Designation and description of a neotype of *Sclerophrys maculata* (Hallowell, 1854), and reinstatement of *S. pusilla* (Mertens, 1937) (Amphibia: Anura: Bufonidae)

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**Abstract**

Molecular analysis indicates that African material previously referred to *Amietophrynus maculatus* (Hallowell, 1854; now *Sclerophrys maculata*), is divisible into two distinct clades: a Western Clade from Cameroon westwards and an Eastern Clade from Central African Republic eastwards, and Uganda southwards to South Africa, extending to Angola-Namibia. Preliminary morphological and bioacoustic data support this division. The two clades are recognised here as two separate species. The Western species retains the name *S. maculata*, with Hallowell’s designated type locality of Liberia. The Eastern Clade retains the name published by Mertens (1937), *S. pusilla*. It is noted that a type specimen of *S. maculata* cannot be traced and is presumed lost; the so-called syntypes in the Philadelphia Academy of Natural Sciences are not the material described by Hallowell. None of these have been designated as a neotype, consequently a specimen from Liberia in the collection of the Natural History Museum, London, is designated here as the neotype of *S. maculata*.

**Key words:** Africa, Toad, *Bufo*, *Amietophrynus*, holotype

**Introduction**

One of the most common toad species complexes encountered across the African continent is currently named *Sclerophrys maculata*, following the recent relegation by Ohler & Dubois (2016) of species included in *Amietophrynus* (Frost et al., 2006) to the genus *Sclerophrys* Tschudi (1838). It occurs across a wide range of habitats and is one of the most widely distributed on the continent, ranging from West Africa to East and Southern Africa (Tandy et al., 2004). However, the taxonomy is confused due to a number of unresolved issues, including the synonymy of a species and uncertainty in the allocation of type material. Sampling of molecular, morphological and acoustic data in this study warrants the reconsideration of these problems, to establish an updated and comprehensive understanding.

Hallowell (1844) described what appears to be a single specimen from Liberia as *Bufo cinereus*; later renamed by him as *Bufo maculatus* Hallowell, 1854. The 1844 description reads, “Head small, body of moderate size, covered with numerous small warts; head and body of light ash colour, with numerous dark-coloured spots or blotches; under surface of chin, abdomen and limbs, light yellow; irides –. ” The “length of body” was given as two inches (c. 50 mm). Subsequently (Hallowell, 1854), when renaming the specimen as *B. maculatus*, the only further detail he gave was, “*Bufo maculatus* resembles somewhat *Bufo pantherinus* [‘S. regularis’], but differs from it,
according to the description of Dumeril and Bibron, in the size of the tympanum, the breadth of which is considerably larger than the length of the upper eyelid.” The last statement is ambiguous, but whichever (if any) species answers to this description, it is not the species currently referred to as *S. maculata*, where the breadth (presumably horizontal diameter) of the tympanum does not exceed 75% of the length of the upper eyelid. All in all, his descriptions do not amount to an effective diagnosis.

Poynton (1964) treated Mertens’s (1937) “*Bufo regularis pusillus*”, a morphologically similar toad, as a full species, differing in call, habitat preference and morphology from *S. regularis* (at that time including *S. gutturalis*). He noted that “this form has been referred to the Liberian *B. maculatus* Hallowell by various authors,” but he did not suggest synonymy, observing that the type of *B. maculatus* cannot be traced, and that four specimens labelled as syntypes in the Academy of Natural Sciences, Philadelphia (now the Academy of Natural Sciences of Drexel University, hereafter ANSP), were never designated as such in the literature, and cannot be regarded as type material of *S. maculata*. Poynton (1964) based this conclusion on communications with E.V. Malnate of the ANSP, and examination of the material. Hallowell’s (1854) *Bufo maculatus* is in fact a replacement name of a specimen described by him (Hallowell, 1844) as *Bufo cinereus*, a name already occupied. It was part of a collection made by a Dr. Goheen from “Liberia, West coast of Africa”. The “syntypes” in ANSP were not collected by Dr. Goheen.

Despite the confusion over alleged type material, Tandy & Keith (1972) placed Mertens’ *S. pusilla* tentatively in the synonymy of *S. maculata* (Hallowell, 1854) as a member of a “*Bufo maculatus* group”, “which may be monotypic.” They wrote, “The status of *B. pusillus* is difficult to evaluate, especially since no crosses were made between it and *B. maculatus*, but there are no significant differences between the mating calls of populations which represent the two forms” (p. 160). On this rather slender evidence it became common practice to treat Mertens’ form as a synonym of what was conceived to be *S. maculata*. The procedure was adopted with misgivings by Poynton & Broadley (1988), who noted contra-indications and points of uncertainty but that “the principle of least disturbance of the nomenclature has to be conformed with” (p. 461).

Molecular analysis reported in this study indicates that widespread African material generally referred to *Sclerophrys maculata* (Hallowell, 1854), is divisible into at least two distinct clades. One clade comprises material from Sierra Leone, Ivory Coast, Senegal, Benin, Ghana, Nigeria and Cameroon (herein described as the ‘Western Clade’); the other comprises material from Angola, the Republic of the Congo, Central African Republic, Uganda, Kenya, Tanzania, Malawi, Zambia, Mozambique, Swaziland and South Africa (herein described as the ‘Eastern Clade’). The molecular evidence questions the common practice of treating eastern and southern material as conspecific with western material. The locality of Hallowell’s type of *S. maculata* is Liberia; therefore an east-west taxonomic separation raises the question of referring eastern and southern material to a species originally described from the Kruger National Park, South Africa as *Bufo regularis pusillus* Mertens, 1937. In this paper we analyse molecular, morphological and preliminary bioacoustic data in specimens currently ascribed to the species *S. maculata*. We aim to resolve issues regarding the naming of populations consistent with the data and provide descriptions of the relevant material to allow formal identification of animals in the field and from museum specimens.

**Methods**

**Molecular phylogenetic reconstruction.** Genetic data from 44 individuals from 18 countries were included in a multi-locus molecular phylogenetic reconstruction. Three mitochondrial and two nuclear markers were used: two ribosomal RNA genes 12S and 16S (c. 380 and c. 575 bp), and three coding regions; cytochrome-oxidase subunit 1 (COI; mitochondrial; c. 840 bp), C-X-C chemokine receptor type 4 (CXCR4; nuclear; c. 711 bp) and recombination activating gene-1 (RAG1; nuclear; c. 933 bp). Additional sequence data were generated for *Bufo bufo* to serve as an outgroup, and *Sclerophrys regularis*, *S. gutturalis* and *S. latifrons* to add context to the phylogenetic positioning of specimens. All sequence data were taken from Liedtke’s PhD thesis (2014; GenBank accession numbers in Appendix 1) and supplemented with *S. maculata* sequences available on GenBank (Benson et al., 2013). The sequence data for DQ158469 were not included because the concatenated 12S and 16S fragments appear to originate from different species. Data were processed in the bioinformatics platform Geneious Pro v5.6.7 (www.geneious.com; Kease et al., 2012).

Coding loci were aligned using the auto settings in MAFFT v7.017 (Katoh & Standley, 2013) and SATé (Liu et
al., 2012) was used to align 12S and 16S, using MAFFT as the aligner and RAXML with a GTRCAT model as the tree estimator, limiting the sub-problem size to maximum 50% of the dataset and implementing a stopping rule of ten iterations after the last improvement, and returning the best alignment. To find the open reading frame for coding genes, COI, CXCR4 and RAG1 were realigned and translated using TranslatorX (Abascal et al., 2010). Optimal partitioning scheme and nucleotide substitution models were determined for a dataset where all five genes were concatenated, and for the concatenated mtDNA and CXCR4 and RAG1 separately, using the greedy search algorithm in PartitionFinder v1.1.1 (Lanfear et al., 2012) based on Bayesian Information Criterion (BIC) scores, with branch lengths unlinked and testing only MrBayes-compatible models. Non-coding genes and each codon position for coding genes were treated as individual partitions (totalling to eleven partitions for concatenated dataset, five for the mtDNA and three for each of the nuclDNA datasets).

Phylogenetic reconstruction using Bayesian Inferences implemented in MrBayes v3.2.2 (Ronquist et al., 2012) was carried out on the four datasets, using the best partitioning scheme and substitution models suggested by PartitionFinder for each (concatenated dataset: [COIp3]-HKY+Γ; [12S, 16S, COIp1, COIp2, CXCR4p1, CXCR4p2, CXCR4p3, RAG1p1, RAG1p2, RAG1p3]-GTR+I; mtDNA: [12S, 16S, COI, COI, COIp2, COIp3]-GTR+Γ; CXCR4: [CXCR4p1, CXCR4p2, CXCR4p3]-HKY+Γ; RAG1: [RAG1p1, RAG1p2, RAG1p3]-HKY+Γ). For all inferences, four runs, each with one cold Markov chain and seven heated chains were executed for 10 million generations, sampling every 1000 generations on a Linux-based HPC cluster (Universitätsrechenzentrum, Basel). The metropolis coupling behaviour of the MCMC was optimized for the use of eight chains by increasing the number of chain swaps to four and reducing the temperature of the heating parameter to 0.05. An unconstrained branch length prior was used, sampled from the default exponential distribution with a mean of ten. The parameter traces and interactions as well as the convergence of the four runs were inspected using Tracer v1.6 (Rambaut & Drummond, 2007) and 1 million generations were discarded as burn-in before generating a 50% majority-rule consensus tree from the posterior tree samples, adding all compatible groups to the tree (contype=allcomp).

Intra- and inter-clade uncorrected pairwise-distances for 16S were calculated using the ape package v3.1-4 (Paradis et al., 2004), in R v3.0.2 (R core team, 2013).

Molecular species delimitation. *BEAST (Heled & Drummond, 2010) was used to estimate a species tree based only on samples for which all five genes were available (20 samples). Species assignments were based on the phylogenetic groupings recovered from the MrBayes inference (12 individuals for the Western Clade *S. maculata* and 4 for the Eastern Clade *S. pusilla*, see results). Site models and partitioning schemes were the same as for the MrBayes analysis, and clock and tree models were estimated separately for mtDNA and each of the two nuclear genes. Strict clocks were used, fixing the rate for the mtDNA clock to 1 and allowing the CXCR4 and RAG1 clocks to be estimated from an exponential prior distribution with a mean of ten. Yule process species tree priors were used with piecewise linear and constant root population size model priors. All *Sclerophrys* species were constrained to be monophyletic, to fix *Bufo bufo* as the outgroup.

Bayesian species delimitation was conducted using Bayesian Phylogenetics and Phylogeography (BPP) v3.1 (Yang & Rannala, 2014) to validate the proposed division of *S. maculata* and *S. pusilla*. The *BEAST* species tree was used as the guide tree and the MCMC was run for 20 000 iterations, sampling every 5th iteration after a burn-in of 10000. We used both the rjMCMC algorithm0 (c=2) and algorithm1 (α=2, m=1) and we tested four combinations of two sets of shape parameters for the population size (θ) and divergence time at the root (τ) priors [θ1; G(α=2, β=2000), θ2; G(α=2, β=200); τ1; G(α=27, β=1000), τ2; G(α=5, β=185)]. The different shape parameters for θ reflect small and large population sizes, and the τ priors both have roughly the same means to reflect a root height of 27 million years (Liedtke, 2014), but the degree of diffusion around this mean is greater for τ. For all analyses, sequences were split into three loci: a single concatenated mtDNA locus (12S, 16S and COI), CXCR4 and RAG1, a Dirichlet distribution for strong rate variation between loci (α=10) was used, auto fine-tuning parameters were selected (GBttj: 0.5, GBspr: 0.005, θ: 0.0006, r: 0.0004, mix: 0.06, locusrate: 0.2, seqerr: 1.0) and ambiguous sites were removed (cleandata=1). BPP was run twice per prior-settings to check consistency of posterior probabilities.

Morphological methods. The discovery of molecular differences between eastern and western individuals previously referred to as *S. maculata* raises the question of possible differences in morphology. To investigate this quantitatively, we performed linear morphometric analyses on 48 measured specimens assigned to either the Western Clade or Eastern Clade based on the genetic groupings recovered by the MrBayes analysis on the concatenated sequence alignment. Non-genotyped samples were assigned to either clade based on their relative
geographic location to the nearest genotyped sample. Nineteen adults from Sierra Leone, Senegal, Benin (genotyped), Liberia, Ghana and Nigeria were included in the Western Clade; and the Eastern Clade included 29 adult specimens from Tanzania, Zambia and Malawi, Mozambique (genotyped), South Africa (genotyped), Central African Republic (genotyped), Democratic Republic of the Congo and Namibia. Furthermore, nine adults were measured from the Blue Nile in Ethiopia. Representatives of Ethiopian material are not in our molecular tree and preliminary morphological comparison suggests strong deviations in certain characters (see results). These toads were therefore not grouped as either east or west and largely excluded from the analyses. The Western Clade material included two specimens from Monrovia, Liberia, collected by M.T. Tandy and identified by him as "B. maculatus". The Eastern Clade material includes the Senckenberg Museum (SMF) holotype and museum-designated paratypes of B. regularis pusillus, comprising five adults and two juveniles (SMF 22247, 30591-30596). Data were processed from only the five adults. Additional measurements for juveniles, not included in the analyses are also provided in Appendix 2. Apart from the SMF material, all specimens were selected from the collection of the Natural History Museum, London and the Museum für Naturkunde, Berlin.

Measurements were made by a single person (JCP) with mechanical callipers accurate to 0.02 mm, rounded to the first decimal. The following measurements were taken: snout-vent length (SVL), snout-urostyle length (SUL), tibia length (Tib); foot length (FL); head width (HW); internarial distance (InD); horizontal diameter of tympanum (Ty); width of parotoid glands (PgW); length of parotoid glands (PgL); width of the glandular part of upper eyelid (EW; which was taken to be a reliable measure of the length of the upper eyelid). The parotoid glands tend to be indistinct, making measurement problematic. The glands were measured as the continuous field of distinct large pores, which does not necessarily coincide with the complex surface topography. The dimensions are not always exactly the same on either side of the animal; the larger values were recorded. The snout-vent value was not used in calculations, since the position of the vent relative to the urostyle tip varies according to the state of preservation. The ‘urostyle tip’ measurement was taken from the skin surface where the tip was felt. Measurements were log10 transformed to better conform to normality. Nine morphological ratios were calculated and used as size-corrected morphometric characters: Tib/SUL, FL/SUL, HW/SUL, InD/SUL, Ty/SUL, HW/Tib, Ty/Tib, PgW/PgL and Ty/EW. Means, standard deviations and ranges for the Eastern and Western Clade were calculated and an Analysis of Covariance (ANCOVA) with sex (male or female) and group (east or west as determined by the molecular phylogeny) as covariates was used to determine statistically significant differences in ratios.

Bioacoustic methods. A comparison of the mating call structure of Western and Eastern clades was limited to a single published recording for each clade, one from Comoé National Park, Ivory Coast (S. maculata; Rödel 2000) and one from South Africa (S. pusilla; Preez & Carruthers, 2009). This is therefore not an extensive bioacoustic analysis, but meant to serve as a preliminary comparison of this important character complex, which should be investigated further in the future. Acoustic analysis was carried out in the R package seewave v1.7.6 (Sueur et al., 2008) with default, hanning window settings with 85% window overlap. Signal and pause period durations were computed with amplitude smoothing as well as an amplitude threshold to filter out background noises (10% threshold for S. pusilla and 40% for S. maculata). As only one calling individual of each clade and no further information was available, e.g. temperature during the recording, single calling male or calling while being surrounded by other males, the following comparison is kept purely descriptive and should be interpreted with caution.

Results and discussion

Molecular phylogenetics and species delimitation. Cunningham & Cherry (2000) found low mtDNA divergence (1.2%) between Swaziland and Ugandan material referred to S. maculata, but noted, “It remains to be seen whether a species boundary exists between Uganda and West Africa.” Our molecular data support evidence of a degree of divergence between Ugandan and Swaziland material, but a much more profound divergence is shown between eastern material (from Central African Republic east and southwards) and western material (from Cameroon westwards).
Bayesian Phylogenetic Reconstructions of Pan-African Sampling of Individuals of the Western (Sclerophrys maculata; Light Grey) and Eastern (S. pusilla, Dark Grey) Clade. Node labels refer to posterior probabilities (only shown if above 0.9) and specimens marked with an asterisk are also included in the morphological analysis. Tree A is inferred from a five-gene concatenated alignment and Trees B, C, and D are inferred from subsets of this alignment where Tree B includes only mtDNA (12S, 16S, and COI), and Tree C exclusively CXCR4 and Tree D exclusively RAG1.

Bayesian inference on the concatenated sequence data was able to recover a well-supported divergence between an Eastern and a Western Clade (Fig. 1A). The former comprise individuals from South Africa (Nelspruit, 165 km south of the S. pusilla type locality), Swaziland, Mozambique, Zambia, Angola, Republic of the Congo, Malawi, Uganda, Tanzania, Kenya, and Central African Republic, and the latter comprise individuals from Cameroon, Nigeria, Benin, Ghana, Ivory Coast, Sierra Leone, and Senegal. The Eastern Clade shows geographic structuring with geographic proximity of collection sites corresponding to phylogenetic proximity, but in the Western Clade, no strict geographic partitioning is evident. Maximum uncorrected pairwise differences in 16S nucleotide substitutions were 2.12% and 1.98% within the Western and Eastern Clade respectively and 3.18% between the two clades (Fig. 1A). Malone & Fontenot (2008) who analysed results of inter-species crossing experiments of a large number of toad species (original data from Blair, 1972), showed that post-zygotic isolation
can be achieved between species with as little as 2.7% pairwise differences in 12S and 16S sequences, which is more than the genetic distances recovered between the two clades here. The genetic differences recovered may thus reflect sufficient isolation for species-level distinctions. It should be noted however, that the analyses of Malone & Fontenot (2008) also highlight that hybridization in bufonids is common and possible even between distantly related species, with viable offspring resulting from parents with up to 12.3% pairwise differences.

The phylogenetic reconstruction on only the mitochondrial DNA confirms the division of the Eastern and Western Clades (Fig. 1B), however, neither of the two nuclear markers (CXCR4 and RAG1) were able to recover satisfactory support for this division (Fig. 1C and D). The lack of node support may largely be due to the fact that the alignments of both markers show low site heterogeneity, with only 31 segregating sites (out of 711) for CXCR4 and 24 for RAG1 (out of 933).

The *BEAST species tree strongly supports the division of the Western, *S. maculata* clade and the Eastern, *S. pusilla* clade as proposed by the MrBayes inference on the concatenated dataset (Fig. 2). Likewise, all BPP analyses, regardless of prior settings, strongly support the division of these two clades, in no case favouring a tree model where *S. maculata* and *S. pusilla* are collapsed into a single clade (Fig. 2, Table 1). The *S. regularis*-*S. gutturalis* relationship as sister taxa as proposed by the *BEAST* reconstruction is not supported and may need more extensive taxon-sampling to be resolved.

**TABLE 1.** BPP species delimitation algorithms and prior settings used, and supported topologies (with posterior probabilities). Tree topology 11111 refers to the fully resolved *BEAST* guide tree and topology 11110 is the same topology, but with the node for *S. gutturalis* and *S. regularis* not being supported.

<table>
<thead>
<tr>
<th>Algorithm</th>
<th>$\theta$ prior</th>
<th>$\tau$ prior</th>
<th>Best tree topology</th>
<th>Posterior probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>G(2 2000)</td>
<td>G(27 1000)</td>
<td>11111</td>
<td>0.999</td>
</tr>
<tr>
<td>0</td>
<td>G(2 200)</td>
<td>G(27 1000)</td>
<td>11110</td>
<td>0.599</td>
</tr>
<tr>
<td>0</td>
<td>G(2 2000)</td>
<td>G(5 185)</td>
<td>11111</td>
<td>0.998</td>
</tr>
<tr>
<td>0</td>
<td>G(2 200)</td>
<td>G(5 185)</td>
<td>11110</td>
<td>0.595</td>
</tr>
<tr>
<td>1</td>
<td>G(2 2000)</td>
<td>G(27 1000)</td>
<td>11111</td>
<td>0.999</td>
</tr>
<tr>
<td>1</td>
<td>G(2 200)</td>
<td>G(27 1000)</td>
<td>11110</td>
<td>0.601</td>
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<tr>
<td>1</td>
<td>G(2 2000)</td>
<td>G(5 185)</td>
<td>11111</td>
<td>0.999</td>
</tr>
<tr>
<td>1</td>
<td>G(2 200)</td>
<td>G(5 185)</td>
<td>11110</td>
<td>0.608</td>
</tr>
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**Morphological data.** Means, standard deviations and ranges of ratios obtained from the Western Clade, Eastern Clade and Ethiopian (Blue Nile) samples are given in Table 2 and visualized in Fig. 3. Standard deviations are included to indicate variability in the characters sampled. Measurements for all specimens including juveniles (not included in the data analysis) are given in Appendix 2.

Eastern and Western Clade animals show overlap in the ranges of all ratios (Table 2; Fig. 3). ANCOVA analyses, excluding Ethiopia, indicate sexual dimorphism in a number of characters (Table 3; Fig. 3; Appendix 3). Females tend to be larger in overall body size (SUL), but also in body proportions with a significantly wider head (HW/SUL; HW/Tib), intermandibular distance (InD/SUL), tympanum (Ty/SUL; Ty/Tib; Tymp/EW) and parotoid gland (PgW/PgL). Sexual dimorphism in parotoid gland width (compared to length) is weak compared to the inter-group effect, with Western Clade animals having significantly wider glands. This may prove to be a useful diagnostic character in the field. There is also a significant difference in the head width to tibia length (HW/Tib) ratio, which is a good indicator of overall body shape; the Western material can be described as tending to be more slender, although this is not clearly evident in handling individual specimens.

**FIGURE 3.** Plots of snout–urostyle length (SUL) and nine morphopometric ratios. Plots are grouped according to species (West = *Sclerophrys maculata*, light grey; East = *S. pusilla*, dark grey; and Ethiopia, black) and sex. All measurements were log10 transformed.

Morphometrically, the Ethiopian samples vary in relation to either Eastern or Western Clades, depending on the character examined (Fig. 3 and Table 2). Due to low sample sizes (3 males; 6 females), the Ethiopian specimens were excluded from significance testing, however Figure 3 suggests that these animals tend to have longer feet (FL/SUL), wider heads (HW/SUL) and smaller tympana (Ty/SUL; Ty/Tib; Ty/EW) than both the Western and Eastern animals. Although ratios tend to be more similar to Eastern material, we do not consider the material to show clear morphometric intergrading between east and west, and in the absence of molecular data we treat material from Ethiopia as *S. cf. pusilla*. In summary, taking into account overlap in the range of ratios among *S. maculata* and *S.*
It is possible that a single specimen cannot be definitively allocated to one of the species – and therefore the morphological data remains problematic for taxonomic diagnosis.

TABLE 2. Mean (x̄), standard deviation (σ) and range of morphometric ratios per clade. N denotes sample sizes.

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Western Clade (N=19)</th>
<th>Eastern Clade (N=29)</th>
<th>Ethiopia samples (N=9)</th>
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<tbody>
<tr>
<td></td>
<td>x̄</td>
<td>σ</td>
<td>range</td>
</tr>
<tr>
<td>SUL</td>
<td>1.742</td>
<td>0.082</td>
<td>1.592–1.872</td>
</tr>
<tr>
<td>Tib/SUL</td>
<td>0.778</td>
<td>0.007</td>
<td>0.768–0.793</td>
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<tr>
<td>FL/SUL</td>
<td>0.748</td>
<td>0.012</td>
<td>0.726–0.767</td>
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<tr>
<td>HW/SUL</td>
<td>0.741</td>
<td>0.025</td>
<td>0.266–0.355</td>
</tr>
<tr>
<td>Tb/SUL</td>
<td>0.366</td>
<td>0.029</td>
<td>0.32–0.419</td>
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<tr>
<td>HW/Tibia</td>
<td>0.972</td>
<td>0.017</td>
<td>0.941–1.006</td>
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<tr>
<td>Ty/Tibia</td>
<td>0.476</td>
<td>0.038</td>
<td>0.412–0.546</td>
</tr>
<tr>
<td>PgW/PgL</td>
<td>0.728</td>
<td>0.078</td>
<td>0.572–0.935</td>
</tr>
<tr>
<td>Ty/EW</td>
<td>0.747</td>
<td>0.058</td>
<td>0.676–0.849</td>
</tr>
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</table>

TABLE 3. Response variables (body size and morphometric ratios) that are significantly different between sexes and groups (Western versus Eastern Clade). P values correspond to F statistics of ANCOVAs where sex is the covariate (full table in Appendix 3).

<table>
<thead>
<tr>
<th>Response</th>
<th>Western Clade</th>
<th>Eastern Clade</th>
<th>Ethiopia samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUL</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>HW/SUL</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>InD/SUL</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>Ty/SUL</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>HW/Tibia</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Ty/Tibia</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>PgW/PgL</td>
<td>p&lt;0.05</td>
<td>p&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Ty/EW</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
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</table>

Non-morphometric morphological features. Non-morphometric characters that seemed potentially informative were: surface texture of parotoid glands, shape of parotoid and rictal glands, general skin texture, shape of the outer metatarsal tubercles, degree of webbing, and markings. These were chosen as an initial survey suggested they were of value in distinguishing Eastern from Western populations. Their diagnostic value diminished as the study progressed on account of variation shown in both Eastern and Western material. The only character that had moderate (but not absolute) diagnostic value is the shape of the parotoid and rictal glands. Generally the Western material presents a ventral bulge of the parotoid gland behind the tympanum, while in Eastern material the ventral border of the gland generally forms a more-or-less straight line. There may be a separated glandular mass in Eastern material that occupies the post-tympanic bulge characteristic of Western material, but this mass is not included in the measurement of the parotoid gland width.

In Western specimens the rictal glands tend to be larger; an upward curve of the glands generally terminates at the ventral-most level of the parotoid glands, a level generally not reached in Eastern material, but again this is not a constant difference. All that can be said is that the lateral parotoid and rictal glands are generally better developed in Western specimens. The surface texture of the parotoid glands varies from being smooth to spinous in both Eastern and Western material. The parotoid glands in the majority of specimens examined present a smooth surface (70% in east, 60% in west); where spine-tipped warts are present, the spines tend to be better developed in Eastern material but the east-to-west difference is not marked. The spines are more developed in females, which typically have several complete rosettes of spines in the urostylar region. Well-developed, dark-tipped para-urostylar rosettes are only rarely presented in males.
The outer metatarsal tubercle varies from being rounded to oval in both Eastern and Western material. No other east-to-west differences in subarticular tubercles were noted. Determining the extent of webbing is made imprecise by the edge of the web being wavy and irregular. Using the reach of the web between the fourth and fifth toes against the position of the distal tubercle of the outer toe, the edge just passes the tubercle in 25% of Eastern specimens, 35% in Western specimens. The extent of webbing cannot be used as a clear differential character in all the material. Therefore there appear to be no non-morphometric features that can be used to separate Eastern and Western specimens decisively.

Bioacoustics. In contrast to Tandy’s (1972) assessment, we noted potential differences in calls among the two clades. A comparison of the advertisement calls of an animal from Ivory Coast (Western Clade: S. maculata) and from South Africa (Eastern Clade: S. pusilla) suggest that the dominant frequency in S. maculata might be higher than in S. pusilla (median: 2.153 kHz versus 1.808 kHz, respectively). The S. maculata call might further consist of shorter notes and shorter inter-note intervals (mean±SD note: 0.463±0.160 sec; mean inter-note interval: 0.414±0.036 sec) compared to S. pusilla (mean note: 0.628±0.033 sec; mean inter-note interval: 0.713±0.100 sec). Notes of both species are pulsed, with S. maculata having a higher mean number of pulses per note (mean±SD of 121.0±6.557) than S. pusilla (101.8±5.263; based on the 5 notes of the same individuals per species). A spectrogram and oscillogram of 10 sec windows of the two recordings is shown in Figure 4. However, as both recordings include only fragments of continuously repeated calls without metadata (temperature; distance from calling individual, make and type of recording device), a more detailed bioacoustics investigation is needed to formally compare and describe the calls of S. maculata and S. pusilla, a potentially promising endeavour based on this preliminary result.

Distribution. The molecular data suggests the geographic separation of the Eastern and Western Clade lies between western Cameroon and western Central African Republic (Figs. 1A; 5). This roughly demarcates the western Lower Guinean Region (eastern Nigeria, Cameroon) from the eastern Lower Guinean Region (eastern Democratic Republic of the Congo), a boundary of biogeographic significance for other taxa as well (Mayr & O’Hara, 1986; Mausfeld-Lafdhiya et al., 2004; Köhler et al., 2006; Lorenzen et al., 2012), including congeneric species of the Sclerophrys superciliaris complex (Barej et al., 2011). For lowland forest taxa, the eastern and western Lower Guinean regions are thought to represent centers of Pleistocene refugia (Mayr & O’Hara, 1986) and it is interesting that S. maculata and S. pusilla, which are habitat generalists not restricted to tropical lowland forest, show similar biogeographic patterns. There are a number of substantial river systems opening into the Gulf of Guinea that have been shown to interrupt gene flow (Anthony et al., 2007) and more extensive sampling around this region may provide support for the importance of rivers as biogeographic barriers. Within each species there is substantial genetic structure as well (Fig. 1). In the case of S. pusilla, there is concordance with genetic and geographic clustering, with a north versus south differentiation. However, in S. maculata a geographical clustering is not evident despite the range traversing known biogeographic barriers such as the Dahomey Gap (Penner et al., 2011). Further research on these populations will be necessary to understand the phylogeographical patterns.

Taxonomy

It is possible to select a specimen from the Western and Eastern Clades and note a difference in, for example, shape of the parotoid and rictal glands; but on the other hand it is possible to select a specimen from each population and observe no clear difference between them. Broadly speaking then, morphological variation within the Eastern and the Western samples is about as great as the variation within the whole sample, which allows only limited separation at a statistical level (see details above). Nevertheless the morphological and bioacoustical evidence does give support to the molecular evidence of an east-west difference, and based on this combined evidence, we believe the division of the species range to occur in southern Cameroon (Fig. 5), but more extensive sampling is needed to draw conclusions on the precise biogeographic delineation. The differences in certain characters (e.g. parotoids) are significant between east-west populations, as well as general body proportions (head/tibia). This is in agreement with Hulselmans’ (1970) assessment of what he took to be S. pusilla: “In general it is more stockily built than maculatus.” He cited head width and length of the hind-limb, but he did not provide any measurements. In some “preliminary notes” on African bufonids, Hulselmans (1970) stated that S. maculata and S. pusilla “meet in Uganda and northeastern Congo where they intergrade.” This was based on “certain morphological and
biometrical characters,” which were not specified and so cannot be commented on. He also examined a series from the Ugandan highlands (BMNH 1934.12.15 244-247 & 249-251), which he identified as *S. maculata*. The Ugandan series is problematic in that the males have red spots on the thighs, which could indicate introgression with *S. gutturalis*. This series was thus not included in our study. One of the Ethiopian specimens included in this study (BMHN 1969.86) was examined by Hulselmans in 1970 and identified as *S. maculata*. Our results show this designation is questionable.

FIGURE 4. Ten-second windows of spectrogram and oscillogram of an *Sclerophrys pusilla* (above; South Africa, from audio CD in Du Preez & Carruthers, 2009) and *S. maculata* (below, Ivory Coast, from audio CD in Rödel, 2000). Inserts show magnification of a single pulsed note.

In tentatively synonymising *S. pusilla* with *S. maculata*, Tandy & Keith (1972) did not comment on contra-indications in the same volume (Blair, 1972) that blood proteins (transferrins) were “markedly different” (Guttman, 1972) and that there were differences in parotoid secretions (Low, 1972). The contra-indications were later reinforced by immunological evidence, which led Maxson (1984) to conclude that the *pusilla* and *maculata* gene pools have been separated for some five to six million years. This view was also recorded by Bachmann *et al.* (1980). In his unpublished Ph.D. thesis, Tandy (1972) did not refute the findings of Guttman (1972) and Low (1972), although he pointed out that Low’s material (misidentified as *S. latifrons*) included “a hybrid between *B. gutturalis* and *B. rangeri*” (p. 190). He did not add any more morphological data, yet concluded, “I have recordings of *B. maculatus* from more than twenty-five localities, including two in Rhodesia [Zimbabwe], and I have no evidence that indicates that *B. pusillus* is a species distinct from *B. maculatus*” (p. 195). Overall, the molecular, morphological and preliminary acoustic data we present provide evidence that two taxa are discernible in the available material. We thus advocate recognition of separate specific status. It seems advantageous at the present
time to assign material at least south of Ethiopia to *S. pusilla* (Mertens). But the name of Western material is problematic. The fullest discussion of the status of *S. maculata* was presented in Tandy’s unpublished Ph.D. thesis (Tandy, 1972). In summarising the confused history he pointed out that the four ANSP specimens designated in their collection as syntypes were not ever published as such, and no lectotype or neotype was subsequently published. Of the four, ANSP 2622-2625, #2625 is referable to *S. regularis*, i.e. possessing prominent parotoid glands. One of us (JCP) has been able to confirm this, also that the remaining three, while in poor condition and desiccated, especially ANSP 2623 and 2624, are probably referable to the species currently named *S. maculata*. None of these can be Hallowell’s specimen, having been collected by a different person. Hallowell’s specimen was not accessioned in the ANSP collection. Hallowell (1844) records material being “Put in the possession of S.S. Haldeman” and appears to be lost without trace.

In considering the validity of the name, Tandy (1972) made three main points. He noted that Hallowell’s description is “applicable but not diagnostic” (p. 192) regarding material currently named *S. maculata*. Secondly, regarding the type locality of “Liberia, West coast of Africa,” he wrote, “*B. maculatus* is the only species [of large *Sclerophrys*] known from the coastal zone,” so “It is therefore highly probable that Hallowell’s material was *B. maculatus*” (p. 193). Thirdly, since the time of Loveridge’s revival of the name in 1932 for Liberian material, the name has been in current use, so according to the principle of least disturbance of the nomenclature, it should be conserved. He concluded that the name *maculatus* should be used for the species, and wrote, “I plan to redescribe the species from Liberian material and to designate a neotype” (p. 193). Yet in the 42 years that have elapsed, the commonly used name is still not anchored in a type. We accept Tandy’s arguments for the conservation of the name, and we have taken it upon ourselves to designate a neotype from Liberian material that he collected and identified.

**Designation and description of a *Sclerophrys maculata* neotype**

*Bufo cinereus* Hallowell, 1844

*Bufo maculatus* Hallowell, 1854

**Neotype:** a mature male in the collection of The Natural History Museum, London, BMNH 1984.163, collected and identified by Mills Tandy from Monrovia, Liberia, 21 June, 1970, field number MT 918 (Fig. 6a).

**Dimensions** (in mm). Snout-vent length 68.8; snout-urostyle length 64.7; tibia length 24.4; length of foot 25.0; head width 22.6; horizontal diameter of tympanum 4.7; vertical diameter 5.2; length upper eyelid gland 8.4; internarial distance 3.5; length of parotoid gland 11.2; width of parotoid gland 6.9.

**Surface features.** Surface of parotoid glands barely raised above the general surface, with small dark-tipped asperities scattered between large glandular pits; glands separated from eyelids by dark-tipped warts; ventral edge roughly straight, without a clear post-tympanic bulge; a prominent rictal gland is curved upwards at its posterior end but separated from the parotoid gland by isolated dark-tipped warts; snout smooth, small glands with dark tips scattered over rest of head; back with larger dark-tipped glands; increasing in size posteriorly; some forming rosettes with small dark-tipped asperities; ventral surface without asperities; gular region with small rounded warts; remainder of ventral surface a flattened pavement; metacarpal tubercles flattened, outer larger, from which a row of light-coloured glands runs along the ventral surface of the forearm; a large patch of minute dark asperities on the inner surface of the first finger; inner metatarsal tubercle elongated, continuous with a tarsal fold; outer metatarsal tubercle elongated; edge of webbing irregular, passing well beyond the subarticular tubercle of the outer toe, just passing the distal subarticular tubercle of the third toe on the outer side.

**Markings.** Generally indistinct; a light interocular bar with incomplete anterior and posterior dark borders; a pair of incomplete dark patches in the scapular region, and scattered dark patches in the sacral region; no light patch above arm insertion, although rictal glands are lighter in colour; ventral surface immaculate but darkened in gular region.
FIGURE 5. Map showing the distribution of morphological (blue circles) and molecular (green squares) samples across Africa. Symbols with darker outlines were geo-referenced based on anecdotal collection information and are thus only approximations of the true collection site. The grey overlay depicts the IUCN distribution map of *Sclerophrys maculata*, partitioned into a Western (dark grey) and Eastern (light grey) clade, reflecting the phylogenetic divergence between *S. maculata* and *S. pusilla*. The precise contact zone is unknown and is only estimated here based on the available genetic data.

**Comparative description of the *Sclerophrys pusilla* holotype**

*Bufo regularis pusillus* Mertens, 1937

*Holotype*: A mature male in the collection of the Naturmuseum Senckenberg, Frankfurt, Germany, SMF 22247, collected by F. Haas on 4th September 1931, from ‘Letaba Camp’ in Kruger National Park, South Africa (Fig. 6b). Paratypes comprise two mature males (SMF 30592, 30594), two mature females (SMF 30591, 30593) and two juveniles (SMF 30595, 30596), all from the same locality in Kruger National Park.
Dimensions (in mm). Snout-vent length 49.9; snout-urostyle length 46.6; tibia length 18.2; length of foot 18.3; head width 18.2; horizontal diameter of tympanum 3.2; length upper eyelid gland 6.8; internarial distance 3.4; length of parotoid gland 9.8; width of parotoid gland 4.1.

Surface features. Surface of parotoid glands barely raised above general surface, no dark-tipped asperities over most of the glandular surface, a few on the periphery; glands separated from eyelids by small warts, some dark-tipped; ventral edge roughly straight, no post-tympanic bulge; rictal glands weakly developed, continued upwards by two isolated glands, but widely separated from the parotoid glands; dorsal surface of head with small, conical warts, mostly not dark-tipped; back with larger dark-tipped conical glands, increasing in size posteriorly, some formed into rosettes with small, dark-tipped asperities; ventral surface without asperities, covered with a pavement of small, rounded warts; metacarpal tubercles flattened, outer larger, from which a row of light-coloured glands runs along the ventral surface of the forearm; a patch of minute dark asperities on the inner surface of the first finger; inner metatarsal tubercle elongated, continuous with a tarsal fold; outer metatarsal tubercle elongated; edge of webbing irregular, passing well beyond the subarticular tubercle of the outer toe, level with distal subarticular tubercle of the third toe on the outer side.

Markings. A light interorbital bar without distinct dark borders, continuous with a light vertebral line extending to the sacral area; a pair of small, poorly defined darker patches in front of the interorbital bar, a pair of larger patches behind the bar and on scapular and sacral regions; a small light patch above the arm insertion, but not extending to the parotoid gland; rictal glands are lighter in colour; ventral surface immaculate but darkened in gular region.

Paratypes. Rictal glands variable in size, continued upwards by up to three isolated glands, but falling far short of the parotoid glands; females with larger dorsal glands than males, formed into rosettes from the scapular region to either side of the urostyle, where they are almost contiguous; no dark-tipped asperities on the whole of the back, including parotoid glands; a light patch may be present above the arm insertion, but never extending towards the parotoid gland; light vertebral line distinct to indistinct.

Remarks. The holotypes of *S. pusilla* and *S. maculata* were included in the morphometric analysis presented above. The hylopharynx of the *S. maculata* neotype was dissected by A.J.L. Lambiris on 27 July 1991, and described in Lambiris (1994). He noted, ‘There appear to be sufficient differences between the male and female hylopharynaxes of South African *Bufo pusillus* and of West African *B. maculatus* to support recognition of the two taxa as separate entities.’ (p. 284). Similarities between the larynx in a female from Lilongwe, Malawi, and that of a Liberian female led him to suggest that *S. maculata* extends southwards to Malawi; further examination by JCP and by Lambiris shows the Lilongwe specimen to be *S. gutturalis*.

As noted above, it is not always possible to distinguish specimens of *S. maculata* and *S. pusilla* by sight. Comparing the type specimens of the two species side by side, both males, size difference is the most obvious feature, but the *S. pusilla* male does not give the appearance of being fully grown, although it is sexually mature. An age difference is suggested by cornified tips on the fingers and toes of the *maculatus* type, while the fingers and toes of the *pusilla* type are quite delicate.

Related species. Perret (1977) and Bachmann et al. (1980) considered *S. danielae* Perret, 1977 from the Ivory Coast and *S. daniellae* (Perret, 1977) from Sierra Leone, as synonyms of *S. maculata*.
Coast to show closest affinity with *S. maculata* and *S. pusilla*. Perret distinguished *S. danielae* from *S. maculata* on its large, raised parotoid glands, and its smaller size. The prominent glands also separate it from *S. pusilla*. In micro-complement fixation analyses reported by Bachmann *et al.* (1980), the immunological distance between *S. maculata* from Ivory Coast and *S. danielae* was 12 units, and 10 units between *S. maculata* and *S. pusilla* from Kenya. They suggested that the three are more closely related to each other than to any other toad species studied, notably *S. regularis* and *S. gutturalis*, and that the immunological distance “between maculatus and danielae (or pusillus) corresponds to a period of 5–6 million years” since last sharing a gene pool (p. 182). *Sclerophrys regularis* and *S. gutturalis* tend to be regularly confused with *S. maculata* and *S. pusilla*. The former two are distinguished by a usually well-defined and often dark-bordered light band passing from the prominent parotoid gland to near the arm insertion. In the latter two there is at most a light patch above the arm insertion, not forming a band to the flattened parotoid gland.

**Conclusions**

The designation of a type specimen for *S. maculata* has never been clearly established in the literature. Here we designate a neotype. Furthermore, given the differences between Eastern and Western material in molecular, morphological and preliminary acoustic data, we resurrect the species *S. pusilla*. Based on current evidence, the species are found in non- or marginally-overlapping distributions (Fig. 5). *S. maculata* is found in western Central and West Africa whereas *S. pusilla* is found in eastern Central, East and Southern Africa. Compared to *S. maculata*, *S. pusilla* is stockier, with wider parotoid glands relative to their lengths and *S. maculata* has a higher pitched mating call with noticeably shorter note and inter-note durations, but a higher number of pulses per note. Populations from Ethiopia could not be assigned to either species due to a lack of genetic sequence data, but may prove to be a third species.

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SCLEROPHRYS MACULATA (HALLOWELL, 1854) REVISITED

http://dx.doi.org/10.1093/nar/gks1195


http://dx.doi.org/10.1080/21564574.2000.9650012


http://dx.doi.org/10.1093/molbev/msq027


http://dx.doi.org/10.1093/molbev/mst010

http://dx.doi.org/10.1093/bioinformatics/bts199


http://dx.doi.org/10.1093/molbev/mss020


http://dx.doi.org/10.1093/sysbio/syr095

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http://dx.doi.org/10.1371/journal.pone.0003900


http://dx.doi.org/10.2307/2408603


https://doi.org/10.7717/peerj.1553


SCLEROPHRYS MACULATA (HALLOWELL, 1854) REVISITED

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**APPENDIX 1. Specimen information and Genbank accession numbers for the molecular sampling in this study**

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### APPENDIX 2.

External morphological measurements (in mm) of snout-vent and snout-urostyle length (SVL; SUL), tibia and foot length (Tib; FL), head width (HW), internarial distance (InD), horizontal diameter of tympanum (Ty), width and length of parotoid glands (PgW; PgL), and length of the glandular part of upper eyelid (EW). Museum abbreviations are as follows: British Museum of Natural History (BMNH), Naturmuseum Senckenberg, Frankfurt, Germany (SMF) and Museum für Naturkunde, Berlin, Germany (ZMB). Specimens were assigned to either *Sclerophrys pusilla* or *S. maculata* based on genetic groupings (see methods section) and type material is indicated by superscript *(holotype)* and *(paratype)*. Where possible, sex of specimens is given (m=male, f=female) and whether or not the specimen is juvenile (juv) is also stated, along with geographic information for where individuals were collected.

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APPENDIX 2. (continued)

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ZOOTAXA
APPENDIX 3. ANCOVA tables for each morphometric variable/ratio for groups (west and east, excluding Ethiopia) with sex as a covariate. Level of significances correspond to *** <0.0001 ** < 0.001 and * <0.05.

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