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Absence of Extensive Introgression between *Xenopus gilli* and *Xenopus laevis laevis* (Anura: Pipidae) in Southwestern Cape Province, South Africa

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The evolutionary consequences of hybridization, including introgression, have been studied in many anuran amphibians (Lamb and Avise, 1986; Szymura and Barton, 1986), particularly in the African clawed frogs of the genus *Xenopus* (Tinsley, 1981; Kobel, 1996a; Picker et al., 1996). Species of *Xenopus* are found over much of sub-Saharan Africa and are mostly allopatric, though zones of sympatry and naturally occurring hybrids have been described (Picker, 1985; Tinsley et al., 1996; Yager, 1996). Although hybridization has been linked to allopolyploid speciation in *Xenopus* (Carr et al., 1987, Kobel, 1996b), the extent to which hybridization has facilitated genetic introgression among species has not been assessed.

The zone of sympatry between *Xenopus gilli* and *Xenopus laevis laevis* is of interest because *X. gilli* is a vulnerable species (International Union for Conservation of Nature, 1994) whose evolutionary integrity has been hypothesized to be at risk as a result of hybridization and competition with *X. l. laevis* (Kobel et al., 1981; Tinsley, 1981; Picker and de Villiers, 1989). These species are sympatric throughout the distribution of *X. gilli*, which is found in blackwater seepages in lowland-coastal fynbos habitat along the southwestern coast of Cape Province, South Africa (Picker and de Villiers, 1989). The range of *X. laevis*, in contrast, extends throughout much of Africa south of the Sahara. Because the breeding seasons of these taxa coincide, hybridization potentially could occur anywhere in the distribution of *X. gilli* (Kalk, 1960; Picker, 1985). Hybridization could contribute to a decline of *X. gilli* through loss of reproductive potential and dilution of the *X. gilli* genome with that of *X. l. laevis* (Tinsley, 1981; Picker and de Villiers, 1989; Picker et al., 1996). Habitat loss and resource competition with *X. l. laevis*, which has a wider ecological tolerance than *X. gilli*, may also have a deleterious effect on *X. gilli* (Simmonds, 1985; Picker et al., 1996).

To better understand hybridization between these species, we evaluated genetic introgression between *X. l. laevis* and *X. gilli* by using molecular and morphological markers. These data extend a previous project in which we examined the distribution of species-specific mitochondrial DNA (mtDNA) in these species (Evans et al., 1997), by providing a survey of nuclear DNA (nDNA) markers. We also have examined body patterning, coloration, size, and shape for each species, and for a naturally occurring hybrid and three laboratory F_1 hybrids.

Materials and Methods

The same individuals used in the previous study (Evans et al., 1997) from the Cape Point Peninsula, near Kleinmond, and near Pearly Beach served as subjects here (Fig. 1). *Xenopus l. laevis* also was sampled from a site north of Cape Town, outside the distribution of *X. gilli*. Wild individuals were caught with seine nets and collapsible funnel traps baited with bones. After blood samples, morphological measurements, and pictures were taken, frogs were released into their collection site. Voucher specimens were made for two *X. gilli* and seven *X. l. laevis* and contributed to the American Museum of Natural History (AMNH A153026–A153034). Preliminary species designations were made on wild-caught specimens and were based on presence or absence of dorsal patterning characteristic of *X. gilli* (Kobel et al., 1981; Picker, 1985). One individual that was much larger than a typical adult *X. gilli* female but that had dorsal patterning of *X. gilli* was identified as a putative hybrid. Tadpoles of each species collected near Pearly Beach were distinguished by patterning of melanophores on the head (Rau, 1978). Tadpoles were not included in morphological analysis.

To quantify introgression between nuclear genomes of *X. gilli* and *X. l. laevis*, we used taxon-specific restriction enzyme sites in two nuclear loci: the androgen receptor (AR) and the estrogen receptor (ER). The hypervariable region of the AR was amplified by polymerase chain reaction (PCR) with primers XLARbegin (ATG GCG GTG CAC ATA GGG CT) and XLARdwn2 (CGG GGG TCT CTT CGC TCT CCA), designed from AR mRNA sequence of *X. laevis* (GenBank U67129). Primers also were designed to amplify the hypervariable region in the first exon of the ER, based on sequence of ER mRNA from *X. laevis* (Weiler et al., 1987): XLERF1 (ACA AAC TAG CTG GAA CAG TGG

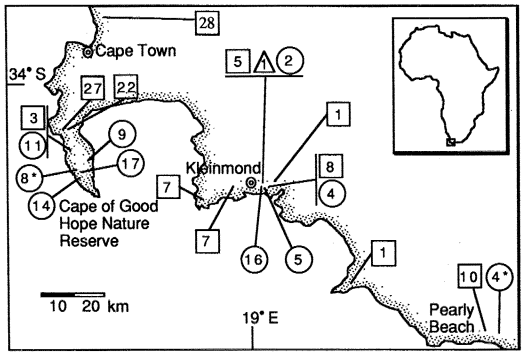

Fig. 1. Number of individuals and sampling localities in southwestern Cape Province. Circles (*Xenopus gilli*); squares (*X. l. laevis*); and a triangle (putative natural hybrid). Asterisk indicates *X. gilli* ponds where *X. l. laevis* has not been found.

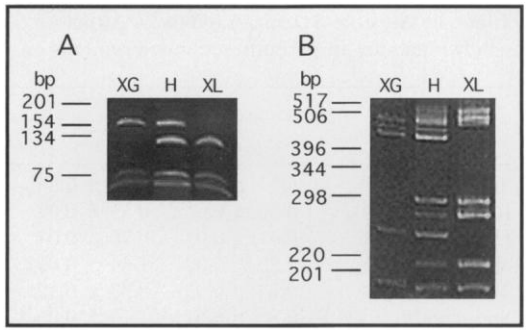

Fig. 2. Species-specific restriction enzyme digestion patterns of nuclear loci of *Xenopus gilli* (XG), a laboratory hybrid (H), and *X. l. laevis* (XL). (A) *Mnl* I digestion of hypervariable region of the androgen receptor. (B) *Rsa* I digestion of hypervariable region of the estrogen receptor.

ACA G) and XLERb7 (TTC CCT TAC TGC GAA AGT GCC CTG). Taxon-specific restriction sites were identified in *X. gilli* and *X. l. laevis* by screening each amplified locus with a battery of restriction enzymes. Complete digests were run on 5% polyacrylamide gels and stained with Cybergreen (Molecular Probes, Inc.). After identifying a species-specific *Mnl* I site in the AR and a species-specific *Rsa* I site in the ER, homozygosity and hybridization-induced heterozygosity could be determined by digesting amplified loci with the appropriate enzyme (Fig. 2).

Hybrid animals were generated in the laboratory using in vitro fertilization (Tabtl and Poo, 1991). Crushed testes from male *X. l. laevis* were mixed with eggs from female *X. gilli*. Ovulation was induced with 300 IU of human chorionic gonadotropin (Sigma). Amplified PCR product from laboratory hybrids generated from three different crosses was used as a control to ensure taxon-specific alleles could be amplified concurrently from hybrid DNA in a single PCR reaction and to ensure that alleles of each locus were inherited biparentally in hybrids.

Because the intermediate nature of hybrid morphology can be diminished by unidirectional backcrossing (Kobel, 1996a), we used molecular data to identify "pure" individuals from hybrid and backcrossed individuals. Photographs of dorsal and ventral surfaces of 86 pure *X. gilli* (64 females and 22 males) and 109 pure *X. l. laevis* (43 females and 66 males) were examined for interspecific variation in body coloration and patterning. Nine morphological measurements were obtained to evaluate interspecific differences in body size and shape. Measurements around the head were taken to reflect differences in shape. Mean differences in size were assessed with Student's *t*-tests (characters normally distributed) or Welch ANOVAs (characters nonnormally distributed), using the Statistical Analysis Systems JMP program, version 3.1 (SAS Institute, Inc., Cary, NC, unpubl., 1995). Groupwide type I error was controlled after Rice, 1989. Measurements that were normally distributed in both species, as determined by the Levene's test, were subjected to principal components analysis (PCA) for each sex. Because a major component of the variation was represented along the first eigenvector, an indication of the effect of size differences (Voss et al., 1990), we divided measurements by snout–vent length to standardize for size before again carrying out PCA (Atchley et al., 1976).

Three F_1 hybrids (one female, two male) generated in the laboratory survived metamorphosis and were included in morphological analysis. Measurements were taken on the wild-caught putative hybrid when it was captured in 1994 and again in 1997 after 2.5 years of laboratory care.

Results

Results from assays of nuclear loci were similar to those from mtDNA analyses (Evans et al., 1997): all 90 *X. gilli* were homozygous for *X. gilli*-specific alleles at the AR and ER loci; and all 119 *X. l. laevis* were homozygous for *X. laevis*-specific alleles. The morphologically intermediate individual was heterozygous at both loci and had *X. gilli* mtDNA (Evans et al., 1997). We did find an uncommon AR allele in some tadpoles of *X. gilli* collected near Cape Agulhas that was not detected in other *X. gilli* or in *X. l. laevis*. In one individual, this allele was heterozygous with the *X. gilli* allele found west of Pearly

TABLE 1. MEAN ± STANDARD ERROR OF MORPHOLOGICAL MEASUREMENTS (cm) AND WEIGHT (gm). Numbers of individuals are in parentheses; measurements on wild-caught female hybrid taken twice at different times.

	Females			Males		
	X. gilli (64)	Hybrids (2)	*X. l. laevis* (43)	*X. gilli* (22)	Hybrids (2)	*X. l. laevis* (66)
Snout vent	5.11 ± 0.08	6.43 ± 0.38	8.63 ± 0.23	3.86 ± 0.05	3.56 ± 0.52	6.35 ± 0.13
Interocular	0.50 ± 0.01	0.55 ± 0.01	0.94 ± 0.03	0.40 ± 0.01	0.34 ± 0.02	0.73 ± 0.02
Eye width	0.51 ± 0.01	0.76 ± 0.04	0.80 ± 0.02	0.46 ± 0.01	0.50 ± 0.07	0.67 ± 0.01
Mouth	1.22 ± 0.02	1.64 ± 0.09	2.25 ± 0.05	1.00 ± 0.02	0.95 ± 0.07	1.77 ± 0.04
Torso	2.64 ± 0.05	3.35 ± 0.12	4.59 ± 0.13	1.71 ± 0.03	1.63 ± 0.25	3.11 ± 0.06
Snout width	0.82 ± 0.01	1.10 ± 0.05	1.48 ± 0.04	0.70 ± 0.01	0.71 ± 0.08	1.19 ± 0.03
Snout–front eye	0.34 ± 0.01	0.40 ± 0.01	0.53 ± 0.02	0.29 ± 0.01	0.27 ± 0.01	0.44 ± 0.01
Eye length	0.31 ± 0.01	0.49 ± 0.07	0.48 ± 0.01	0.25 ± 0.01	0.29 ± 0.02	0.40 ± 0.01
Left elbow–finger	1.59 ± 0.02	2.14 ± 0.07	2.81 ± 0.07	1.33 ± 0.02	1.33 ± 0.12	2.45 ± 0.05
Weight	11.93 ± 0.48	31.90 ± 3.32	60.63 ± 4.04	4.91 ± 0.14	4.20 ± 1.22	26.86 ± 1.42

Beach. Two other individuals (of four tadpoles analyzed from Pearly Beach), were homozygous for the uncommon allele. Based on sizes of digested fragments, the uncommon allele has at least one less restriction site than the common AR allele of *X. gilli* and at least two less than the AR allele of *X. l. laevis*. Five F_1 hybrids (including the three that survived metamorphosis plus two tadpoles) each had *X. gilli* mtDNA and were heterozygous at both nuclear loci. These results confirm the sensitivity of the techniques used to evaluate heterozygosity and homozygosity.

Ventral patterning of *X. l. laevis* and *X. gilli* can be similar and thus did not serve as a good marker for assessing introgression. In three, molecularly identified pure *X. gilli*, the yellow with black vermiculation coloring typical of *X. gilli* was faded in the anterior of the animal, resembling a shade commonly seen in *X. l. laevis*. Ventral coloration of molecularly identified pure *X. l. laevis* usually was silvery-yellow over most of the belly and the ventral surface of the head. However, silver-yellow or pink with yellow vermiculation occurred in the inguinal region on 54 *X. l. laevis* and on the entire ventrum in 11 *X. l. laevis*. The field-collected putative F_1 hybrid had *X. gilli*-like dorsal patterning observed also in laboratory-generated hybrids and *X. gilli*-like patterning on the ventrum.

Xenopus gilli and *X. l. laevis* differ in size (Table 1). Females and males of *X. l. laevis* were significantly larger than those of *X. gilli* in every measurement (Student's *t*-test, or Welch ANOVA, $P < 0.05$). Measurements of intraocular distance, eye width, mouth, snout to front of eyes, eye length, and torso were normally distributed in females of both species; intraocular distance, eye width, mouth, snout to front of eyes, eye length, and snout width were normally distributed in males of both species. In PCA of normal measurements, members of each taxon clustered in overlapping but discrete groups, with most of the variation represented along the first eigenvector (Fig. 3). When measurements were divided by snout–vent, all female ratios except eye width/snout–vent and snout width/snout–vent were normal, as were all male ratios. PCA of ratios had greater species overlap, particularly among female ratios (data not shown), though these distributions were still suggestive of a distinct morphology in each species.

The wild-caught, putative F_1 hybrid and a female, laboratory hybrid occupied intermediate positions between females of each species in both unstandardized (Fig. 3A) and standardized analyses. Measurements taken on the wild-caught female hybrid after 2.5 years in captivity, during which period significant growth had occurred, were closer to those of *X. l. laevis*. The laboratory males that were 2.5 years old, and possibly not fully grown, appeared intermediate and/or *X. gilli*-like in both unstandardized (Fig. 3B) and standardized analyses.

DISCUSSION

Even low levels of genetic introgression are expected to prevent different neutral alleles at the same locus from becoming fixed in two populations (Slatkin, 1987). However, with the exception of a single putative F_1 hybrid, mtDNA haplotypes in our study were partitioned in a nonoverlapping, species-specific manner (Evans et al., 1997), and two nuclear loci were fixed for species-specific alleles in both species. The naturally occurring putative F_1 hybrid had a *X. gilli* mtDNA haplotype, indicating it had a *X. gilli* mother. Though these molecular markers will not detect all backcrossed hybrid progeny, morphological analyses presented here also suggest

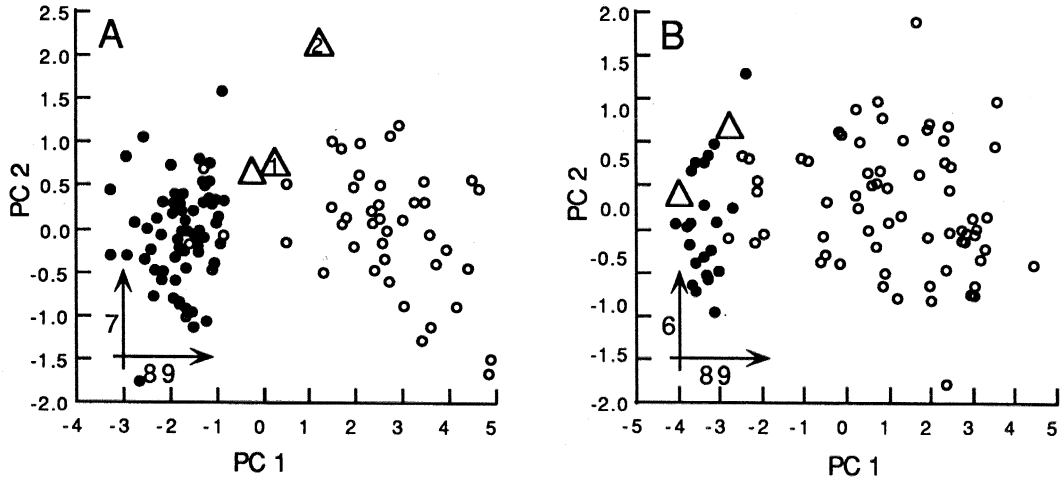

Fig. 3. Principal components analysis of unstandardized morphological data of females (A) and males (B). Individuals of *Xenopus gilli* are designated by (●), natural and laboratory hybrid individuals by (△), and *X. l. laevis* by (○). Measurements taken at time (1) and (2) on the same naturally occurring female hybrid are depicted inside triangles. Percent of the variation represented by each axis is shown in the bottom left corner of each graph.

that *X. gilli* and *X. l. laevis* are distinct species with respect to size and shape. Taken together, these results suggest that introgression between these taxa does not, at present, make a significant contribution to the genome of either.

Environmental factors may facilitate hybridization between these species. *Xenopus l. laevis* and *X. gilli* respond differently to water pH and human disturbance, and hybrids can tolerate more acidic water than can *X. l. laevis* (Picker et al., 1993). However, the frequency of hybrids reported here is much lower than those of previous studies (Picker, 1985; Simmonds, 1985; Picker et al., 1996). One possible explanation for the latter is that researchers and conservation officials have been removing naturally occurring putative hybrids and *X. l. laevis* from ponds in *X. gilli* habitat to preserve species integrity of *X. gilli*. Trapping of these animals has been going on since 1983 in the Cape of Good Hope Nature Reserve and in some areas east of False Bay. Every pond in which only *X. gilli* was caught in 1994, except Gilli Dam in the Cape Point Nature Reserve and a pond near Pearly Beach, is known to have contained *X. l. laevis* and/or hybrids in the recent past (Picker, 1985; Simmonds, 1985; Picker and de Villiers, 1989; unpubl. data). Though both parental species are long lived (15+ years in captivity; Tinsley et al., 1996), yearly fluctuation in hybrid populations could account for variable frequencies of hybrids over time. Another explanation for the difference in hybrid frequency is incorrect classification of hybrids, especially backcrossed hybrids, in other studies. "*Xenopus gilli*-like" and "*X. laevis*-like" hybrids reported in Picker et al. (1996), for example, could be variants within each parental species, rather than backcrossed intermediates. Additionally, low fitness of hybrids may provide an obstacle to hybrid backcrossing and introgression. First-generation hybrid males are known to be sterile, though both sexes of F_2 progeny can be fertile (Kobel, 1996a). Furthermore, different mating calls of hybrid males, relative to those of the parental species (Picker et al., 1996), may not attract phonolocating females as efficiently as the calls of parental species. Sexual dimorphism within species (*Xenopus* females are larger) and size differences between species could also prevent amplexus between large females of *X. l. laevis* and small males of *X. gilli*.

Although survival of an intact *X. gilli* genome does not appear at this time to be threatened by hybridization with *X. l. laevis*, the two taxa may compete for limited resources in habitat of *X. gilli* (Rau, 1978; Simmonds, 1985). Hybridization with *X. l. laevis* may reduce reproductive potential of *X. gilli*, especially since populations of *X. l. laevis* are generally much larger than *X. gilli*. Cannibalism of eggs and tadpoles by *X. l. laevis* also could reduce *X. gilli* survivorship. For these reasons, we recommend continued removal of *X. l. laevis* from *X. gilli* habitat in management areas such as the Cape of Good Hope Nature Reserve.

To best preserve this species, further protec-

tion of *X. gilli* habitat is imperative. Unique blackwater ponds of this habitat are threatened by construction and drainage projects, housing, and farming activity (Kobel et al., 1981). Conservation of populations near Kleinmond and Pearly Beach would protect much of the genetic diversity seen in mtDNA of *X. gilli* (Evans et al., 1997) and nDNA. Such efforts also would protect other endangered species (e.g., the micro frog, *Microbatrachella capensis*) that share the lowland fynbos habitat with *X. gilli.*

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