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DNA EVIDENCE FOR NONHYBRID ORIGINS OF PARTHENOGENESIS IN NATURAL POPULATIONS OF VERTEBRATES

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Naturally occurring unisexual reproduction has been documented in less than 0.1% of all vertebrate species. Among vertebrates, true parthenogenesis is known only in squamate reptiles. In all vertebrate cases that have been carefully studied, the clonal or hemiclonal taxa have originated through hybridization between closely related sexual species. In contrast, parthenogenetic reproduction has arisen in invertebrates by a variety of mechanisms, including likely cases of "spontaneous" (nonhybrid) origin, a situation not currently documented in natural populations of vertebrates. Here, we present molecular data from the Neotropical night lizard genus *Lepidophyma* that provides evidence of independent nonhybrid origins for diploid unisexual populations of two species from Costa Rica and Panama. Our mitochondrial and nuclear phylogenies are congruent with respect to the unisexual taxa. Based on 14 microsatellite loci, heterozygosity (expected from a hybrid origin) is low in *Lepidophyma reticulatum* and completely absent in unisexual *L. flavimaculatum.* The unique value of this system will allow direct comparative studies between parthenogenetic and sexual lineages in vertebrates, with an enormous potential for this species to be a model system for understanding the mechanisms of nonhybrid parthenogenesis.

KEY WORDS: ***Lepidophyma*, lizard, microsatellites, nonhybrid origin, parthenogenesis, phylogenetics.**

The evolutionary success of sexual reproduction has been attributed to two factors: sexuals can adapt more rapidly to changing environments and they are less prone to the accumulation of deleterious mutations (Kondrashov 1993). Unisexual organisms present a unique opportunity to test these two hypotheses (Simon et al. 2003). Unisexual reproduction falls into one of three categories: hybridogenesis (hemiclonal reproduction where the female half of the genome is passed intact to the next generation following fertilization), gynogenesis (reproduction in the presence of sperm, but no fertilization of the egg), or true parthenogenesis (no participation or requirement from a male). Hybridogenesis and gynogenesis are known in fish and amphibians, and all-female clones are dependent to some degree on males of a closely related bisexual species. Although unisexual invertebrates have been known for centuries and are relatively well studied (summaries in Suomalainen et al. 1987; Cuellar 1987), unisexual vertebrates were not discovered until the mid-twentieth century (summary in Dawley and Bogart 1989). In subsequent decades, the number of known unisexual vertebrates has increased considerably and stands at more than 70 "biotypes" of parthenogenetic reptiles and gynogenetic or hybridogenetic amphibians and fish (Vrijenhoek et al. 1989; Kearney et al. 2009). Although unisexuals

constitute less than 0.1% of all vertebrates, they continue to attract considerable attention, in part due to a fascination with the strange and novel, but also from a recognition that the exception provides a vantage point from which to evaluate the norm (Dawley and Bogart 1989; Vrijenhoek 1994, 1998; Beukeboom and Vrijenhoek 1998; Barraclough et al. 2003; Kearney et al. 2009).

The critical question of how unisexuals originate has important implications regarding the roles of genetic variability and the evolution of sex (Barton and Charlesworth 1998). Historically, two competing hypotheses have been proposed for the origin of natural unisexual populations; interspecific hybridization and (spontaneous) mutational hypotheses. To date, all natural populations of unisexual vertebrate taxa investigated, regardless of whether they reproduce by gynogenesis, hybridogenesis, or parthenogenesis, show patterns of genetic variation consistent with an origin by interspecific hybridization between closely related species (Dawley and Bogart 1989; Simon et al. 2003; Korchagin et al. 2007; Kearney et al. 2009). Here, we present phylogenetic (mitochondrial and nuclear sequences) and allelic (microsatellite) data that are consistent with a nonhybrid origin for unisexuals in two species of night lizards (*Lepidophyma*: Xantusiidae) from Central America.

The Hybrid Origin Hypothesis (HOH) postulates that two closely related gonochoristic ("bisexual" hereafter) species interbreed and successfully produce viable offspring that then reproduce parthenogenetically. Vertebrate parthenogens are normally diploid or triploid and commonly reflect high levels of nuclear gene diversity due to "fixed heterozygosity" at codominant loci for which the two hybridizing parental species segregate for alternative alleles (Simon et al. 2003). Despite the high nuclear gene heterozygosity, parthenogens are generally thought to be ephemeral over evolutionary time (Moritz 1993; but see Fontaneto et al. 2007). An alternative origin for parthenogenetic forms is through the accumulation of spontaneous mutation(s) or the Mutational Origin Hypothesis (MOH) (Suomalainen et al. 1987; Bullini 1994; Johnson and Leefe 1999). Little is understood about the mechanisms of such an origin, but the most obvious difference is detectable genetic evidence for a single parental species under the MOH, whereas a unique combination of divergent genotypes from two distinct parentals is required to support the HOH (Simon et al. 2003). With adequate sampling, a combination of mitochondrial and nuclear markers can be used to discriminate between these alternatives (Fig. 1).

The best vertebrate candidates for a nonhybrid origin of parthenogenesis involve unisexual populations in two species of night lizards (*Lepidophyma*). The genus extends from northern Mexico through Central America to Panama (Fig. 2), and currently 18 species are recognized (Bezy and Camarillo 2002; Canseco-Márquez et al. 2008). In *Lepidophyma reticulatum* (Costa Rica)

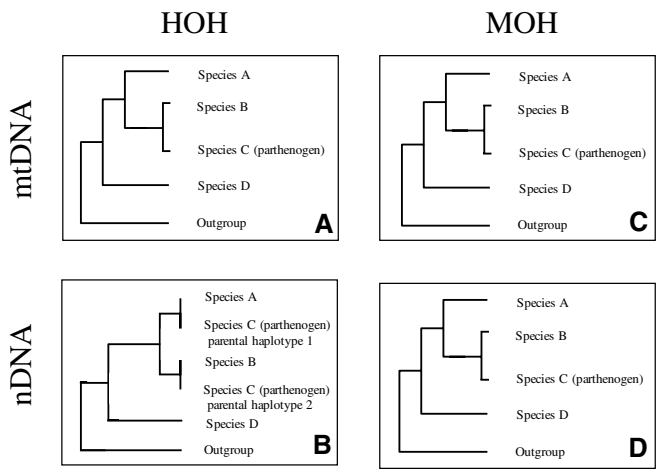

Figure 1. Hypothetical phylogenies under a hybrid (HOH) and mutational (MOH) origin for parthenogens. (A) HOH for a mitochondrial phylogeny; the HOH predicts phylogenetic recovery of mitochondrial haplotypes for the parthenogen with the bisexual descendent of the female parental species. (B) HOH for a nuclear phylogeny; nuclear genes for which alternative alleles segregate in different hybridizing bisexual species are expected to be expressed as fixed heterozygous genotypes in parthenogens. Further, phylogenetic reconstruction should "split" these fixed hybrid heterozygotes into alternative clades (Moritz 1987; Sites et al. 1990), each of which is recovered with the descendants of either the maternal or paternal ancestors, and the maternal clade should match that recovered in the mtDNA analysis. This test will be strongest if the nuclear alleles are synapomorphic characters in the phylogeny, because this eliminates the possibility that heterozygous genotypes in the parthenogens represent shared ancestral polymorphisms (Murphy et al. 2000). A multivariate analysis of microsatellite genotypes from parthenogens should form a distinct cluster that is intermediate to those representing the two bisexual species. (C) MOH for a mitochondrial phylogeny; the MOH predicts an mtDNA phylogeny in which the parthenogens are recovered with their sister bisexual species. (D) MOH for a nuclear phylogeny; the nuclear gene tree should recover a similar or identical topology to the mtDNA tree. Any nuclear heterozygotes in the parthenogens would reflect the idiosyncratic history of mutations shared with the single bisexual ancestral lineage, or alleles of mutational origin that define clonal diversity in parthenogens. Clustering of multilocus microsatellite genotypes from parthenogens should form one group embedded within the single parental species.

all known individuals are female; *L. flavimaculatum* populations in Panama and south-eastern Costa Rica lack males, whereas northern populations contain both sexes and appear to be bisexual. Limited available data from morphology, karyotypes, and allozymes (summaries in Bezy 1972; Bezy and Sites 1987; Bezy and Camarillo 2002) do not provide evidence for a hybrid origin of the all-female populations of *L. flavimaculatum*. To date, all unisexual populations studied are diploid (except one 2N/3N

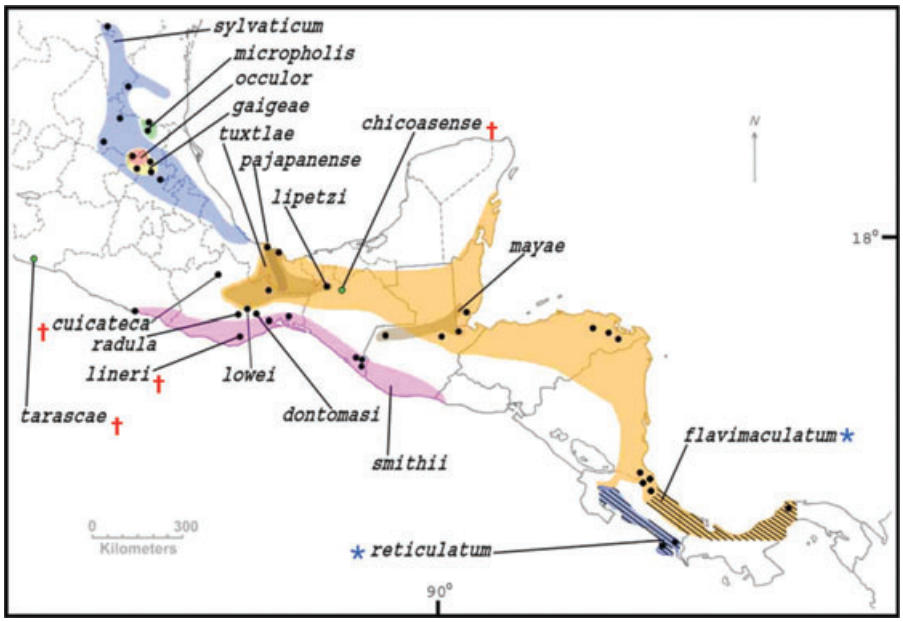

Figure 2. **Map of Mexico and Central America showing the global distribution of *Lepidophyma* species. Sampling localities are indicated by black filled circles (Appendix S1); species not sampled (red crosses), and parthenogens (blue stars); distribution of parthenogenetic forms (hatched area); region of sympatry between *L. reticulatum* and bisexual *L. flavimaculatum* (unshaded blue).**

mosaic individual; Bezy 1972). The earlier studies, however, were limited in taxon sampling and resolution of the genetic characters. Here, we test the HOH and MOH for the unisexual species *L. reticulatum* and unisexual populations of *L. flavimaculatum* using phylogenetic and population genetic methods, including dense population sampling, high-resolution molecular data, and sufficient taxonomic sampling to include all likely parental taxa.

Materials and Methods

SAMPLE COLLECTION

Fourteen of the 18 recognized *Lepidophyma* species (Bezy and Camarillo 2002; Canseco-Márquez et al. 2008) were collected for DNA sequencing and subsequent phylogenetic and population genetic analyses (Appendices S1 and S2). Four species not included in this study (*L. lineri, L. chicoasense, L. tarascae*, and the recently described *L. cuicateca*) have extremely restricted distributions in southern Mexico and are either very rare or extinct (Bezy and Camarillo 2002; Canseco-Márquez et al. 2008). Several other species also have very localized distributions and are extremely difficult to collect; out of necessity, five species are represented here by single tissue samples, and two species each are represented by two and three samples, respectively. The collection of individuals used here represents over 30 years of fieldwork by RLB and colleagues. Sequences from five species of the sister genus *Xantusia* (Vicario et al. 2003) were used as outgroups: (*X. bolsonae, X. extorris, X. gilberti, X. riversiana*, and *X. vigilis*).

PCR AND PHYLOGENETIC ANALYSES

Two mitochondrial genes were amplified: the complete Cytochrome *b* (Cyt *b*; 1143bp) and partial 12S rRNA (12S; 924bp). Cyt *b* was amplified in two fragments using the primer combinations L14724 and CB3 and F1 and RD. The 12S region was amplified using 12StPhe and 12Se. Polymerase chain reaction (PCR) conditions are the same as those described in Sinclair et al. (2004). Eight nuclear regions (alpha-enolase [a-Enol; 238bp], oocyte maturation factor [C-mos; 495bp], proopiomelanocortin-A gene [POMC; 516bp], brain-derived neurotrophic factor [BDNF; 708bp], glyceraldehyde-3-phosphate dehydrogenase [Gapdh, intron 11; 305bp], neurotrophin-3 gene [NT-3; 528bp], recombination activating-1 gene [RAG-1; 840bp], and Pinin [PNN; 932bp]) were also amplified using standard PCR conditions. Details of the primer sequences and source are given in Appendix S3. All sequences are available through GenBank (accession numbers given in Appendix S1).

PCR cycling was performed in a 9600 thermocycler (PerkinElmer), and 2 μl of PCR product was run on 1% agarose gels stained with ethidium bromide and viewed under UV light. All PCR products were cleaned using Millipore plates. Sequencing reactions were performed in 4 μl volumes, using the ABI Bigdye Ready-Reaction kit, following the standard cycle sequencing protocol. Double-stranded sequences were generated using an ABI3730XL automated DNA sequencer. Sequences were edited and aligned using Sequencher (Gene Codes Corp.) and checked by eye. Alignment gaps were used to maximize codon identity and minimize the number of insertions or deletions. For the coding

(exon) regions, gaps represented a gain/loss of complete amino acids. These gaps were usually fixed within species. All alignments were relatively unambiguous as sequences were from two closely related sister genera. Reading frames for mitochondrial and nuclear protein-coding regions were checked to guard against pseudogene amplification (Song et al. 2008).

Phylogeny reconstruction for 2067bp of mtDNA sequence was performed using Bayesian Inference (Huelsenbeck and Ronquist 2001) and maximum likelihood (ML) in PAUP* (Swofford 2002). The program ModelTest 3.07 (Posada and Crandall 1998) was used to select the "best-fit" model of evolution for the combined mitochondrial dataset using the AIC criterion. For the Bayesian analysis, four independent searches were performed with three million generations each and four incrementally heated Markov chains were used to enable a more thorough search of the parameter space. "Burnin" plots were examined and the initial 50,000 replicates were excluded from subsequent analysis. A 50% majority-rule consensus tree was generated using the remaining replicates for each of the searches in PAUP* (Swofford 2002). The percentage of samples recovering a particular clade was taken as that clade's posterior probability (Huelsenbeck and Ronquist 2001). ML runs were performed on a 68-node cluster running Debian Linux each with two Intel Xeon quad core processors (E5345) (Intel Corp, Santa Clara, CA) at Brigham Young University. The nuclear gene sequences (4562bp) were analyzed separately using equally weighted Maximum Parsimony in PAUP* and Bayesian analysis incorporating the "best-fit" model of evolution for each gene region. In the parsimony analyses, gaps were coded as a fifth character state, as they were deemed informative. Each gap was treated as a single event. One thousand search replicates were performed using the TBR search algorithm, and nodal support was assessed using 1000 bootstrap replicates. A Shimodaira–Hasegawa test (S–H test; Shimodaira and Hasegawa 1999) was executed in PAUP* (using a RELL distribution with 1000 replicates and separate tests executing the mtDNA and nDNA datasets) to test whether each of the combined mtDNA and nuclear trees were significantly different. The phylogenies (and shared nuclear sequences) were then used to identify potential paternal species under an HOH.

We tested for recombination in each nuclear gene region using the software RDP3 (Martin et al. 2005). The program examines nucleotide sequence alignments and attempts to identify recombinant sequences and breakpoints using 10 published recombination detection methods with a range of performance, including approaches well suited for our observed levels of genetic diversity (Posada and Crandall 2001).

MICROSATELLITE LOCI

Fourteen di- and tetra-nucleotide microsatellite markers were amplified in taxa that were both phylogenetically and geographically proximal to *L. flavimaculatum* and *L. reticulatum* (Appendix S2) using conditions described in Sinclair et al. (2006). By avoiding distantly related, divergent *Lepidophyma* species in this analysis, we reduce the possibility of spurious allelic similarity due to homoplasy. We used GenePop-on-the-web version 3.4 (Raymond and Rousset 1995) to test for Hardy–Weinberg equilibrium and linkage disequilibrium for five samples for which at least eight individuals were genotyped. These were the bisexual populations of *L. flavimaculatum* (Rancho Grande, $n = 11$; Tapezco, $n = 9$), the unisexual population of *L. flavimaculatum* (Escobal, $n = 9$), *L. reticulatum* (Las Cruces, $n = 9$), and *L. sylvaticum* (San Luis Potosi, $n = 10$). Hardy–Weinberg exact tests were performed for each locus where possible, and a Holm–Bonferroni method was used to correct for multiple tests (Holm 1979). A principal coordinate analysis (PCA) was performed in GenAlEx version 6.1 (Peakall and Smouse 2006) to visually represent the relative degree of genetic similarity among individuals and the distinction, if any, among sampled populations/species.

Results

PHYLOGENETIC RELATIONSHIPS

The mitochondrial tree recovered all species of *Lepidophyma* represented by more than one terminal as clades (ML bootstrap = 96–100; Bayesian PP = 1.0), with the exception of *L. sylvaticum*, which is paraphyletic relative to *L. micropholis* (Fig. 3A). All bisexual and unisexual *L. flavimaculatum* terminals were recovered as a clade sister to the unisexual *L. reticulatum* (both well-supported). Both parthenogens are in a highly derived position in the tree, and both show a low level of within-taxon mtDNA variation (<2.0% sequence divergence). The nuclear tree (Fig. 3B) also recovered the *L. flavimaculatum* (including bisexual and unisexual terminals) and *L. reticulatum* clades as sister taxa (MP bootstrap = 99; Bayesian PP = 1.0). The S–H tests indicated there were significant differences between the mitochondrial and nuclear gene trees (mtDNA, $P < 0.001$, nDNA, $P = 0.051$). However, the differences involved several poorly sampled taxa whose phylogenetic position was not well-resolved (*L. occulor, L. pajapanense,* and *L. mayae*). In both trees, the unisexual and bisexual *L. flavimaculatum* terminals formed a clade, sister to the unisexual species *L. reticulatum*, with *L. lipetzi* comprising the sister species to this group (*L. lipetzi* (*L. reticulatum* (*L. flavimaculatum*-bisexual + *L. flavimaculatum*-unisexual))), (mtDNA ML bootstrap = 99; nuclear; MP bootstrap = 100). This topology is consistent with phylogenetic predictions under an MOH for nuclear and mitochondrial sequences (see Fig. 1C,D), but not the HOH.

Of all nuclear genes sequenced, the Gapdh region was the most variable at the species level, and proved to be a good marker for detection of hybridization in *Xantusia* (Leavitt et al. 2007).

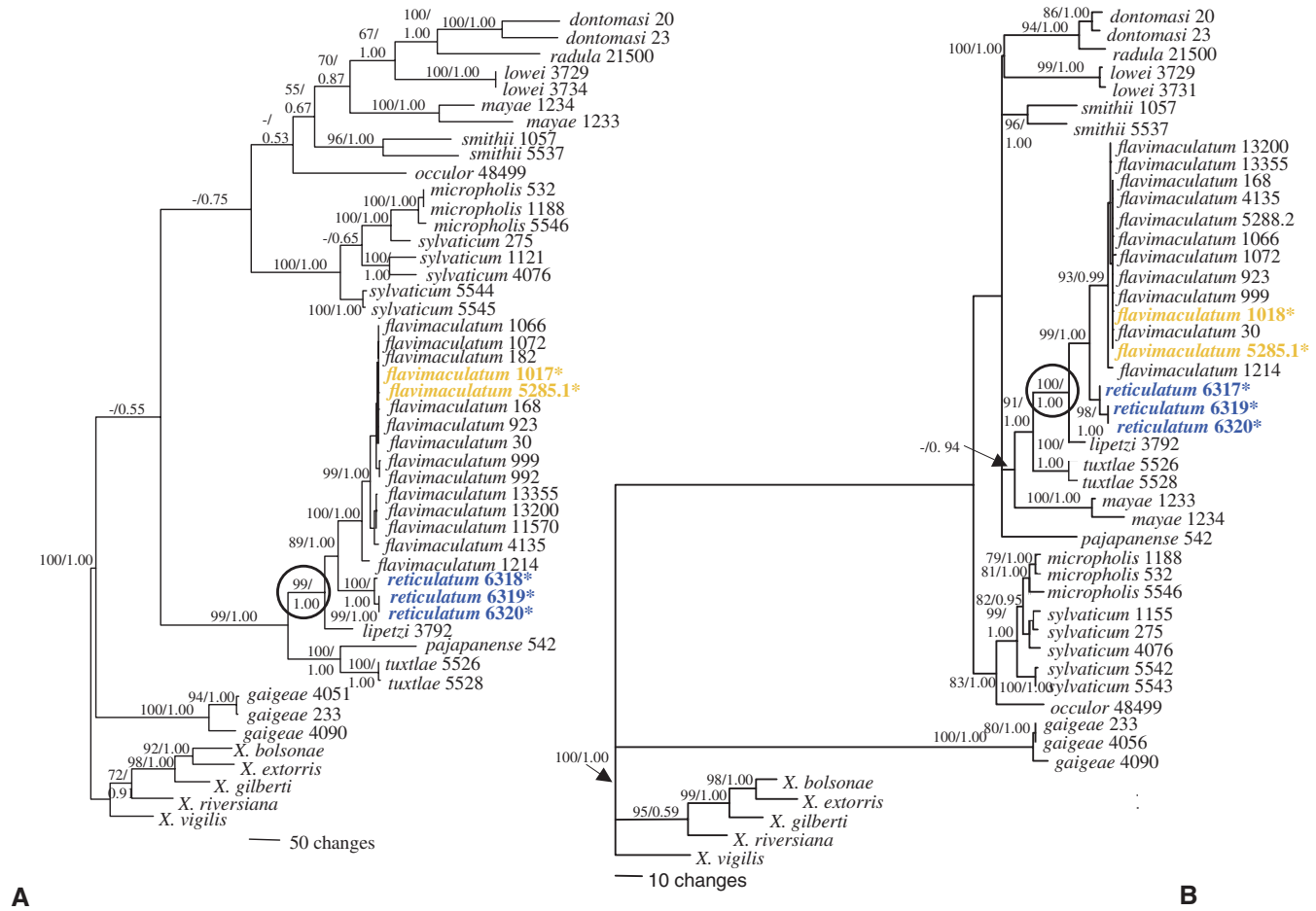

Figure 3. **Phylogeny reconstructions for *Lepidophyma*. (A) Combined mitochondrial phylogeny based on 2067bp of Cyt *b* and 12S, -Llk = −17771.684; the Akaike information criterion (AIC) implemented in ModelTest, selected the General Time Reversible model plus gamma distribution rate heterogeneity (GTR+I+G). The parameters were base frequencies A = 0.3403, C = 0.2989, G = 0.1324, and T = 0.2284, transition rates = (A–G) 9.9071 and (C–T) 12.5819, transversion rates = (A–C) 2.1624, (A–T) 1.7805, (C–G) 0.8232, (G–T) 1.000, gamma distribution shape parameter (G) = 0.9070, and the proportion of invariable sites (I) = 0.3872. Nodal support = ML bootstrap/Bayes PP. (B) combined nuclear sequences (4562bp; 28 equally most parsimonious trees of 629 steps, nodal support = MP bootstrap/Bayes PP; *indicates parthenogenic individuals; terminals are identified by species name and sample number (Appendix S1).**

In *Lepidophyma*, these sequences detected heterozygosity as evidenced by two nucleotides (double peaks in the electropherograms; Brumfield et al. 2003) at seven nucleotide positions in the bisexual species *L. sylvaticum* from Mexico. The heterozygous positions were not fixed in *L. sylvaticum*; only four of the 10 alternative alleles were found in the same or nearby populations. No heterozygous individuals were observed among any of the unisexual or bisexual terminals for *L. flavimaculatum* (n = 13) or *L. reticulatum* (n = 3) at any of these or other nucleotide positions, consistent with an MOH. In fact, all *L. flavimaculatum* sequences from unisexual and bisexual individuals were identical to each other, and with a single nucleotide substitution from unisexual *L. reticulatum* (also identical to each other). This pattern was almost identical for all eight nuclear gene regions. Although these results may be an artifact of limited sample sizes, they are confirmed by a more extensive microsatellite dataset summarized below. Furthermore, for clonal species of hybrid origin, multiple heterozygosities are normally found even with a sample size of one, and large samples show the heterozygosity to be fixed, with very few exceptions.

MICROSATELLITE VARIATION

For the microsatellite data (Appendix S2), we found no evidence of fixed heterozygosity in the *L. flavimaculatum* unisexuals from Panama; all 14 loci were homozygous. Average heterozygosity levels ranged from 25.5% to 30.8% for the two bisexual *L. flavimaculatum* populations from Costa Rica, with no deviation from Hardy–Weinberg ratios (Table 1). The spatial arrangement of multilocus genotypes showed extensive among-population variation within *L. flavimaculatum* (Fig. 4). All unisexual *L. flavimaculatum* form a single cluster, together with the most geographically proximal bisexual populations of El Tigre and Tapezco, Costa

Table 1. Summary of observed heterozygosity levels (%) for each microsatellite locus.

Species	*N*	LflA102	LflA104	LflA107	LflB102	LflC101	LflC102	LflC104	LflC105	LflC109	LflC110	LflC112	LflD103	LflD120	LflD124	Overall
L. flavimaculatum (bi)	35	17.1*	24.2*	2.9*	17.1*	22.9*	8.6*	34.4*	45.7*	42.9	10.0*	11.4*	67.6*	62.9*	38.2*	29.0
Rancho Grande (bi)	11	0.0	40.0	0.0	27.3	0.0	0.0	63.6	54.5	81.8	0.0	0.0	81.8	54.5	27.3	30.8
Tapezco (bi)	9	0.0	12.5	0.0	22.2	44.4	0.0	11.1	33.3	22.2	11.1	0.0	66.7	100.0	33.3*	25.5
Panama (uni)	11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
L. spp3794/5793	2	100.0	100.0	0.0	0.0	50.0	0.0	0.0	50.0	50.0	0.0	50.0	50.0	0.0	100.0	39.3
L. reticulatum (uni)	12	0.0	0.0	9.1	63.6	0.0*	0.0*	0.0	72.7	36.4	11.1*	9.1	8.3*	60.0	0.0*	19.3
Las Cruces (uni)	9	0.0	0.0	0.0	62.5	0.0	0.0	0.0	75.0	12.5	0.0*	12.5	0.0*	42.9	0.0*	14.7
L. lipetzi	1	0.0	50.0	0.0	0.0	0.0	0.0	50.0	100.0	50.0	–	50.0	100.0	–	0.0	33.3
L. sylvaticum																
San Luis Potosi (bi)	10	70.0	70.0	30.0	40.0	0.0*	80.0	20.0	30.0*	0.0	40.0	80.0	22.2	100.0	77.8	50.8

*Deficit in heterozygosity.

Species or population: bi, bisexual; uni, unisexual.

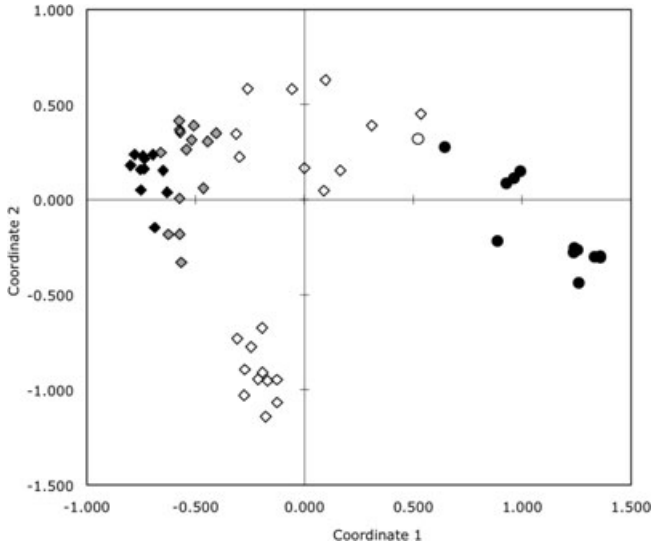

Figure 4. Principal coordinate analysis of multilocus genotypes for the *L. flavimaculatum*/*L. reticulatum* group. The two parthenogens form discrete clusters, one for *L. reticulatum* (solid circles) and a second for unisexual *L. flavimaculatum* (solid diamonds), which are the geographically closest samples from El Tigre and Tapezco (gray diamonds) and well within the spread of all bisexual *L. flavimaculatum* (open diamonds); *L. lipetzi (open circle).*

Rica. Our most interior Costa Rican sample from Rancho Grande formed a separate cluster, and contains private alleles not found elsewhere in bisexual or unisexual *L. flavimaculatum* or in *L. reticulatum*: A104: (144, 148), B102: (258), C104 (186, 191, 192), D120 (328; Table 2). Within *L. flavimaculatum,* alleles present in the unisexual population from Panama are all present in bisexual populations from Costa Rica, with the exception of three rare alleles segregating in Belize (locus D103–227, freq = 0. 059; locus D124–256, freq = 0.015), and Guatemala (locus D103–227, freq = 0.103). All *L. reticulatum* clustered loosely together, but differences between the two populations are evident.

Heterozygosity levels varied across microsatellite loci for *L. reticulatum* (0.0–75.0%, Table 1). The overall heterozygosity was lower in unisexual *L. reticulatum* (14.7–19.3%) than bisexual *L. flavimaculatum* (25.5–30.8%). Five of eight polymorphic loci had a significant deficit in heterozygotes (Table 1). Two populations were sampled within *L. reticulatum*—Las Cruces ($n = 9$) and Rincon ($n = 3$), and despite small samples there were fixed differences at seven loci whereas the other seven loci were heterozygous but shared common alleles between the localities. The pattern of variation in *L. reticulatum* is also inconsistent with hybridization for example, five of eight individuals from the Las Cruces sample are heterozygous at locus B102 (260/262); allele 262 is unique to the population, suggesting that heterozygosity at this locus is best explained by postorigin mutation rather than hybridization. A large number of alleles are shared with *L. flavimaculatum* bisexuals

Table 2. Summary of alleles within *L. flavimaculatum, L. reticulatum,* and *L. mayae*. Alleles shared between unisexuals and bisexuals are in bold.

Locus	*L. reticulatum* unisexual (Las Cruces, Costa Rica)	Het	*L. flavimaculatum* unisexual (Escobal, Panama)	Het	*L. flavimaculatum* bisexual Costa Rica	Het	*L. flavimaculatum* bisexual northern Central America	Het	*L. mayae bisexual* Guatemala	Het
A102	**195**	0.0	**195**	0.0	189, **195**	0.0	**195,** 197, 199, 201, 205	42.9	185, 187, 189	20.0
A104	**124**	0.0	**136**	0.0	126, 130, **136,** 140, 144, 148	23.1	**124,** 128, 134, 140	26.6	**124,** 130, **136,** 137, 140	80.0
A107	148, **157**	9.1	**152**	0.0	**152**	0.0	146, 150, **152,** 154	14.3	135, 142, **152, 157,**159, 167	40.0
B102	252, **254, 256, 260,** 262	63.6	**256**	0.0	248, 250, **256,** 258	21.4	248, **254, 260,** 270, 272, 274	42.9	227, 242	0.0
C101	**176, 183,** 187	0.0	**171**	0.0	155, 163, 167, **171,** 175	14.3	163, 167, **171,** 175, 179, **183**	42.9	143, 164, **176**	20.0
C102	209, **213**	0.0	**197**	0.0	**197,** 217	7.1	**197, 213,** 226	14.3	205, 226, 230, 234, 239	40.0
C104	148	0.0	**176**	0.0	**176,** 180, 183, 186, 188, 191, 192	37.0	156, **176,** 180, 183, 180	16.7	151, 254	25.0
C105	**299, 307, 311,** 315, 323, 397	72.7	**299, 303**	0.0	287, 290, 295, **299, 303**	42.9	290, 295, **299, 303,** 306, **307, 311**	57.1	273, 356, 372, 384, 389, 399	40.0
C109	266, **275, 279, 287,** 291, 303, 311	36.4	**275**	0.0	257, **275, 279,** 283, **287**	42.8	271, **275, 279,** 283	42.9	234	0.0
C110	178, **183,** 191, **216,** 237	11.1	**220, 225, 229**	0.0	179, **220, 225, 229,** 233	7.1	200, 205, **216, 220, 225**	33.3	**183,** 203, 208, 211, 224	80.0
C112	210, 227	9.1	**206**	0.0	**206**	0.0	201, **206,** 214, 219, 221, 230	57.1	203, 219, 222	20.0
D103	155, **207, 211, 219, 227, 231**	8.3	**211, 223, 227**	0.0	203, **207, 211,** 215, **219,** 223, **231, 235**	64.3	203, **207, 219, 223, 227, 231,** 235	66.7	163, 169	0.0
D120	**295, 299, 303**	60.0	**283, 287, 291, 307, 312**	0.0	271, 275, 279, **283, 287, 291, 295, 299, 303, 307, 312,** 316, 320, 328	64.3	279, **287, 291, 307, 312,** 316, 320	66.7	–	–
D124	152, **244, 248, 252, 256**	0.0	**248, 252, 256**	0.0	224, 228, 232, 236, 240, **244, 248, 252**	25.0	224, 232, 236, **244, 248, 256,** 260	33.0	–	–

(25/48 = 52%) and eight alleles are shared with *L. flavimaculatum* unisexuals. The non-*flavimaculatum* alleles present in *L. reticulatum* are shared with some of the other bisexual species (data not shown). Ten private alleles from seven loci are exclusive to *L. reticulatum*. Nine alleles across six loci are shared between bisexual and unisexual populations of *L. flavimaculatum* and at least one of the *L. reticulatum* populations. This pattern supports a spontaneous origin of both unisexuals from a bisexual *L. flavimaculatum*-like ancestor, but also reflects subsequent divergence of the unisexuals. There was no evidence that the genetic variation present in the unisexuals could be split into two separate genomes inherited from divergent parental species or populations.

Discussion

HYBRID VERSUS NONHYBRID ORIGIN

Both the mtDNA tree and the nuclear tree consistently recover the same clade: (*L. lipetzi* (*L. reticulatum* (*L. flavimaculatum*-unisexual + L. *flavimaculatum*-bisexual)). This same topology is also recovered (Bayesian PP = 1.0, and ML bootstrap values ≥ 93) from a much larger dataset (nine nuclear and four mitochondrial genes, 7186 bp) in a phylogenetic study of the family Xantusiidae (J. Pramuk, R. Bezy, B. Noonan, E. Sinclair, K. de Queiroz, and J. Sites, unpubl. ms) and indicates that parthenogenesis most likely evolved twice in the genus *Lepidophyma*: the first origin involving the unisexual species *L. reticulatum* (Pacific Costa Rica), and a later origin within *L. flavimaculatum* involving the unisexual populations in Panama and southeastern Costa Rica.

The unisexual population of *L. flavimaculatum* in Panama is not distinguishable from the bisexual populations that range from northern Costa Rica to Guatemala by any markers in the mitochondrial and nuclear genes sequenced, but they do differ in that the unisexual samples are homozygous at all 14 microsatellite loci. To our knowledge, this is the first report of a complete lack of heterozygosity using microsatellite markers for any unisexual lizard, and does not conform to expectations of the HOH. The Asher Effect (Asher 1970) or "decay" of heterozygosity to homozygosity has been used to explain a complete absence of heterozygosity in hybrids. It can occur under some forms of meiosis in parthenogenetic species (under HOH), making it impossible to identify the two parental species and hence to differentiate between an MOH and HOH. However, if loss of alleles under the Asher Effect is random with respect to parental origin, parthenogens of hybrid origin should be fixed for alternative alleles from each of the parental species at different (unlinked) nuclear loci, and therefore be in strong linkage disequilibrium due to the association of "frozen" alternative homozygous genotypes. This loss (in *L. flavimaculatum* unisexuals) via the decay mechanisms described by Asher (1970) seems unlikely as we do not detect linkage disequilibrium (see below). In such rapidly evolving markers as microsatellites, high levels of heterozygosity are expected under an HOH. However, our findings are consistent with all expectations of the MOH for a recent origin of unisexual *L. flavimaculatum*. We find no evidence of genomes derived from two genetically divergent parental populations, as would be expected under the HOH. This result is also in a stark contrast to studies in the unisexual lizard *Darevskia unisexualis* (Tokarskaya et al. 2004; Badaeva et al. 2008), where variation at a single (GATA)n microsatellite locus has arisen via germline and somatic mutations in an unstable locus.

The picture for *L. reticulatum* is more complex as it involves greater genetic variation, consistent with an earlier origin for this unisexual species. It differs at between 103 and 125 mitochondrial nucleotides (5–6% sequence divergence) and six nuclear substitutions from *L. flavimaculatum*, but is consistently placed sister to *L. flavimaculatum*. At the 14 microsatellite loci examined, a total of 47 alleles are present in *L. reticulatum*, eight of which are unique and presumably represent mutations that occurred within this species. There are 24 alleles shared with *L. flavimaculatum*, and these occur widely, rather than being restricted to any one geographic population. The remaining 14 microsatellite alleles are shared among other *Lepidophyma* species, and *L. reticulatum* does not share an unusually high number of alleles with any one species other than *L. flavimaculatum*. The overall observed heterozygosity for the Las Cruces population of *L. reticulatum* (14.7%) is about half that of the bisexual populations of *L. flavimaculatum* (Table 1).

Given evidence for two spontaneous origins, the most obvious difference between the two parthenogens is in variation across the microsatellite loci; a complete absence of heterozygosity within unisexual *L. flavimaculatum* from Panama compared with 30.8% and 25.5% for the two bisexual populations of *L. flavimaculatum*, and 14.7% for the unisexual *L. reticulatum*. This is not attributed to reduced heterozygosity due to cross-species amplification, as microsatellite loci for a single population of a more distantly related species, *L. sylvaticum*, are highly polymorphic (overall heterozygosity = 51.7%) for the same set of loci and PCR amplification conditions.

DECAY OF HETEROZYGOSITY AND LOSS OF HYBRID SIGNAL?

Heterozygosity in parthenogenetic individuals of hybrid origin can be eliminated either by a terminal fusion in the absence of recombination or the inhibition of meiosis II (Fig. 2 in Asher 1970), and will result in a homozygous individual. A second mechanism, ameiotic recombination (Omilian et al. 2006), has been shown to result in a low rate (<1.2%) of spontaneous loss of heterozygosity (LOH) in asexual *Daphnia* lineages. This process is expected to eliminate heterozygosity much faster than mutation can replenish it, causing clonal lineages to lose allelic variation

over time (Omilian et al. 2006). The less likely third alternative is biased gene conversion; a mechanism demonstrated for the ribosomal DNA repeat complex in the hybrid unisexual gecko *Heteronotia binoei* (Hillis et al. 1990). Yet, this parthenogen still retained a signature-fixed heterozygosity at diagnostic allozyme loci characteristic of vertebrate parthenogens.

The decay of heterozygosity and loss of a hybrid-origin genetic signature by any of the above mechanisms could explain our results if these processes were biased to preferentially eliminate one set of paternal alleles, but for several reasons we think this is unlikely. First, we have no evidence for recombination in nuclear sequences (see Methods), although the power of the tests may be limited by low sequence divergence (<5.0% across the genus). Second, if the 14 microsatellite loci used in this study are unlinked, and maternal and paternal alleles assort randomly at each locus in meiosis, the probability of losing only paternal alleles would be $1/(2)^{14}$. The biased loss of all paternal alleles (by elimination or conversion) might still be possible at a higher probability if most of the microsatellite markers are strongly linked. The loci used in this study have not been mapped and sample sizes generally preclude estimates of linkage disequilibrium, but for the five largest samples, there was no evidence of linkage disequilibrium for any combination of polymorphic loci (not shown). The fixed homozygosity for unisexual populations of *L. flavimaculatum* at all microsatellite loci, the absence of evidence for a paternal lineage in nuclear sequences, and the lack of evidence for any mechanism of heterozygosity decay all favor the hypothesis of a relatively recent mutational origin for these unisexual populations.

The patterns of variation in the unisexual *L. reticulatum* also suggest that none of the three heterozygosity-eliminating processes have operated in this species, for the reasons just given. However, unlike the completely homozygous unisexual populations of *L. flavimaculatum*, varying levels of heterozygosity are observed at the microsatellite loci in *L. reticulatum*. The two unisexuals share about half of their alleles, many of the non-*flavimaculatum* alleles present in *L. reticulatum* are shared with other bisexual species, suggesting either that polymorphisms have persisted through several speciation events, or that allelic homoplasy is common. Of special interest are the 10 private alleles (from seven loci) that are exclusive to *L. reticulatum*. Two mutually compatible explanations for this pattern are that some alleles are present in other species but were missed by limited sampling, and/or that some have originated via subsequent mutation events as clones have diversified. The absence of evidence for a paternal lineage in this species, and the strong support in both gene trees for its phylogenetic position as the sister species of the (*L. flavimaculatum* – bisexual + *L. flavimaculatum* – unisexual) clade, leads us to conclude that this species was derived via spontaneous mutational origin prior to the origin of unisexual *L. flavimaculatum*. This interpretation is consistent with the clonal diversity observed in the microsatellite loci, and the modest mtDNA and nuclear sequence divergence within this clade relative to the near absence of sequence divergence in the bisexual–unisexual clade of *L. flavimaculatum* (Fig. 3).

TWO ORIGINS OR A REVERSAL?

We are interpreting our phylogenetic results as two independent origins of unisexuality, but from a strict parsimony perspective, the alternative is that there was a single loss of sexual reproduction in the ancestor to the (*L. reticulatum* + *L. flavimaculatum*) clade, followed by a reversal of this transition back to sexual reproduction in the ancestor of the bisexual *L. flavimaculatum* populations. Another possibility is the origin of a "parthenogenesis mutation" and its passage as a polymorphism through the *L. reticulatum*–*L. flavimaculatum* speciation event, followed by fixation of the mutation in *L. reticulatum* and its retention as a polymorphism in *L. flavimaculatum*. These complex scenarios are consistent with the following two observations. First, all populations of *L. flavimaculatum* have low levels of geographic divergence compared to other species of *Lepidophyma*, suggesting that the species may represent a recent range expansion of a unisexual lineage into Central America, with a bisexual reversal in the northern part of the range. Second, *L. flavimaculatum* and *L. reticulatum* are largely allopatric, but a sympatric contact between bisexual *L. flavimaculatum* and unisexual *L. reticulatum* does exist in the Volcan Arenal region of Costa Rica; this provides potential for exchange of genes between these two species, therefore retaining polymorphism.

However, neither of these alternatives can satisfactorily explain the observed patterns of variation in all other markers, in the context of the geographic distributions of all populations of both species, without additional assumptions, including ad hoc shifts in geographic range. An ecological observation of interest is that, in contrast to the majority of organisms of hybrid origin which often are associated with environments heavily influenced by Pleistocene glacial cycles and characterized by weak biotic interactions (Kearney 2005), the unisexual *Lepidophyma* show neither. Both species are found in lowland tropical forests in lower Central America where the role of Pleistocene climatic cycles was likely restricted to elevational compression, and the species-rich ecosystems are characterized by strong complex biotic interactions. If the arguments of Kearney (2005) are correct, then *Lepidophyma* represents an example in which parthenogenesis does not serve to stabilize hybrid genotypes (which boost genetic variability and thereby provide an advantage in recolonization), and the depauperate genetic diversity of these unisexuals must be considered in another ecological context.

The presently available data cannot be used to resolve the sequence of events within *Lepidophyma*. It is plausible to

speculate on alternative hypotheses for the origin of unisexuality in *Lepidophyma* as these become starting points for further examination. The uniqueness of this system will allow direct comparative studies between parthenogenetic and sexual lineages in vertebrates. Limited information is available on the success of *Lepidophyma* in captivity, however, there is an enormous potential to develop this species into a model system for understanding the mechanisms of naturally occurring nonhybrid vertebrate parthenogenesis.

Conclusions

The interpretations of the genetic patterns described here, completeness of the taxonomic sampling, and the previous allozyme, chromosome, and morphological data (summarized in Bezy 1989; Bezy and Camarillo 2002) lead us to a strong inference that the all-female populations of *L. flavimaculatum* and *L. reticulatum* have been twice derived independently from *L. flavimaculatum*-like populations, and that these derivations did not evolve via hybridization. The microsatellite results presented here for unisexual *L. flavimaculatum* are unique in that this is the first report of a complete lack of heterozygosity in any naturally occurring population of unisexual lizards. We have no evidence for the mechanisms that might explain these transitions, but mechanisms recently described for aphids by Delmotte et al. (2001) might be relevant. These are either a complete spontaneous loss of males and of sexual reproduction through mutations along the pathways leading to production of unisexual forms, or the derivation of unisexual lineages resulting from "contagious" transmission of alleles favoring parthenogenesis. There is some evidence supporting the latter in the gradual "phasing out" (decrease in frequency) of males in Costa Rican populations of *L. flavimaculatum* near the Nicaraguan border and the contact of *L. flavimaculatum* with the unisexual *L. reticulatum* in this transitional region.

The genetic architecture for microsatellites in the two unisexual *Lepidophyma* stands in a stark contrast to that for allozymes in the well-studied parthenogenetic species of lizards of the genus *Aspidoscelis*. These latter are of hybrid origins having complete sets of alleles from each parental species, resulting in heterozygosity that is fixed and that is among the highest reported in vertebrates (summaries in Moritz et al. 1989; Reeder et al. 2002). The maintenance of fixed heterozygosity in parthenogenetic *Aspidoscelis* is a result of their meiotic mechanism that involves premeiotic endomitosis (Cuellar 1971). Whatever mechanism is responsible for the presumed parthenogenesis in *Lepidophyma,* it has resulted in a complete lack of microsatellite heterozygosity in unisexual *L. flavimaculatum*, and low levels of heterozygosity in the older *L. reticulatum*, presumably resulting from the gradual accumulation of mutations. Regardless of the mechanism involved, it appears that stabilization of hybrid genotypes (Kearny 2005) has not played a role in the transition from sexual to unisexual reproduction in these tropical lizards.

The apparent lack of hybridization in the origin of unisexual *L. flavimaculatum* bears importantly on the constraints of the evolution of parthenogenesis in vertebrates. In the "balance hypothesis" of Moritz et al. (1989), the chances of a hybridization event resulting in the founding of a parthenogenetic lineage depend on the hybrid-mediated disruption of meiosis resulting in the production of unreduced oocytes, but with minimal reduction in fecundity or viability of the hybrid. In the case of unisexual *L. flavimaculatum*, it appears that the production of unreduced oocytes likely occurs via mutation in genes regulating meiosis. Further, the confounding effects of polyploidy appear to be absent as chromosomal, allozyme, and microsatellite data contain no evidence of triploidy. The one exception is an unexplained diploid/triploid mosaic in one *L. flavimaculatum* from Panama, far removed from any known males (Bezy 1972).

The recent reports of "spontaneous" parthenogenetic events in captive squamates might offer clues about MOH mechanisms; automictic restitution leads to increased homozygosity, and in the case of the Komodo dragon (*Varanus komodoensis*; with a ZW sex-determining mechanism), all-male offspring (Watts et al. 2006). Although not conclusive, the case in the Burmese python (*Python molurus*) appears to involve a clonal mechanism (Groot et al. 2003). This example and our data for the night lizards suggest that, contrary to all previous reports, nonhybrid routes to parthenogenesis do occasionally succeed in vertebrates.

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Supporting Information

The following supporting information is available for this article:

Appendix S1. List of taxa Lepidophyma sampled and outgroup species, with location, collection numbers, and Genbank accession numbers.

Appendix S2. List of Lepidophyma individuals, their locations, museum voucher numbers, genotypes for 14 microsatellite loci, and bases at seven nucleotide positions that were heterozygous in *L. sylvaticum*.

Appendix S3. List of 10 mitochondrial and nuclear genes, primer sequences, and source used in this study.

Supporting Information may be found in the online version of this article.
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