology characters, the male specimens that produced these vocalisations are well allocable to species 2, 3, 4 and 5. All individuals recorded, except that of species 4, are of comparable SVL and calls were recorded at comparable temperatures (Tab. 1). The limited number of specimens recorded does not allow for statistical testing; hence, we merely describe apparent differences.

Advertisement calls largely show overlap in the call length and the frequency range. The number of notes per call is almost identical in species 2, 3 and both are similar to species 5, while in species 4 the number of notes per call is considerably higher than in all other species. Likewise, the number of notes per second among species 2 and 3 are equal, while the two others are different from them and from one another. Each of the four species studied displayed two dominant frequencies (Fig. 5). Again species 2 and 3 were similar, with species 3 calling at least at 200 Hz higher in both the lower and the upper

dominant frequencies. The lower dominant frequencies of species 4 and 5 are similar and lower than in species 2 and 3, while the upper dominant frequency in species 4 equals species 1, but that of species 5 is distinct to all other species.

Species 4 and 5 are morphologically well distinguished which is supported by bioacoustic parameters. The other two species mainly differ in their dominant frequencies and even the shapes of their calls in Figures 5a and b look alike. Dominant frequencies are here considered to play a major role. We often observed that *Ptychadena* males call in dense groups (see also Channing & Howell 2006) resulting in a monotonous sound so that single calls can not be identified. If different species which commonly call in choruses co-occur the dominant frequency is the character which helps discriminating them (Ryan 2001). We conclude that also species 2 and 3 were confirmed through bioacoustics.

We lack information about temporary partitioning throughout the year. But due to our preliminary observations, these species appear to be opportunistic breeders, which like many other *Ptychadena* species are calling throughout rainy periods (e.g. Rödel 2000).

As elsewhere in *Ptychadena* (e.g. Passmore 1977), we demonstrated vocalisations to be a useful tool for the discrimination of the species co-occurring in the Kakamega Forest.

DNA barcoding

Table 2 summarizes the uncorrected p-distances for the mitochondrial 16S rRNA gene data set containing four of the proposed species. Individuals used for molecular analyses, by morphometry and non-morphometrical morphology, fell into species 1, 2, 4 and 5, respectively. Their uncorrected p-distances range 8-22 %. Vences et al. (2004) found that within *Ptychadena* species genetic divergence was low while suggested cryptic species within *Ptychadena mascareniensis* sensu lato showed > 5 % divergence in uncorrected p-distances. This strongly indicates the existence of (at least) four syntopic species in the Kakamega Forest. For species 3, no tissue sample was available but this taxon is supported using morphology and bioacoustics (see above).

There is no doubt, DNA barcoding, especially when using the mitochondrial 16S rRNA gene, is a reliable tool in amphibian taxonomy (e.g. Vences et al. 2005). Accordingly, this approach helped to identify different taxa of *Ptychadena* in the Kakamega Forest.

Colonisation of Kakamega Forest by *Ptychadena*

We are still far from a complete phylogeny of the genus *Ptychadena.* However, previous molecular approaches have revealed surprising results and unexpected biogeographic relationships including oceanic dispersal (Vences et al. 2004, Measey et al. 2007). We here deduce from Figure 4 conclusions concerning the colonisation of the Kakamega Forest by the genus *Ptychadena.* Data suggest that the Kakamega Forest was invaded by different *Ptychadena* lineages. As this forest represents a remnant of a once continuous Guineo-Congolian rain forest (e.g. Mutangah et al. 1992, Wagner et al. 2008), little doubt arises about the apparent affinities of species 2, 4 and perhaps 5 to Ridged Frogs from Central or even West Africa (Fig. 4). They may have invaded the Kakamega Forest from or via the Congo basin, as has been proposed for other anuran species (e.g. Schick et al. 2005). At the same time, as evident in species 2, the Kakamega Forest may represent the easternmost area where these forms can be found, since in more central Kenya other *Ptychadena* have been found only (Vences et al. 2004). These circumstances underline the importance of the Kakamega Forest with respect to our understanding of historical biogeography as well as for conservation.

In contrast to the biogeographic affinities to Central and West Africa, species 1 was nested in one clade with *P. anchietae* sensu lato from East and South Africa (Fig. 4). This demonstrates that, besides the Congo basin, *Ptychadena* has colonised the Kakamega Forest from elsewhere.

Unfortunately little inference can be drawn from the type of habitat Ridged Frogs occupy in the Kakamega Forest, as there is no such graduation as pristine habitat, secondary rain forest and disturbed areas. Due to its limited extension and immense human influence basically the whole forest is more or less disturbed and species were found all over the different habitats.

Conclusions

Supporting previous findings throughout tropical Africa, our data show that several *Ptychadena* species can occur synoptically. As in other co-occurring *Ptychadena* species, the five taxa suggested here, are in part difficult to distinguish. Our results propose that morphometrics and non-morphometrical morphology are helpful but are not sufficient to securely define groups. More convincing results were obtained through analyses of advertisement calls and the study of a molecular marker. Depending on season, *Ptychadena* vocalisations may be difficult to obtain but recording requires limited technical efforts and various analysis software is freely available. In contrast, tissue sampling for DNA studies in the field is simpler but DNA barcoding requires access to a molecular lab and is comparatively costly.

As shown above, for the identification – not definition – of species a combination of morphometrical and non-morphometrical morphology characters is suitable. We hope this will stimulate colleagues to further test how far external characters are helpful identification tools for the entire genus *Ptychadena*. We recommend following the standardised diagnostic scheme suggested here and, if necessary, expanding it in order to ease comparisons and identifications of potential taxa. Standardised schemes may be especially important before the background of rapid and easy field identifications for conservation purposes. As anchored in the IUCN Amphibian Conservation Action Plan (Gascon et al. 2007), the ongoing worldwide amphibian crisis requires increased study and documentation of exact species distributions and their Red List status (http://www.iucnredlist.org). This can be performed at best when simple and low cost tools are developed for rapid and easy identification of species even by non-specialists. Well aware that not all amphibian genera and species are suitable for this purpose, Ridged Frogs may serve as an example where this is possible to a high degree.

Not only the identification of species but also their phylogenetic relationships and putative dispersal routes are important for conservation action. Figure 4 shows that in the Kakamega Forest representatives of different *Ptychadena* clades occur with at least two distinct biogeographic affinities. These facts may be considered in future debate of biodiversity management.

Acknowledgements

For logistic support we are grateful to National Museums of Kenya. BIOTA E08, part of the BIOLOG campaign under the Federal Ministry of Education and Research (Germany), kindly provided the financial framework for this study (01LC0025). Permits to perform fieldwork and collections were issued by Kenya Wildlife Service (KWS) under the BIOTA MoU. Special thanks to B. Zimkus and M.-O. Rödel for helpful reviews of the original manuscript.

References

Amiet, J.-L. (1989): Quelques aspects de la biologie des amphibiens anoures du Cameroun. – Année Biologique, **28**: 73-136.

Benson, D. A., I. Karsch-Mitzrachi, D. J. Lippman, J. Ostell & D. L. Wheeler (2004): GenBank: update. – Nucleic Acids Research, **32**: 23-26.

Channing, A. & K. M. Howell (2006): Amphibians of East Africa. – Frankfurt/M.: Chimaira.

Fishpool, L. D. C. & M. I. Evand (2001): Important bird areas in Africa and associated islands: priority sites for conservation. – Newbury and Cambridge: Pisces Publications and BirdLife International.

Frost, D. R. (2008): Amphibian species of the world: an online reference. Version 5.2 (15 July 2008). – New York: American Museum of Natural History. [http://research.amnh.org/herpetology/amphibia/index.php]

Gascon, C., J. P. Collins, R. D. Moore, D. R. Church, J. E. McKay & J. R. Mendelson III (2007): Amphibian Conservation Action Plan. Proceedings: IUCN/SSC Amphibian Conservation Summit 2005. – Gland: IUCN.

Glaw, F. & M. Vences (1994): A fieldguide to the amphibians and reptiles of Madagascar. – Cologne: Vences + Glaw Verlags GbR.

Guibé, J. & M. Lamotte (1957): Révision systématique des *Ptychadena* (Batraciens, Anoures, Ranidés) d'Afrique occidentale. – Bulletin de l'Institute Fondamental d'Afrique Noire, **19:** 937-1003.

Guibé, J. & M. Lamotte (1958): Les *Ptychadena* (Batraciens, Ranidés) du Cameroun. – Bulletin de l'Institute Fondamental d'Afrique Noire, **20:** 1448-1463.

Guibé, J. & M. Lamotte (1960): Deux espèces affines de batraciens africains longtemps confondues: *Ptychadena oxyrhynchus* (Smith) et *Pt. abyssinica* (Peters). – Bulletin de la Muséum national d'Histoire naturelle, **32**: 380-391.

Heyer, W. R., A. S. Rand, C. A. G. da Cruz, O. L. Peixoto & C. E. Nelson (1990): Frogs of Boracéia. – Arquivos de Zoologia, **31**: 237-410.

Huelsenbeck, J. P. & F. Ronquist (2001): Mr.Bayes: Bayesian inference of phylogeny. – Bioinformatics, **17**: 754-755.

Kokwaro, J. O. (1988): Conservation status of Kakamega Forest in Kenya – the easternmost relict of the Equatorial rainforests of Africa. – Monographs in Systematic Botany of the Missouri Botanical Gardens, **25:** 471-489.

Kumar, S., K. Tamura & M. Nei (2004): MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. – Briefings in Bioinformatics, **5**: 150-163.

Lamotte, M. (1967): Le problème des *Ptychadena* (Fam. Ranidae) du groupe *mascareniensis* dans l'ouest africain. – Bulletin de la Muséum national d'Histoire naturelle, **39**: 647-656.

Largen, J. M. (1997): Two new species of *Ptychadena* Boulenger 1917 (Amphibia Anura Ranidae) from Ethiopia, with observations on other members of the genus recorded from this country and a tentative key for their identification. – Tropical Zoology, **10**: 223-246.

Largen, J. M. (2000): A new species of *Ptychadena* Boulenger 1917 from Ethiopia (Amphibia Anura Ranidae). – Tropical Zoology, **13**: 171-178.

Measey, J., M. Vences, R. C. Drewes, Y. Chiari, M. Melo & B. Bourles (2007): freshwater paths across the ocean: molecular phylogeny of the frog *Ptychadena newtoni* gives insight into amphibian colonization of oceanic islands. – Journal of Biogeography, **34**: 7-20.

Mutangah, J. G., O. Mwangangi & M. Mwaura (1992): Kakamega Forest – a vegetation survey report. – Nairobi: National Museums of Kenya.

Passmore, N. I. (1976): Vocalizations and breeding behaviour of *Ptychadena taenioscelis* (Anura, Ranidae). – Zoologica Africana, **11**: 339-347.

Passmore, N. I. (1977): Mating calls and other vocalizations of the five species of *Ptychadena* (Anura, Ranidae). – South African Journal of Sciences, **173**: 212-214.

Perret, J.-L. (1979): Remarques et mise au point sur quelques espèces de *Ptychadena* (Amphibia, Ranidae). – Bulletin de la Société neuchâtelois des Sciences naturelle, **102**: 5-21.

Perret, J.-L. (1981): Le statut de *Ptychadena schubotzi* (Sternfeld) (Amphibia, Ranidae). – Bulletin de la Société neuchâtelois des Sciences naturelle, **102**: 53-57.

Perret, J.-L. (1987): À propos de *Ptychadena schillukorum* (Werner, 1907) (Anura, Ranidae). – Bulletin de la Société neuchâtelois des Sciences naturelle, **110**: 63-70.

Perret, J.-L. (1994): Description de *Ptychadena largeni* n. sp. (Anura, Ranidae) d'Èthiopie. – Muséum d'Histoire Naturelle de Genève, **117**: 67-77.

Perret, J.-L. (1996): Une nouvelle espèce du genre *Ptychadena* (Anura, Ranidae) du Kenya. – Revue Suisse de Zoologie, **103**: 757-766.

Poynton, C. J. & D. G. Broadley (1985): Amphibia Zambesiaca 2. Ranidae. – Annals of the Natal Museum, **27**: 115-181.

Rödel, M.-O. (2000): Herpetofauna of West Africa amphibians of the West African savanna. – Frankfurt/M.: Chimaira.

Ronquist, F. & J. P. Huelsenbeck (2003): MR.BAYES 3: Bayesian phylogenetic inference under mixed models. – Bioinformatics, **19**: 1572-1574.

Ryan, M. (2001): Anuran communication. – Washington D.C.: Smithsonian Inst. Press.

Schick, S., M. Veith & S. Lötters (2005): Geographical range patterns of amphibians from the Kakamega Forest, Kenya. – African Journal of Herpetology, **54**: 185-190.

Swofford, D. L. (2001): PAUP*: Phylogenetic analysis using Parsimony (*and other methods), version 4.06b. – Sunderland, MA: Sinauer Associates.

Vences, M., J. Kosuch, M.-O. Rödel, S. Lötters, A. Channing, F. Glaw & W. Böhme (2004): Phylogeography of *Ptychadena mascareniensis* suggests transoceanic dispersal in a widespread African-Malagasy frog lineage. – Journal of Biogeography, **31**: 593-601.

Vences, M., M. Thomas, A. van der Meijden, A. Chiari & D. R. Vieites (2005): Comparative performance of the 16S rRNA gene in DNA barcoding of amphibians. – Frontiers in Zoology, **2**: 5.

Wagner, P., J. Köhler, A. Schmitz & W. Böhme (2008): The biogeographical assignment of a west Kenyan rain forest remnant: further evidence from analysis of its reptile fauna. – Journal of Biogeography, **35**: 1349-1361.

Appendix 1

List of *Ptychadena* specimens (n = 130) from the Kakamega Forest, Western Province, Kenya (Fig. 1), included in the morphological and non-morphometrical morphology studies: Species 1 (11 specimens) – Buyangu Hill area, NMK A/3845, A/4211, A/4216, A/4220/2, A/4224/1, A/4224/2; Isecheno, A/4226/1, A/4226/2, A/4234/1, A/4234/2, A/4234/3. Species 2 (75 specimens) – Buyangu Hill area, A/4085, A/4086, A/4087, A/4089, A/4090, A/4091,A/4092, A/4093, A/4094, A/4097, A/4099, A/4101, A/4102, A/4103, A/4104, A/4105, A/4106, A/4107, A/4108, A/4109, A/4110, A/4111, A/4112, A/4113, A/4114, A/4115, A/4116, A/4117, A/4118, A/4119, A/4120, A/4121, A/4122, A/4123, A/4124, A/4125, A/4127, A/4128, A/3840/2, A/3840/1, A/1414/1, A/1414/3, A/3573/2, A/1414/2, A/1414/4 A/3573/1, A/4214, A/4217/1, A/4217/2, A/4221, A/4223, A/4227/1, A/4227/2, A/4227/3, A/4227/4, A/4227/5, A/4227/6, A/4227/7, A/4227/8, A/4227/9, A/4227/10, A/4229/1, A/4229/2,A/4235/3, A/4235/4, A/4235/5, A/4235/6, A/4235/7, A/4235/8, A/4235/9; Isecheno, A/4232/1, A/4232/2, A/4232/3, A/4232/4, A/4232/5. Species 3 (6 specimens) – Isecheno, A/3107/2, A/1611/1, A/1611/2, A/3574, A/3107/1, A/4222. Species 4 (6 specimens) – Buyangu Hill area, A/4088, A/3955/1, A/3955/2, A/4213, A/4235/1, A/4235/2. Species 5 (32 specimens) – Buyangu Hill area, A/3849, A/4095, A/4096, A/4098, A/4100, A/4126, A/4211, A/4215/1, A/4215/2, A/4215/3, A/4215/4, A/4218, A/4219/1, A/4219/2, A/4219/3, A/4225/1, A/4225/2, A/4228/1,A/4228/2, A/4228/3, A/4230/1, A/4230/2, A/4231/1, A/4231/2, A/4236/1, A/4236/2; Isecheno, A/103/1, A/103/2, A/4233/1, A/4233/4, A/4233/2, A/4233/3.

Appendix 2

Factor loadings of principal components of PCAs, their explained variance and proportion of total variance; for abbreviations see Material and methods. Bold indicates that values are significant ($p > 0.05$).

Morphometry (see Fig. 6a)

	factor 1	factor 2
FL	-0.007136	**-0.912247**
TL	**0.890906**	0.116180
HW	**0.825106**	0.245776
HL	0.224672	0.300943
SN	**0.718349**	0.371110
EN	**0.839460**	0.007490
TD	**0.834013**	-0.198253
ED	**0.823229**	0.042019
ID	0.627293	0.418950
explained variance	4.512541	1.351033
proportion of total variance	0.501393	0.150115

Non-morphometrical morphology (see Fig. 6b)

	factor 1	factor 2	factor 3
ridges on legs	**-0.750763**	0.512234	0.019379
ridges on lateral sides of body	-0.224544	0.490730	0.236417
warts on lateral sides	0.499755	0.258699	0.485852
light tibial line	**0.831210**	-0.410489	-0.034760
light bands on posterior face of femur	0.405792	**-0.842314**	0.005393
dark bands on posterior face of femur	0.350553	**-0.876641**	0.106227
dark mottling on posterior face of thigh	-0.379459	**0.865355**	-0.101689
pale triangle on dorsal snout	**-0.843938**	0.452191	0.031430
whitish ring around tympanum	**-0.729897**	0.215073	0.114113
green or brown median dorsal band	**0.846223**	-0.399729	-0.024547
outermost dorsal ridge whitish	0.504205	-0.470736	-0.191481
outer metatarsal tubercle	0.140686	0.216008	**-0.840148**
explained variance	4.221364	3.644226	1.072309
proportion of total variance	0.351780	0.303685	0.089359

Morphometry and non-morphometrical morphology combined (see Fig. 6c)

	factor 1	factor 2	factor 3	factor 4
FL	-0.500389	0.085266	0.452639	0.244764
TL	0.263702	**0.822119**	-0.129133	-0.017426
HW	0.584093	0.623307	-0.219797	-0.025447
HL	0.157647	**0.895183**	0.129998	-0.000878
SN	0.584093	0.623307	-0.219797	-0.025447
EN	0.157647	**0.895183**	0.129998	-0.000878
TD	0.079919	**0.811378**	0.027989	0.128543
ED	0.253385	0.585570	-0.335473	-0.136320
ID	0.239024	**0.749019**	-0.103325	0.053315
ridges on legs	**0.855453**	0.285817	0.083961	0.022555
ridges on lateral sides of body	0.458415	0.177461	0.061528	0.099562
warts on lateral sides	-0.183111	-0.014325	**-0.752456**	0.240725
light tibial line	**-0.890960**	-0.157897	-0.107308	-0.085549
light bands on posterior face of femur	**-0.829653**	-0.196247	0.241844	0.203972
dark bands on posterior face of femur	**-0.799275**	-0.207398	0.225611	0.310412
dark mottling on posterior face of thigh	**0.811406**	0.217574	-0.213930	-0.299147
pale triangle on dorsal snout	**0.915764**	0.203938	0.101375	0.075033
whitish ring around tympanum	0.673304	0.148000	0.235479	0.248619
green or brown median dorsal band	**-0.895185**	-0.160797	-0.120544	-0.085259
outermost dorsal ridge whitish	-0.682521	-0.112894	-0.071058	-0.157318
outer metatarsal tubercle	0.010105	-0.067232	0.140115	**-0.830100**
explained variance	7.493768	4.998936	1.324615	1.191122
proportion of total variance	0.356846	0.238045	0.063077	0.056720

Manuscript received: 18 December 2007

Authors' addresses: Beryl A. Bwong, Department of Herpetology, National Museums of Kenya, P.O. Box 00100 40658, Nairobi, Kenya; Robert Chira, School of Biological Sciences, University of Nairobi P.O. Box 301970, Nairobi, Kenya; Susanne Schick, Zoology Department, Mainz University, Saarstraße 21, 55099 Mainz, Germany; Michael Veith & Stefan Lötters, Institute of Biodiversity and Ecosystem Dynamics, University of Amsterdam, P.O. Box 94766, 1090 GT Amsterdam, The Netherlands; current address: University of Trier, Biogeography Department, 54286 Trier, Germany, E-Mail: loetters@uni-trier.de.