Phylogeography of the genus *Xenopus* in southern Africa

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Abstract. *Xenopus laevis* (Daudin, 1802) has been the subject of numerous studies but is taxonomically poorly understood. Part of the Cytochrome *b* gene in mitochondrial DNA was sequenced from 50 individual *Xenopus* from three currently recognised species, taken from 28 localities in southern Africa, and from an outgroup of two West African *X. epitropicalis.* Phylogenetic analyses of these data reveal well-supported tree structure, demonstrating three clades within what is currently regarded as *X. laevis*: *X. l. laevis* from the Cape, *X. l. petersii*, and *X. l. laevis* from further north. This evidence agrees with other studies (on morphology, call and parasite data) which suggest that what is currently regarded as *X. laevis* encompasses more than one species. Workers using *Xenopus* collected away from the Cape of South Africa are not safe in assuming that they are using *X. laevis*.

Introduction

One species, *Xenopus laevis* (Daudin, 1802), has dominated the choice of developmental, cell and molecular biologists, although interests in its phylogeny have by comparison been neglected (Gurdon, 1996). Studies of *Xenopus* phylogeny have used interspecific hybridisation, analysis of hybrid chromosomes, karyological comparisons, immunological distances, osteology, restriction mapping of mitochondrial DNA (mtDNA), albumins, isozyme patterns, and electrophoretic patterns of sperm nuclear proteins (Graf, 1996). The results of these studies are not in complete agreement but have produced a good foundation for understanding the evolution and allopolyploid speciation within the group. In the most recent taxonomic review of the genus, which drew on a wide range of data including morphology, calls and parasite compatibility, Kobel et al. (1996) recognised 16 species. These were placed into four species groups: the sub-genera *Silurana* and *Xenopus*, within *Xenopus* the '*muelleri*' and '*laevis*' groups; with '*laevis*' comprising two main subgroups: '*fraseri*' and '*laevis*'.

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Xenopus laevis was described from what is now Western Cape Province of South Africa, and is currently regarded as having a wide distribution in central and southern Africa, made up of several putative subspecies. Kobel et al. (1996) recognised six *Xenopus laevis* subspecies (*laevis*, *petersii*, *poweri*, *victorianus*, *sudanensis*, *bunyonensis*). However, a later molecular study (Kobel et al., 1998), found that *X. l. laevis* was basal in the clade containing the other subspecies, concluding that it should placed at the species level. The rDNA of the other subspecies was so similar that subspecies status seemed adequate.

Southern Africa, as defined by Poynton (1964), is an area comparatively well sampled for pipids with two wide ranging species and a restricted endemic: *X. laevis*, *X. muelleri* and *X. gilli*, respectively (Poynton, 1964). Recent molecular studies have examined population variation within South African *X. laevis* and the comparative molecular phylogeny of *X. laevis* and *X. gilli*; Evans et al. (1997) used mtDNA, and found long-term isolation between two groups of *X. gilli* which they attributed to ancient habitat fragmentation by ocean transgression, whereas *X. laevis* from the same localities showed no such isolation trends. Grohovaz et al. (1996) found evidence to suggest that the southern winter-rainfall and northern summer-rainfall areas divided two groups of *X. laevis*, plus an isolated third group which displayed "the most divergent" mtDNA.

The genus *Xenopus* has an unusual mode of allopolyploid speciation through hybridisation which may be considered to be a confounding effect in molecular studies using mtDNA. However, this mechanism is only known to occur in central African *Xenopus,* where it has lead to 'reticulate evolution' and many polyploid species (Tymowska, 1991). In southern Africa, all *Xenopus* are tetraploid ($n = 36$) (Kobel et al., 1996) and hybrids of *laevis/muelleri* (Poynton and Broadley, 1991; Fischer et al., 2000) and *gilli/laevis* (Picker, 1985) are reported, but are sterile, and hence do not cause problems for a maternally inherited mtDNA study.

Xenopus laevis remains the central model amphibian in many biological studies, but the issues raised from recent mtDNA studies challenge its current specific status. Phylogeographic studies attempt to discover patterns of inter- or intra-specific genetic polymorphism, and using this approach, we sampled members of the genus *Xenopus* from an extensive area of southern Africa, to address the phylogenetic issues raised by Kobel et al. (1998) and Grohovaz et al. (1996): the relationship between *X. laevis laevis* and its neighbouring subspecies *X. l. petersii*, and the variation within the range of *X. l. laevis.* Sampling of all members of the genus from the region also allows confirmation of the relationship of *X. laevis* with sympatric *X. muelleri* and *X. gilli.* We use parsimony methods to analyse sequence data from the Cytochrome *b* (Cyt *b*) gene of mtDNA to propose a revised phylogeny for southern African *Xenopus.*

Materials and methods

Animals were collected at 28 localities from June 1998 to June 2000 using simple funnel traps (see fig. 1). Adult *Xenopus* were identified with the aid of Kobel et al. (1996). Toe clips were placed into 98% ethanol; toe clipping

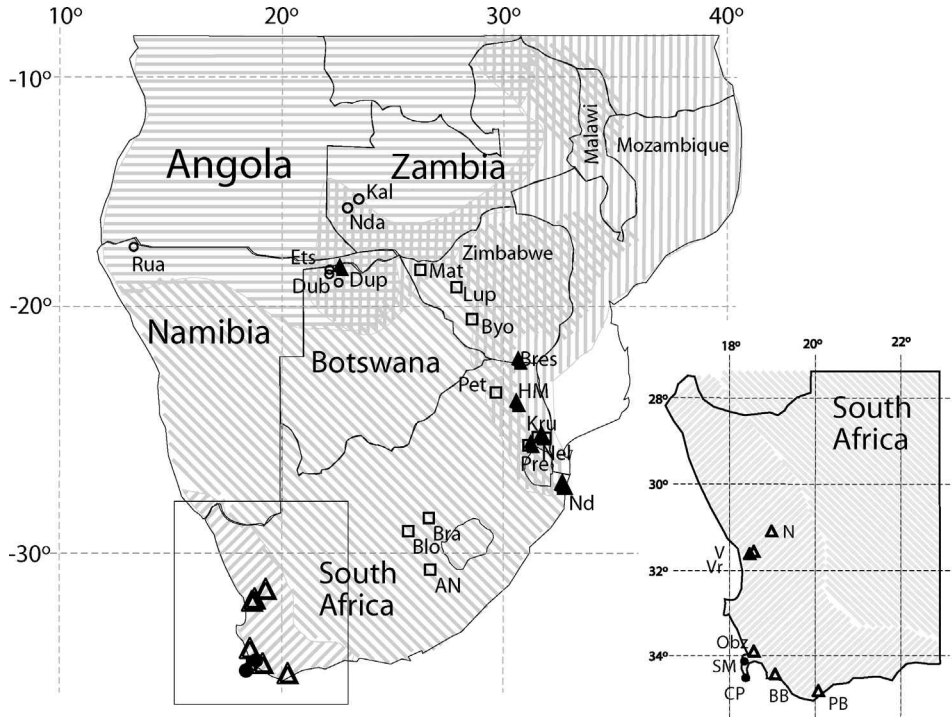

Figure 1. Map of southern Africa (inset of Cape region) showing collection localities and the suggested distribution of the genus *Xenopus* according to this study, Poynton and Broadley (1991) and Tinsley et al. (1996). *Xenopus l. laevis* (north) open square and left shading; *X. l. laevis* (Cape) open triangle and right shading; *X. gilli* closed circle; *X. muelleri* closed triangle and vertical shading; *X. l. petersii* open circle and horizontal shading. See fig. 2 legend for site abbreviations.

is the least damaging way to obtain tissue from live adults, and individuals are known to re-grow toes within two months (Measey, 2001). Tail clippings were made of larval *X. laevis* when adults could not be obtained. DNA from the endangered *X. gilli* was obtained with permission from South African National Parks. Tissues of *X. epitropicalis* were donated by M. Burger from a recent expedition to Gabon.

DNA Preparation, Amplification and Sequencing

In the laboratory, tissue was digested using extraction buffer and DNA extracted using standard phenol chloroform methods (see Hillis et al., 1996), which yielded adequate quantities of DNA suitable for PCR. Extracted DNA was dried, re-suspended in TE buffer, and stored at −20° C.

Sequences were amplified using the polymerase chain reaction (PCR) (94°C 30 s, 56°C 45 s, 72°C 1 min) for 34 cycles using a pair of 12S universal primers (12Sa 5′AAA CTG GGA TTA GAT ACC CCA CTA T 3′ and 12Sb 5′ GAG GGT GAC GGG CGG TGT GT 3′) and for 25 cycles (96°C 10 s, 50°C 5 s, 60°C 4 min) using a pair of Cyt *b* universal primers (Cyt *b* I 5′ CCA TCC AAC ATC TCA GCA TGA TGA AA 3′ Cyt *b* II 5′ CCC TCA GAA TGA TAT TTG TCC TCA 3′ — IDT®) in a Perkin-Elmer Geneamp 9600. This spans approximately half of the 12S gene and half of the Cyt *b* gene and yielded a sequence homologous to codons 2584 to 2904 (320 bp) and 16388 to 16677 (290 bp) respectively, in the published sequence of the complete *Xenopus laevis* mitochondrial genome (Roe et al., 1985). We consistently found the same four codons absent in all 20 specimens

sequenced of our 12S than that published in Roe et al. (1985). These base pairs were deleted when aligning the Roe et al. (1985) sequence.

PCR products were purified using a QIAquick PCR purification kit; cycle sequenced using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit; and the extension products purified using Centri-Sep™ columns (Princeton Separations). Products were sequenced on an ABI377 automated DNA sequencer (Applied biosystems) using BigDye terminator chemistry.

We undertook a pilot study of 20 samples, including all *Xenopus* species in southern Africa, based on 320 bp 12S and 290 bp Cyt *b*, to determine the suitability of these genes.

Data Analysis

We coded each alignment gap as a 'fifth base', and performed either Branch-and-Bound searches, or Exhaustive searches for the smaller data matrices. Bootstrap values were obtained within PAUP* (Swofford, 2000), and Bremer decay indices were derived using the program TREEROT (Sorenson, 1999). We have attempted to evaluate previous studies on the relationships of the species of *Xenopus*, using the maximum parsimony approach (see below). The trees were rooted on *X. epitropicalis* (following Cannatella and de Sá, 1993; Evans et al., 1997; Kobel et al., 1998).

Results

We first performed a parsimony analysis separately for the 12S and Cyt *b* data sets from the pilot study. Further sequencing of 12S was abandoned due to the low level of sequence divergence in this gene and further sequencing effort was concentrated on Cyt *b*. The Cyt *b* sequences obtained for all individuals were submitted to Genbank and assigned Accession Numbers AY217671 to AY217722 inclusive.

The maximum parsimony analysis produced 60 trees of 221 steps, with a Consistency Index (CI) of 0.61 and a Retention Index (RI) of 0.87. The bootstrap tree does not have exactly the same topology as the consensus tree (fig. 2), but the values indicate support for the appropriate clades. Likewise Bremer decay indices are only indicated for branches for which bootstrap values are available. Many branches were assigned values of one, but only some of these are indicated where bootstrap values are present.

The tree can be written as a statement of the relationships of the taxa (((*X. l. laevis*-Cape + *X. l. petersii* + *X. l. laevis*-North), *X. gilli*), *X. muelleri*), rooted on *Xenopus epitropicalis*. Starting at the base, the *Xenopus muelleri* clade is well supported with a decay index of seven, and bootstrap of 99%. This clade shows some resolution, with more eastern specimens from South Africa forming a monophyletic group (bootstrap of 83% and decay index of two). The single animal from Okavango, Botswana, is the sister group to the rest of this clade. The next clade consists of specimens of *X. gilli*, supported by a decay index of seven, and bootstrap of 100%. *Xenopus gilli* is the sister group to the terminal polytomy. The terminal polytomy consists of three well-supported clades. These represent *X. l. laevis* from the Cape (see fig. 1), *X. l. petersii*, and *X. l. laevis* from further north.

The Cape *X. l. laevis* clade is supported with a bootstrap value of 69%, but the decay index value of one indicates that the arrangement of terminal taxa is unstable. Two subgroups are recognised: a polytomy of specimens from Cape Town and its surrounds

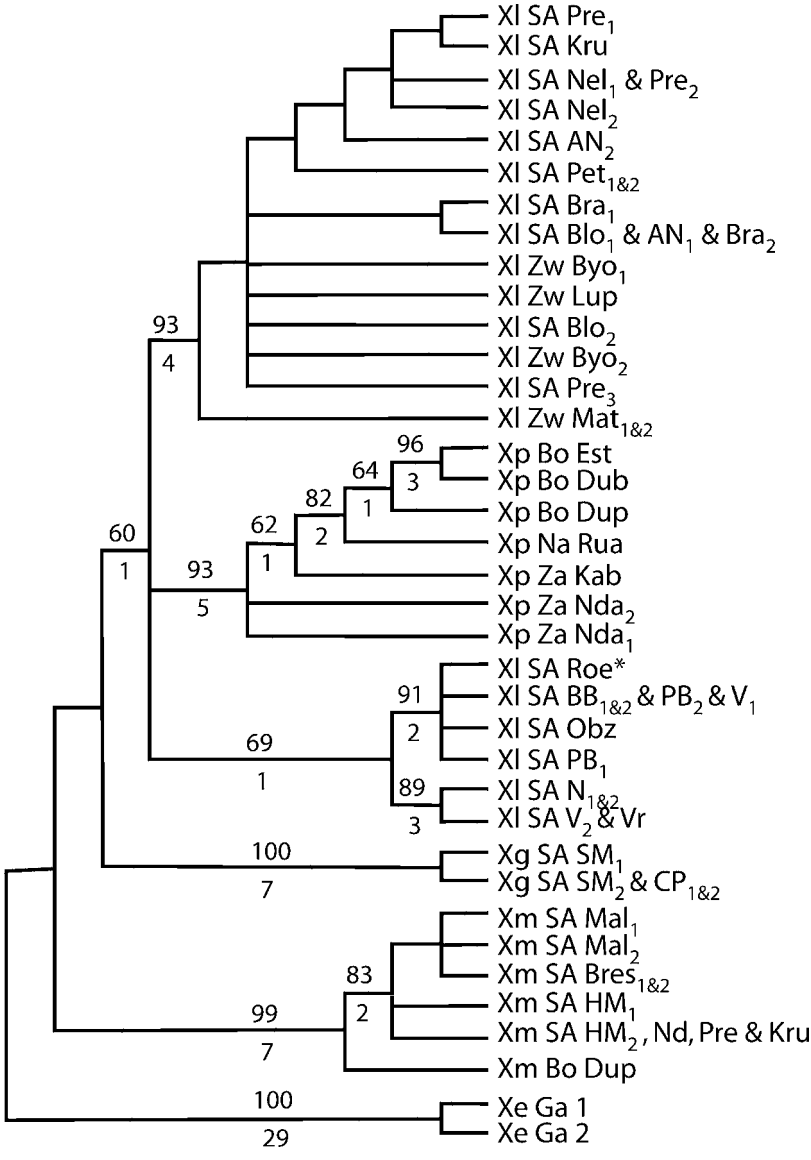

Figure 2. The consensus of 60 trees with 221 steps from maximum parsimony analysis (CI = 0.61 and RI = 0.87). Bootstrap values above 50% from 5000 replicates are shown above the branch, and the Bremer decay indices are shown below. Rooted on *X. epitropicalis.* (*Xl, Xenopus l. laevis*; *Xp, Xenopus l. petersii*; *Xm, Xenopus muelleri*; *Xg, Xenopus gilli*; *Xe, Xenopus epitropicalis.* SA, South Africa: Obz, Observatory; N, Nieuwoudtville; Vr, Vredendorp; V, Vredendal; SM, Silvermine; CP, Cape Point; BB, Betty's Bay; PB, Pearly Beach; AN, Aliwel North; Blo, Bloemfontein; Bra, Brandfort; Nd, Ndumu; Mal, Malelane; Pre, Pretorius Kop; Nel, Nelspruit; Kru, Kruger; HM, Hans-Merensky; Pet, Petersburg; Bres, Breslau; Roe* sequence from Roe et al. (1985). Zw, Zimbabwe: Byo, Bulawayo; Lup, Lupane; Mat, Matetsi. Bo, Botswana: Dup, Sepopa; Dub, Okavango; Ets, Etsutsa, Okavango. Na, Namibia: Rua, Ruacana. Za, Zambia: Nda, Ndau; Kal, Kalabo. Ga, Gabon.)

(including the sequence from Roe et al., 1985) supported by a bootstrap of 91% and a decay index of two, and a group from Niewoudtville supported by a bootstrap of 89%, and a decay index of three. Note that individuals from Vredendal (100 km southwest of Niewoudtville) occur in both subgroups.

The *X. l. petersii* clade is supported by a bootstrap of 93%, and a decay index of five. Within this group, some resolution is apparent, with the strongest support for the Okavango animals with a bootstrap of 96% and a decay index of three.

The third clade consisting of specimens of *X. l. laevis* from further north is also well supported with a bootstrap of 93% and a decay index of four. There is little resolution within this group, although the specimens from Bloemfontein and surrounds form a monophyletic group, and the specimens from Kruger Park and Petersburg form a second monophyletic group. Animals from Aliwal North fall into both of these groups. The specimens from Matetsi (near Victoria Falls), Zimbabwe are the sister group to the rest of this clade.

Discussion

Except for *X. laevis*, all of the currently described species are well supported as monophyletic groups (fig. 2). Very small variation within the Cyt *b* gene was found in *X. gilli* sampled from two sites in the Cape Peninsula National Park; this represents one of the two distinct clades, flanking False Bay, as described by Evans et al. (1997). Kobel et al. (1996; 1998) demonstrate that *X. muelleri* falls into two distinct groups, 'east' and 'west'. Variation in Cyt *b* for *X. muelleri* is small for individuals in South Africa which form a well supported monophyletic group. One other individual from Okavango, Botswana, falls outside this group, although we presume that all of these animals are *X. muelleri* 'east'. Our tree (fig. 2) is consistent with Kobel et al. (1998) with regard to the shared taxa.

Within what is currently regarded as *X. laevis*, we find a trifurcation of well supported clades, each with internal structure. This in itself does not suggest that each of these groups should be raised to specific level. However, the taxonomic status of *X. l. petersii* has long had a history of change. Bocage (1895) described it as *Xenopus petersii* in 1895, noting it as having three principal varieties based on the degree of ventral patterning: one of these (Var. B) was later described as *X. poweri* Hewitt (1927), without considering *X. petersii.* Use of ventral patterning is not reliable as a distinguishing character as even *X. l. laevis* shows a tendency to develop ventral spotting (Poynton, 1964). This is consistent with our own experience; that ventral patterning is highly variable both within and between populations. Parker (1936) described four races or groups of *X. laevis*, reducing *X. petersii* to a subspecies of *X. laevis* and suggesting that *X. poweri* is a synonym of *X. petersii.* Data from this study and that of Kobel et al. (1998) show that *X. l. laevis* and *X. l. petersii* (we include *X. l. poweri* in *X. l. petersii*) are sister taxa, and that *X. muelleri* is the sister group to this '*laevis*' group. Although Poynton and Broadley (1991) denote intergrades, our results

suggest that *X. l. laevis* and *X. l. petersii* are behaving like parapatric species rather than freely interbreeding subspecies. The remaining taxa, presently considered subspecies of *X. laevis*, could be a polytypic species complex with significant divergence occurring between the southern and northern forms (*petersii* and *sudanensis*).

The mtDNA gene tree should reflect population history where local populations are reciprocally monophyletic for their mtDNAs and diversity within populations is much less than among populations (Avise, 1989). The evaluation of mitochondrial DNA represents the relationships of female haplotypes and therefore the genealogical history of the populations sampled. However, Moritz et al. (1992) cautioned against using mtDNA patterns as the sole criterion for determining species boundaries. Kobel et al. (1996), in their review of *Xenopus* alpha-taxonomy, note that morphology, calls and genetic differentiation of *X. laevis* subspecies also suggest separation at the species level. *Xenopus l. laevis* is by far the largest subspecies with females on average being 36% larger than females of any other *Xenopus* species. The fundamental frequency and pulse rate of calling males differs from all other known *X. laevis* subspecies. Lastly, Jackson and Tinsley (1997) found that egg size of *Dollfuschella rodhaini*, a digenean parasite of *Xenopus* spp. was significantly different between *X. l. laevis* (collected from the Cape and Transkei of South Africa and Mazoe in Zimbabwe) and all other *Xenopus* species or subspecies examined. They concluded that parasite divergence demonstrates evolutionary distance as a relative measure of *X. l. laevis* from the other (more northerly) *X. laevis* subspecies. Our analysis is consistent with all available data from previous studies, despite the range of characters employed.

Xenopus l. laevis has a distribution encompassing much of southern Africa, over temperate, sub-tropical and tropical biomes (see fig. 1). In comparison to native *X. l. laevis*, feral populations of this subspecies have been relatively well studied and are known from United Kingdom, United States of America and Chile (McCoid and Fritts, 1980; Measey, 1998; Lobos and Measey, 2002), where conditions and temperatures also vary considerably. Climatic factors recorded for *X. l. laevis* in the Cape coincide with winter rain (July to September), and are opposite to those in Gauteng (September to the end of January), or further north in the tropics (Berk, 1938; Kalk, 1960). Grohovaz et al. (1996) who also found distinct mitochondrial haplotypes between these regions in *X. laevis* suggested that the isolation of northern and Cape types may have arisen from these opposing wet seasons. If this was the case, then we might expect animals from tropical regions to differ once again. However, our data show that northern *X. l. laevis* passes into Zimbabwe extending right up to the Zambezi River. The *X. l. laevis* distribution is reported to continue north into Malawi (Poynton and Broadley, 1991; Tinsley et al., 1996), which is also the locality for an unnamed subspecies (Vonwyl and Fischberg, 1980). No animals from this most northerly part of the distribution are included in this study and yet would be of great importance in any determination of taxonomic status of northern *X. l. laevis*.

Grohovaz et al. (1996) presented data from restriction enzyme analysis of mtDNA, and their analysis suggested an isolated group of *X. l. laevis* from Nieuwoudtville. We

performed an exhaustive search on their data matrix, which produced only one most parsimonius tree of 22 steps, with CI of 0.95 and RI of 0.95. Our analysis suggests a different arrangement of the five groups from the neighbour-joining dendrogram than they presented, where animals from the 'Nieuwoudtville pocket' (Cape 3) fall within the Cape *X. l. laevis* group. Our analysis of their data is consistent with that in fig. 2, showing a clade of northern *X. l. laevis*, not part of the Cape populations. We find that the population from Vredendal, which is situated 100 km away and 700 m down an escarpment, has the Nieuwoudtville haplotype as well as the more southerly haplotype. Animals from higher populations may have found their way down the escarpment and across the previously arid flats; Vredendal has recently been subject to increased irrigation due to growth in the wine industry. Thus we may expect that the Nieuwoudtville haplotype is spread throughout this area, and potentially further north and south. In this way the present distribution of *X. l. laevis* may have been significantly enlarged by human habitation. In this study, *X. muelleri* and *X. l. petersii* from Okavango were both found to have phylogenetic separation from the rest of their respective groups (fig. 2), and may signify that Okovango *Xenopus* are relatively isolated.

In conclusion, within southern Africa, there are three clades in what is currently regarded as *Xenopus laevis* which are strongly supported with Bremer decay indices (fig. 2). These represent *X. l. petersii*, *X. l. laevis* from the Cape and more northerly *X. l. laevis*. The consensus from literature (see above) indicates that *X. l. poweri* Hewitt 1927 is a synonym of *X. l. petersii* Bocage, and that distinctions on the basis of ventral patterning are not valid. Molecular evidence from this and other studies suggests that it is appropriate to re-instate *X. l. petersii* back to specific level: *X. petersii* Bocage 1895. The status of the type of *X. petersii* should be ascertained and a neo-type designated if necessary.

There is a deep split in what is currently considered *X. l. laevis*. From this analysis and that of Grohovaz et al. (1996), the split seems to coincide with rainfall patterns from Cape *X. l. laevis* (as defined by Daudin, 1802 and used by Roe et al., 1985) and a more northerly form which we have shown extends from at least Aliwal North (South Africa) well into the tropical range of *X. l. laevis* at Victoria Falls (Zimbabwe). This clade seems to represent a distinct taxon. However, it is not possible to conclude that there are two taxa within *X. l. laevis* on mtDNA evidence alone (Frost and Hillis, 1990), and further studies are needed to investigate the distribution and boundaries of these clades. On this evidence, we feel that it would not be prudent for investigators currently using *Xenopus* collected away from Daudin's type locality, in the winter-rainfall area of South Africa, to consider such materials *X. laevis*.

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