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Population-specific incidence of testicular ovarian follicles in *Xenopus laevis* from South Africa: A potential issue in endocrine testing

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ABSTRACT

The African clawed frog (*Xenopus laevis*) has been identified as an appropriate sentinel for testing endocrine activity of existing chemicals in North America and Europe. Some reports suggest that the herbicide, atrazine (CAS Number [1912-24-9]) causes ovarian follicles to form in the testes of this frog. *X. laevis* collected from North East (NE) sites in South Africa had testicular ovarian follicles, irrespective of exposure to atrazine, while frogs from Southwest Western (SW) Cape region sites had none. Phylogenetic analysis of mitochondrial and nuclear genes indicates that frogs from the SW Cape are evolutionarily divergent from those from NE South Africa and the rest of sub-Saharan Africa. These findings provide a possible explanation for why conflicting results have been reported concerning the impact of atrazine on amphibian sexual differentiation and highlight the importance of understanding taxonomic status of the experimental animal. Even in common laboratory animals, there is a need for their correct taxonomic characterization before their use in tests for endocrine disruption.

1. Introduction

Testicular ovarian follicles (TOFs, also reported in the literature as testicular oocytes; TOs) are a phenomenon that can be induced by exposure to estrogens such as 17β-estradiol and which have been suggested as a potential endpoint for characterizing exposures and responses to estrogenic compounds (Hecker et al., 2006; Jobling et al., 1998; Mackenzie et al., 2003). TOFs are female reproductive cells with an intact nucleus, nucleoli, and a surrounding squamous epithelial layer embedded in testicular tissue (Hecker et al., 2006) and can occur naturally but to varying degrees in the testes of some frog species (Reeder et al., 2005; Witschi, 1929, 1930, 1942). Results of studies on exposure to atrazine and its linkage to the incidence of TOFs fall into three categories. Some researchers report no incidence in unexposed animals but increased incidence of TOFs in developing larval frogs exposed to concentrations of ≥0.1 μg atrazine/L (Hayes et al., 2003). Others report the presence of testicular oocytes in unexposed as well as atrazine-exposed frogs in laboratory and field studies with no relationship to exposure concentration (Coady et al., 2005; Jooste et al., 2005; Murphy et al., 2006; Smith et al., 2005). Other studies report no incidence in frogs, whether they are exposed to atrazine or not (Kloas et al., 2009; Oka et al., 2008). In an ecoepidemiological study of the grey tree frog, *Acris crepitrans*, TOFs were observed in specimens collected both before and after the introduction of atrazine to the market (Reeder et al., 2005). The number of TOFs has been reported to decrease with age in *Xenopus laevis* (Everson, 2006; Jooste et al., 2005) and regressed TOFs are more frequently observed in older animals (Jooste et al., 2005). An EPA Science Advisory Panel recommended that, because of the uncertainty and differences in observations between laboratories and in the field, a definitive study of the phenomenon should be conducted (USEPA, 2003).

For many years, exports of *X. laevis* as laboratory animals took place from the W Cape area of South Africa (SA) (Tinsley and McCoid, 1996), but the origins of the many colonies of *X. laevis* in laboratories around the world are, for the most part, unknown. The taxonomic relationships among all species of *Xenopus*, including morphologically differentiated populations of *X. laevis*, have been characterized by both mitochondrial (mtDNA) and nuclear DNA (Evans et al., 1997, 2004, 2005, 2008; Grohovaz et al., 1996; Measey and Channing, 2003) and multiple putative subspecies

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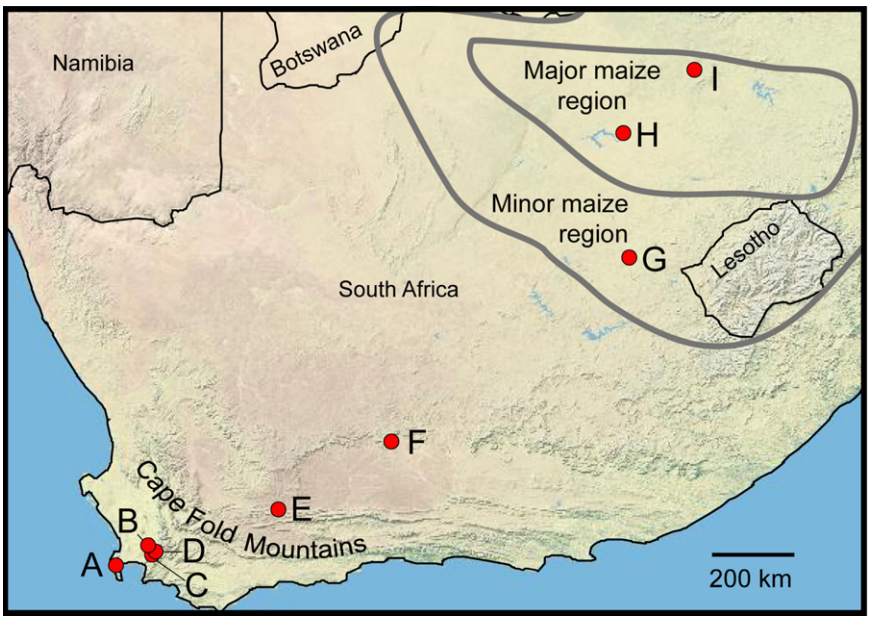

Fig. 1. Map of South Africa with collection sites A–I, major, and minor maize regions, indicated. For more details on the sites, see SI.

of *X. laevis* have been identified (Tinsley et al., 1996). Based on mtDNA, there are at least three divergent lineages in *X. laevis* within South Africa (SA) (Grohovaz et al., 1996). The distribution of these mtDNA lineages is potentially consistent with a role played by the Cape Fold Mountains (Fig. 1), which separate the southern winter-rainfall and northern summer-rainfall areas of SA, in restricting gene flow within *X. laevis*. However, relationships between these mtDNA lineages and the incidence of TOFs or molecular variation in nuclear DNA have not been investigated. As has been pointed out, correct identification of test organisms provides a baseline for extrapolation as well as avoiding the propagation of conceptual and methodological errors (Bortolus, 2008).

In this study, we explored the morphology, atrazine exposure, molecular diversity, and incidence of TOFs in *X. laevis* individuals collected in habitats in SA with little or no atrazine exposure, ranging from downwind and near to the major atrazine use-area in maize crops to areas that are upwind, far from this region, and that have no atrazine exposure—current or historical (Sites A–I, Fig. 1, see SI for details on sites). DNA sequences were obtained from representative individuals from these localities and from additional samples from throughout sub-Saharan Africa. We included other African species (*X. gilli*, *X. muelleri*, and *Scathophaga tropicalis*) in our molecular analysis as outgroups. In addition to these wild-collected frogs, we analyzed DNA from *X. laevis* from *Xenopus*-1 Inc. and *Xenopus* Express who supply this species to the north-American market and maintain colonies developed from *X. laevis* originally from suppliers in the W Cape (Weldon et al., 2007).

2. Methods

2.1. Collection of test animals and residue analyses

Four to 10 baited bucket *Xenopus* traps were set in the water bodies at the selected collection sites (B–I; see Fig. 1). Up to a maximum of 50 male frogs were collected at each site. Actual number of males collected varied between 12 and >50 (see SI). Females were also collected for the purposes of morphological characterization. Sediment and water samples were taken in the shallow water in the each of four quadrants of the pond. Water samples were pooled into two 1 L sub-samples collected in 1 L solvent rinsed (acetone and hexane) glass bottles. Water samples were stored at 4 °C (Eisenreich et al., 1994) until analysis of atrazine and terbuthylazine and chloro-metabolites in environmental samples, as well as other pesticides. Analyses for triazines and metabolites at Sites B–G were conducted with a method detection limit (MDL) of 0.025 μg/L by Dr. Robert Yokley (Syngenta Laboratories). Pesticide residues at Site I were characterized in a previous study (Du Preez et al., 2005a,b). Other pesticides and elements in sediment and water were analyzed with a MDL of 0.1 μg/L by the South African Bureau of Standards, a certified laboratory located in Pretoria, SA, using standard methods (AOAC, 1998). Briefly, the pH of the sample was adjusted to pH 7, extracted 3-x with dichloromethane and the extract dried over anhydrous sodium sulphate. The extract was evaporated to dryness and redissolved in hexane. Organophosphorus compound and triazines were quantified using GC-NPD; organochlorine pesticides, pyrethroids, and PCBs using GC-ECD. Identity was confirmed using GC-MS and duplicate recovery determinations were performed by adding known amounts of pesticides to a laboratory control sample and analysing these concurrently with the samples. The MDL was 0.1 μg/L. Water quality parameters were measured as previously Du Preez et al. 2005a; Du Preez et al., 2005b) and recorded (see SI for details).

2.2. Analyses of morphology, histology, and skeletochronology

Frogs were weighed (±0.01 g) and the snout-vent length measured. Malformations and other abnormal morphological characteristics were recorded. Male frogs were then dissected, the gonads examined for anomalies, measured, and photographed. One gonad was placed in a biopsy cassette and fixed in Bouin's fixative for 48 h then transferred to 70% ethanol for storage. Preserved testicular tissue was embedded, sectioned, and stained as previously described (Jooste et al., 2005) to produce serial sections (7 μm) of the entire testis. Every section was examined for the presence of TOFs. Age profile of specimens was determined by skeletochronology as previously described (Du Preez et al., 2005b).

2.3. DNA sequencing and phylogenetic analysis

Total genomic DNA was extracted according to Ivanova et al. (2006). The mitochondrial DNA barcodes (Hebert et al., 2003a,b) were generated as follows: PCR products were generated from the

5′ region of the cytochrome C oxidase I gene as 652 bp amplicons (corresponding to base positions 6474–7126 of the *Danio rerio* mitochondrial genome) using a pair of primer cocktails as detailed by (Ivanova et al., 2007). Cocktail components are modifications of the primers used by Folmer et al. (1994).

C_FishF1t1
VF2_t1 (Ward et al., 2005)
5′GTAAAACGACGGCCAGTCAACCAACCACAAAGACATTGGCAC3′

FishF2_t1 (Ward et al., 2005)
5′TGTAAAACGACGGCCAGTCGACTAATCATAAAGATATCGGCAC3′ (1:1 ratio)

C_FishR1t1
FishR2_t1 (Ward et al., 2005)
5′CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA3′

FR1d_t1 (Ivanova et al., 2007)
5′ CAGGAAACAGCTATGACACCTCAGGGTGTCCGAARAAYCARAA3′ (1:1 ratio)

The primers in these primer cocktails were tailed with:
M13F (Messing, 1983) 5′TGTAAAACGACGGCCAGT3′
M13R (Messing, 1983) 5′CAGGAAACAGCTATGAC3′

The recombination activating gene 2 (RAG2) was amplified with these primers:
Rag2.for.45 (5′CTGGGAGTAATA CATCACTGATC 3′)
Rag2.rev.1149 (5′CCTCGTCAAAAATGTTCCCGTCTCTG3′)

The hypervariable position of the androgen receptor (XLAR) was amplified with these primers:
XLAR.for.40 (5′AGGGCTCGGCGGGGTATACAAACAGC 3′)
XLAR.rev.431 (5′GGCGCTATCAGAGATGCCTTCG3′)

PCR reaction mixtures consisted of 6.25 μL of 10% trehalose, 3.0 μL of ultrapure ddH_2O, 1.25 μL of 10× PCR buffer, 0.625 μL of 50 mM $MgCl_2$, 0.125 μL of each primer (10 μM), 0.0625 μL of 10 mM dNTP mix, 0.06 μL of Platinum® Taq DNA polymerase (Invitrogen, Inc.), and 1.0 μL of template DNA. PCR amplification reactions were conducted on Eppendorf Mastercycler® gradient thermal cyclers (Brinkmann Instruments, Inc.). The mitochondrial PCR reaction program consisted of 2 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 40 s at 52 °C, and 1 min at 72 °C. The PCR program for both nuclear markers consisted of 35 cycles with the following profiles: 94 degrees 1 min, 55 degrees 1 min, 72 degrees 1 min, followed by a one cycle extension at 72 degrees. Upon completion of the 35 cycles, the thermal program concluded with 10 min. at 72 °C and then held at 4 °C.

PCR products were visualized on 2% agarose E-gel® 96 plates (Invitrogen, Inc.) and labeled using the BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.). Bi-directional sequencing reactions were carried out with the M13 resequecing primers for the mitochondrial DNA barcodes and with the regular PCR primers for the nuclear loci using an ABI3730 capillary sequencer.

Sequences were obtained from representative individuals with the same provenance as those in our morphological analyses. We also sequenced samples from additional location (Sites A and H within South Africa, Fig. 1, and other countries in Africa) where no morphological analyses were performed.

Data were aligned using SeqScape 2.1.1 (Applied Biosystems, Inc.) and MacClade (Maddison and Maddison, 2000). Specimen provenance and sequence data were organized and analyzed using the barcode of life data systems (BOLD Ratnasingham and Hebert, 2007). Heterozygous positions in the nuclear loci were resolved into individual alleles before analysis by assuming observed alleles in homozygous individuals were present in heterozygotes whenever possible (Clark, 1990).

Bayesian analysis was performed on each locus with MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001) using a model selected by MrModeltest (Nylander, 2004), which for CO1 was the general time reversible model with gamma distributed rate heterogeneity (Γ), for RAG2 was the Hasegawa, Kishino, and Yano model (HKY) + invariant sites (I) + Γ, and for AR was the HKY + Γ model. For RAG2, the Γ parameter was not used because this parameter distorted the scaling of branch lengths (although the tree topology was the same). Marcov Chain Monte Carlo runs were performed for 5,000,000 generations with a burnin of 1,000,000 generations, which was conservative based on analysis of posterior distribution of tree likelihoods. *S. tropicalis* was selected as an out-group based on previously published relationships (Evans et al., 2004).

2.4. Statistical analysis

Mann–Whitney rank sum test and Kruskal–Wallis one-way analysis of variance on ranks and Dunn's method for multiple comparisons were conducted with the aid of SigmaStat (Systat Software Inc., 2004).

3. Results

3.1. Pesticide exposures

Organochlorine, organophosphorus, and pyrethroid pesticides were not detected at any of these locations (see SI). Atrazine, its metabolites, and other triazines also were not detected, except at Site I, where low concentrations of atrazine, simazine, and terbuthylazine were observed (0.1, 0.4, and 0.3 μg/L, respectively). Because of the persistence of atrazine in surface waters (Giddings et al., 2005), these samples are representative of use within the previous 2 years in the watershed of lotic sites such as these. Exposure at Site I is consistent with other observations in this and other nearby sites (Du Preez et al., 2005a). Lack of measured exposures at the time of sampling at other sites is consistent with upwind location and lack of local maize production (and use of atrazine) in these areas where climate is unsuitable for this crop.

3.2. Morphology of frogs

There were morphological differences between the two major populations of frogs in SA and among sites within these regions. Male frogs collected in the W Cape region (Sites B–D) were significantly heavier, were longer, and had greater testis mass than those from the NE region (Sites E–G, and I; Fig. 2A and B, and SI Fig. 4, respectively, $p \leq 0.001$ Mann–Whitney rank sum test) but there were also significant differences among sites within this region (see SI). This is consistent with results of another study where intra- and inter-site variation was observed (Everson, 2006). Median gonadosomatic index (GSI—the weight of the testes as a percentage of the total body weight) of male *X. laevis* from W Cape region was significantly greater than those from NE region ($p \leq 0.001$ Mann–Whitney rank sum test, Fig. 2C). The masses of testes of male *X. laevis* at Sites G and D were significantly less than at other sites (see SI), resulting in small GSIs at these sites. Ages of frogs ranged from 1 to 6 years with a median age of 2 year in the NE region and 1 year at the W Cape region. Frogs from the NE region were significantly older than those from the W Cape region ($p \leq 0.001$ Mann–Whitney rank sum test, see SI Fig. 2, Table 2), due primarily to the values from Site E, which had more older *X. laevis* and where 1-year-old frogs comprised only 12% of the sample (see SI).

3.3. Incidence of TOFs

TOFs (Fig. 3) were observed in frogs from the mostly atrazine-free NE sites (Sites E, F, G, and I) but none were observed at the W Cape sites (Sites B–D). The total number of mature and regressed TOFs per individual varied among NE sites (Fig. 4), although not significantly ($p \geq 0.05$, Mann–Whitney rank sum test).

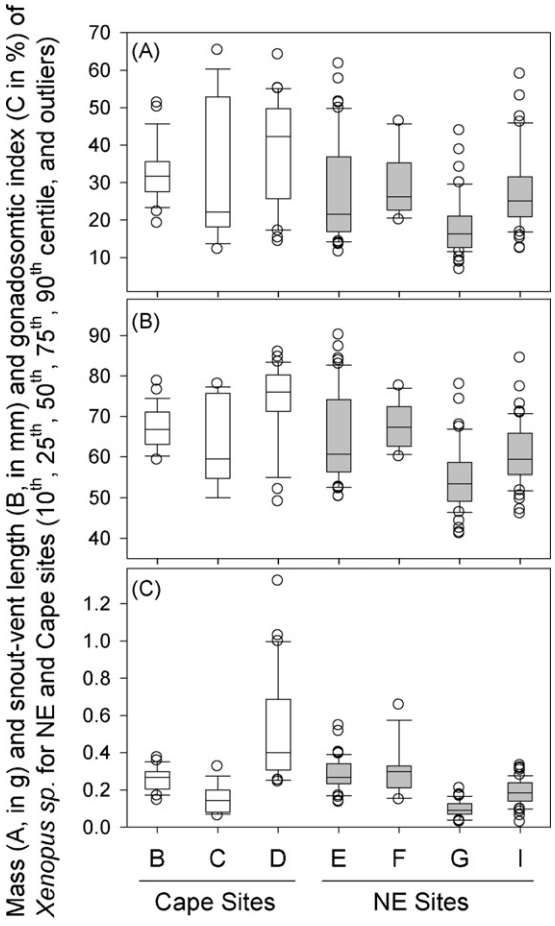

Fig. 2. (A) Mass, (B) snout-vent length, and (C) gonado-somatic index of male *Xenopus laevis* from the collection sites. Measurements were not taken on frogs from Sites A or H, or other locations in sub-Saharan Africa.

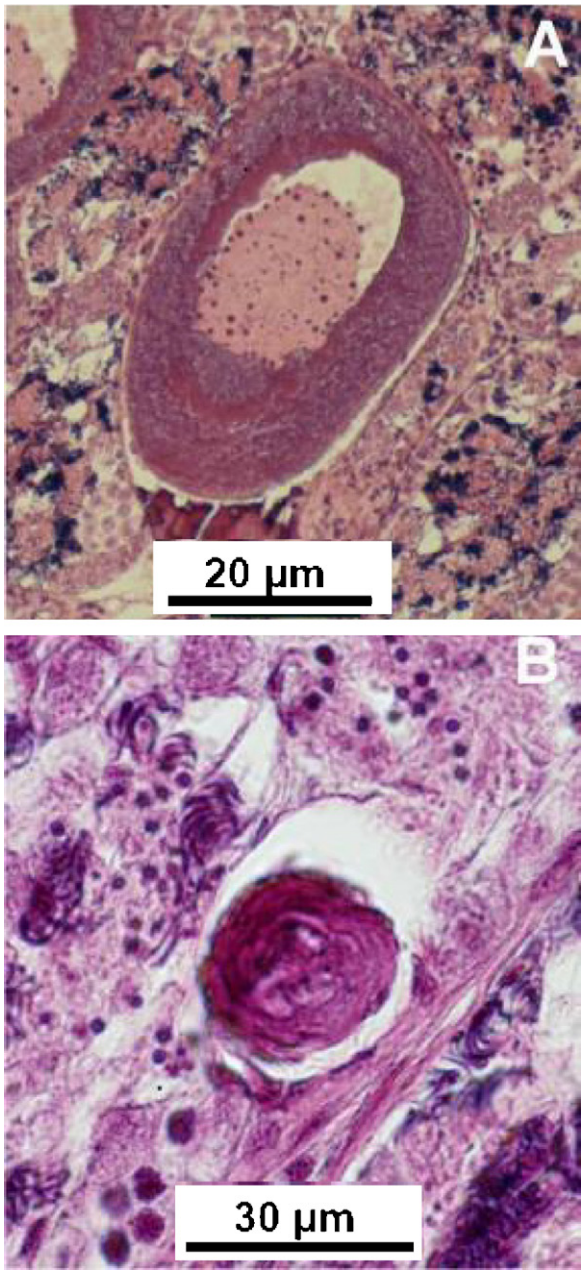

Fig. 3. Testicular ovarian follicles: (A) mature testicular ovarian follicle from a 2-year-old adult male *X. laevis* and (B) a regressed testicular ovarian follicle from a 4-year-old adult male.

3.4. DNA phylogeny

Molecular variation was geographically structured in *X. laevis* (Fig. 5). Within SA, at least two divergent populations existed, one in the W Cape at Sites A–D and the other in NE SA at Sites F–I, and there was a contact zone or cline between them at Site E. Over the entire range of *X. laevis*, four diverged mtDNA lineages were observed and were carried by (a) individuals from Sites A–D, and six individuals from Site E, (b) four individuals at Sites E and all from Site F, (c) individuals from Sites G–I and Malawi, and (d) individuals from the rest of the sub-Saharan samples (Botswana, Tanzania, Rwanda, Uganda, DRC, Congo Brazzaville, Cameroon, and Nigeria). The recombination activating gene 2 (RAG2) locus also has unique alleles in each of these four general regions and admixed variation at Site E is revealed by heterozygous individuals. The geographic distribution of variation in the hypervariable region of the androgen receptor (AR) locus is similar to RAG2, including heterozygous individuals at Site E, but with the exception that AR alleles at Site F and some individuals at Site E were identical to those found at Sites G, H, and I in NE SA. All sequences and specimen provenance data are archived in a publicly accessible project on BOLD titled: "*Xenopus laevis* geographical variation" (http://www.barcodinglife.org).

4. Discussion

Phylogenetic analysis of mitochondrial and nuclear genes indicates that frogs from the SW Cape are evolutionarily divergent from those from NE South Africa and the rest of sub-Saharan Africa. Site E was either a point of secondary contact or a cline between a population in the W Cape (Sites A–D) and a population in the rest of SA (Sites F–I) plus Malawi. Six mtDNA haplotypes from Site E were closely related to those from the W Cape and four were closely related to those from Site F (Fig. 5). Of nine genotypes at RAG2 and AR, none were homozygous at both loci for alleles from only one of these populations, one individual was homozygous at each locus for alleles from different populations and the others were heterozygous at one (one individual) or both loci (seven individuals) for alleles from both of these populations (Fig. 5). This indicates that these populations are reproductively compatible in nature but it is notable that the mitochondrial divergence values observed across these populations are characteristic of divergences observed between some Xenopus species (e.g., Evans et al. 2008). All commercially purchased individuals that we analyzed appear to be derived

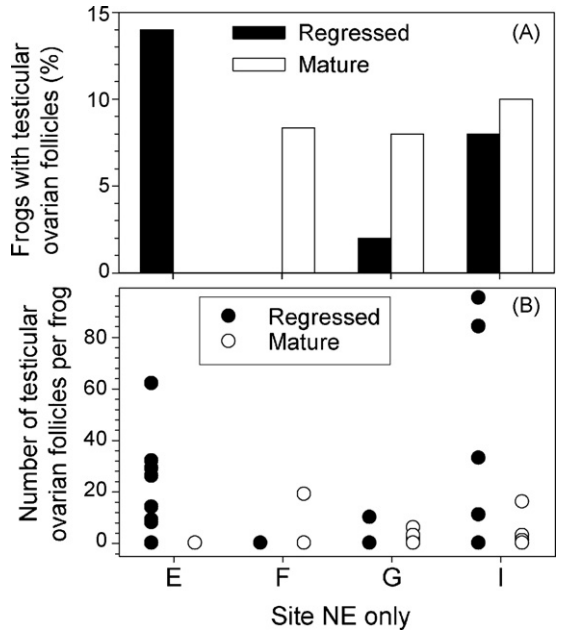

Fig. 4. (A) Prevalence of testicular ovarian follicles (TOFs) in *Xenopus laevis* from the NE sites. (B) Numbers of TOFs per frog. Atrazine was detected at concentrations ≥ MDL (0.025 μg/L) at Site I only. Mature and regressed TOFs were not observed in frogs from the W Cape sites (Sites B–D). Data on the incidence of TOFs were not obtained from frogs from Sites A or H, or other locations in sub-Saharan Africa.

from the W Cape population in that their mtDNA haplotypes and alleles at both nuclear loci clustered with wild-caught individuals sampled in this region.

This study has demonstrated a marked geographic variation in the incidence of TOFs in *X. laevis*. Specifically, mature and regressed TOFs were observed in frogs from NE sites but none were observed from W cape sites. Previous studies have reported that older frogs have fewer TOFs (Everson, 2006; Jooste et al., 2005), which may explain the presence of only regressed TOFs in *X. laevis* from Site E, where frogs were significantly older (see SI). These data, taken together with molecular divergence in mitochondrial and nuclear DNA, highlight geographic variation with respect to natural occurrence of TOFs in the absence of atrazine. The incidence of TOFs at Site I, where atrazine was detected, was not greater than incidence at the other atrazine-free NE sites. This is consistent with other observations on the effects of atrazine in *X. laevis* in laboratory studies (Coady et al., 2005), semifield studies (Everson, 2006; Jooste et al., 2005) and field studies conducted in locations close to and including Site I (Smith et al., 2005) and does not support an association between atrazine exposures and the incidence of TOFs in the portion of the range of *X. laevis* that is NE of the Cape Fold Mountains. Our findings also are consistent with the observations of others (Reeder et al., 2005; Witschi, 1929, 1930, 1942) that the occurrence of TOFs can be a natural phenomenon, and that its prevalence varies between species or populations, even those that are closely related. Because atrazine was not detected in sites from the W Cape, it was not possible to test for a relationship between incidence of TOFs in adults in this population and atrazine exposures in the field. However, TOFs were not observed in recent laboratory studies on *X. laevis* derived from this area and exposed to concentrations of atrazine between 0.01 and 100 μg/L from Nieuwkoop and Faber (NF) stage 46 to completion of metamorphosis (Kloas et al., 2009) or in *X. laevis* exposed to atrazine at a concentration range of 0.1–100 μg/L from NF stage 49 to completion of metamorphosis in a static renewal exposure design (Oka et al., 2008). The lack of response of TOFs in the NE and W Cape populations of X. laevis to exposure to atrazine is consistent with the lack of a plausible mechanism of action, either directly as a hormone mimic or indirectly. This and other possible effects of atrazine on frogs and other aquatic organisms is discussed in a recent review (Solomon et al., 2008).

Atrazine is not considered highly acutely toxic to larval *X. laevis* (Table 1). Based on the dates of these studies, the relatively few tests that have been reported in the literature were most likely conducted on *X. laevis* exported from the W. Cape region. The acute toxicity of atrazine in *X. laevis* from the NE has not been reported and their relative sensitivity in terms of traditionally measured responses is unknown. However, no adverse effects were observed at concentrations up to 30 μg atrazine/L in semifield studies (Jooste et al., 2005).

The absence of naturally occurring TOFs from *X. laevis* in the W Cape region must be considered when assessing the significance of reproductive/developmental endpoints in *X. laevis*. There is developmental variation between *X. laevis* populations in different parts of its sub-Saharan range, and this variation corresponds with evolutionary divergence between them (this study) (Evans et al., 2004; Grohovaz et al., 1996; Measey and Channing, 2003). That recent exports of *X. laevis* from SA only take place from the W Cape and Southern Cape region (Weldon et al., 2007) implies that recently established stock in the USA and other parts of the world is *X. laevis*

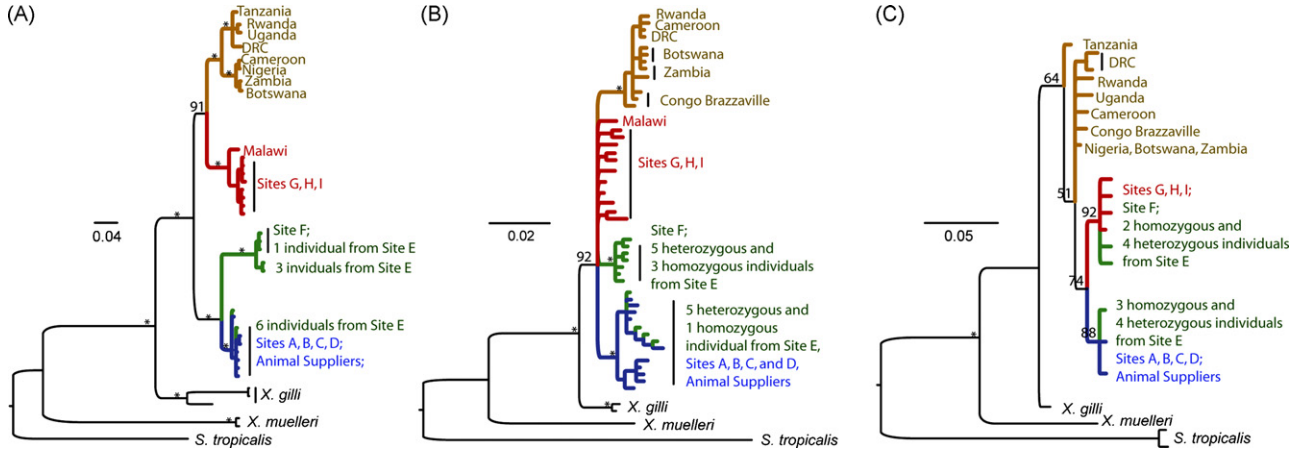

Fig. 5. Bayesian consensus phylograms of relationships between unique haplotypes and alleles of (A) mitochondrial DNA (mtDNA), (B) the recombination activating gene 2 (RAG2), and (C) the hypervariable region of the androgen receptor (AR). A total of 87, 75, and 119 *Xenopus laevis* individuals, respectively, were sequenced for each of these genes. Branches with posterior probabilities of interest that are over 90% are labeled and those greater than 95% are indicated with an asterisk. Sampling sites refer to those depicted in Fig. 1.

Table 1
Acute and chronic toxicity of atrazine to larval and tadpole stages of *Xenopus laevis*.

Effect measure (μg/L)	Endpoint[a]	Duration of study (d)	Response	Reference
100	LOEC	28	Increased time for development	Freeman and Rayburn (2005)
800	NOEC	35	Lethality	
100	LOEC	21	Uptake of propidium iodide in nuclei from exposed larvae	
1,100–3,030	LOEC	4	Larval development (FETAX)[b]	Morgan et al. (1996)
<8,000–33,000	EC50	4	Larval development (FETAX)[b]	
10,000–35,000	LOEC	2	Organogenesis[c]	Lenkowski et al. (2008)
100,000–126,000	LC50	4	Larval development (FETAX)[b]	Morgan et al. (1996)

[a] LOEC = lowest observed effect concentration; NOEC = no observed effect concentration.
[b] Values are the range of effect concentrations in buffered and natural water.
[c] Values are the range over which responses in various organ systems were observed.

laevis. This was confirmed by our multi-locus analyses of molecular variation in *X. laevis* obtained from the two major suppliers in the US. However, the provenance of older stocks is less clear and domesticated stocks potentially could even be derived from multiple geographic sources or their offspring. Moreover, laboratory crosses of different subspecies of *X. laevis* produce fertile and apparently normal progeny (Blackler and Fischberg, 1968; Blackler et al., 1965) and we detected evidence of genetic exchange between molecularly divergent populations at Site E. This may explain why studies in different laboratories have produced different results. The importance of correct identification of organisms used in ecological studies has been emphasized (Bortolus, 2008). This was the case for earlier onset of mammary tumors and dietary exposures of rats to atrazine, where a strain-specific mechanism was observed in Sprague–Dawley rats only (USEPA, 2000).

Non-lethal endpoints in chronic bioassays often have not taken differences between strains into consideration. Because responses in these types of assays may be dependent on physiological and developmental processes as well as toxicokinetic processes related to adsorption, distribution, metabolism, and excretion, it should be required that the test species be genetically characterized before extrapolating conclusions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2009.07.018.

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